Udo Blum

Plant-Plant Allelopathic Interactions II

Laboratory Bioassays for Water-Soluble Compounds with an Emphasis on Phenolic Acids

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This volume is dedicated to researchers and their graduate students who are interested in studying plant-plant allelopathic interactions.

Preface

In a previous volume (Blum 2011) the author suggested that we could improve our understanding of plant-plant allelopathic interactions in the field by making laboratory bioassays more holistic. The comments and suggestions in that volume as to how to go about that were rudimentary at best. Reflections after the volume was published lead the author to conclude that a more detailed analysis of the factors making up laboratory bioassays was needed in the hope that such an analysis would provide clearer and more useful directions on how to design more holistic or more relevant laboratory bioassay systems. The more holistic being a theoretical goal and the more relevant being a more pragmatic goal.

More specifically this volume presents a detailed description and discussion of the underlying features, issues, and suppositions associated with seed and seedling laboratory bioassays presented in the earlier volume. It also continues the retrospective analysis of seed and seedling laboratory bioassays begun in the previous volume. It is, however, broader in scope and substance in that the information provided is relevant to all water-soluble compounds released to soil by putative allelopathic living plants and their litter and residues. It is ultimately an attempt to update and expand the practical guidelines for designing laboratory bioassays that have previously been provided in the literature with the hope that the designs of future seed and seedling laboratory bioassays will become more relevant to field systems. This volume like its predecessor does not provide a comprehensive review of the literature. The literature about designing and implementing laboratory bioassays of water-soluble allelopathic compounds is much too extensive for that. Standard references have been included to provide background and additional details.

Chapter 1 provides a general introduction to this volume, discusses the nature of plant-plant allelopathic interactions, describes the nature and sources of allelopathic compounds in soils, discusses the concepts of holism and reductionism as it relates to laboratory bioassays, provides a listing of benefits, limits, and common pit falls for laboratory bioassays, and answers or sets the stage for answering the following questions: (a) Why is it important to design laboratory bioassays that are more holistic or, stated in a more pragmatic way, more relevant to field environments? (b) What can be done to make laboratory bioassays more relevant to field environments? and (c) Is it always necessary to make laboratory bioassays relevant

to field environments? Chapter 2 describes and provides comments on the following basic features of laboratory bioassays: (a) biotic and physicochemical factors, (b) test materials, (c) measurements, hypotheses, experimental designs, and data analyses, and (d) basic information that should be provided for all laboratory bioassays. Chapter 3 discusses a number of issues and challenges associated with creating more relevant model laboratory bioassays including the following: treatment concentrations, mobility and transport, species density, symbiotic relationships, microorganisms, controls or references, and measurements among others. Chapter 4 describes a set of standard hypothetical laboratory bioassays that may be used to screen for stimulatory or inhibitory effects of identified putative allelopathic compounds, leachates, exudates, litter, residues, and soils. Comments regarding potential benefits and limitations of these bioassays are provided. Chapter 5 provides an abridged version of the known effects, the physicochemical and biotic factors that modify effects, and the modes of action of allelopathic compounds using phenolic acids as the model compound. Chapter 6 describes a number of standard laboratory bioassays for identifying and characterizing the modes of action by which identified putative allelopathic compounds, mixtures of allelopathic compounds, mixtures of organic and/or inorganic compounds and residues may stimulate or inhibit sensitive weed species. Five different approaches will be described: (a) bioassays for simple mixtures of identified putative allelopathic compounds, (b) bioassays for residue leachates plus or minus XAD-4 resin, (c) the application of regression analysis to data from residues and soil extract bioassays, (d) bioassays for determining the role of treatment surface area, and (e) using omics methods as tools to determine modes of action. Chapter 7 compares field systems with past and present laboratory bioassay systems, provides some thoughts on ways to minimize the impacts of atypical factors in seed and seedlings laboratory bioassays, points out which factors limit our ability to design field-relevant model systems, suggests future directions for laboratory and field research on plant-plant allelopathic interactions in a question format, and outlines the central tenets (i.e., opinions, doctrines, or principles) articulated in this volume.

Finally, this volume has been written specifically for researchers and their graduate students who are interested in studying plant-plant allelopathic interactions. The author hopes that this retrospective and at times critical analysis of past standard laboratory bioassays will provide a foundation for better and more field-relevant laboratory designs in the future.

Reference

Blum U (2011) Plant-plant allelopathic interactions. Phenolic acids, cover crops, and weed emergence. Springer Science and Business Media, Dordrecht

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Reference

Blum U (2011) Plant-plant allelopathic interactions: Phenolic acids, cover crops, and weed emergence. Springer Science and Business Media, Dordrecht.

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Abbreviations

AIW	Average individual weight
AM	Arbuscular mycorrhizae
ATP	Adenosine triphosphate
BOA	Benzoxazolin-2-one
CFU	Colony-forming units
C/N	Carbon/Nitrogen ratio
DIBOA	2,4 dihydroxy-1,4-benzoxazin-3(4H)-one
ECM	Ectomycorrhizae
ED	Effective dose
EDTA	Ethylenediamine tetraacetic acid
FA	Ferulic acid
F-C	Folin-Ciocalteau
FER	Ferulic acid
GB	Glass beads
IPA compound	Identified putative allelopathic compound
MES	2(N-morpholino)-ethanesulfonic acid
NMR	Nuclear magnetic resonance
NPK	N, NO_3, NH_4, P and K unless otherwise indicated
PCO	*p*-Coumaric acid
*p*C-M	*p*-Coumaric acid and Methionine
*p*C-G	*p*-Coumaric acid and Glucose
PPFD	Photosynthetic Photon Flux Density
PVP	Polyvinylporrolidone
PVPP	Polyvinylpolyporrolidone
R	Reference
VA	Vanillic acid
XAD or XAD-4 resin	Amberlite XAD polymeric absorbent

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Chapter 1
Background for Designing Laboratory Bioassays

Abstract This chapter provides a general introduction to this volume, discusses the nature of plant-plant allelopathic interactions, describes the nature and sources of allelopathic compounds in soils, discusses the concepts of holism and reductionism as they relate to laboratory bioassays, provides a listing of benefits, limits, and common pit falls (e.g., false assumptions and misconceptions) for laboratory bioassays, and answers or sets the stage for answering the following questions: (a) Why is it important to design laboratory bioassays that are more holistic or stated in a more pragmatic way more relevant to field environments? (b) What can be done to make laboratory bioassays more relevant to field environments? and (c) Is it always necessary to make laboratory bioassays relevant to field environments?

1.1 Introduction

How plants interact with each other in a positive or negative manner has been of considerable interest to societies as a whole and to foresters, crop and weed scientists, horticulturists, and ecologists in particular. Plant interactions, for example, can influence or at times determine plant community diversity, structure, productivity, reproduction, succession, the establishment and survival of invading or introduced plant species within a community, the management practices of crop and forest production systems, the types of crop rotations and cover crops utilized by farmers, the species combinations used in multi-cropping systems, and the traits selected for or introduced into commercial crop and tree species through breeding and genetic engineering (for examples see Harper 1977; Rice 1984, 1995; Grace and Tilman 1990; Pyke and Archer 1991; Hierro and Callaway 2003; Macías et al. 2007b; Gómez-Aparicio 2009; Thorpe et al. 2011). Characterizing and understanding how plants interact with each other within natural and managed ecosystems are thus important both from a theoretical and practical (or applied) standpoint. The primary tools utilized to characterize and understand the modes of action or mechanisms by which plants interact with each other have been field observations, field and laboratory bioassays (e.g., germination, growth, and reproduction), and the quantification of cellular and plant processes (e.g., nutrient uptake, enzyme activity, synthesis and changes of metabolites, water relations, respiration, and photosynthesis). In the last decade or so, a range of molecular tools such as genomics, proteomics,

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metabolomics, and physionomics have been developed and added to our arsenal for identifying primary sites and modes of action of stressors including allelopathic compounds (Summer et al. 2003; Dunn and Ellis 2005; Grossmann 2005; Macías et al. 2007b; Duke et al. 2008, 2013; Wallenstein and Weintraub 2008; Chen et al. 2009; Metz 2011; Grossmann et al. 2012; also see Sect. 6.6). So far among these tools, metabolomics (metabolic finger printing and profiling) has been found to be particularly useful and has been very effectively utilized to indentify phenotypic responses at the molecular level for a range of stresses including allelopathic compounds (Gidman et al. 2003; Johnson et al. 2003; Grossmann 2005; Leão et al. 2009; Bertran et al. 2010; Sardans et al. 2011; Grossmann et al. 2012; D'Abrosca et al. 2013; Marti et al. 2013; see Sect. 6.6).

Plants within natural and managed (field) systems interact in a variety of ways by:

a. physical force or connections (e.g., negative: strangulation by strangler figs or attachments by parasitic plants, and positive or negative: connections by root graphs and hyphal strands of mycorrhizae),
b. competing for resources such as nutrients, water, light, pollinators, and space (negative: exploitative or resource competition), and
c. releasing chemicals into the environment (i.e., sending chemical messages) that directly or indirectly act as plant promoters, modifiers, or inhibitors (i.e., positive or negative allelopathic interactions).

How important each of these types of interactions may be in influencing or determining the structure, function, and management of natural and managed ecosystems has at times been very contentious among researchers particularly in regard to resource competition and allelopathy (see Harper 1975, 1977; Grace and Tilman 1990). For additional information and details regarding plant-plant interactions and their potential roles in natural and managed ecosystems the reader may wish to read several of the following: Graham and Bormann (1966), Harper (1977), Brownlee et al. (1983), Francis and Read (1984), Grace and Tilman (1990), Molisch (2001), Reigosa et al. (2006), and Smith and Read (2008).

This volume deals with one way in which plants interact, plant-plant allelopathic interactions. More specifically it deals with how to design and use seed and seedling laboratory bioassays to identify and characterize the potential processes and modes of action that bring about positive or negative chemical (i.e., allelopathic) interactions among plants. In a previous volume (Blum 2011) the author suggested that we could improve our understanding of plant-plant allelopathic interactions in the field by making laboratory bioassays more holistic. Reflections after the volume was published lead the author to conclude that a more detailed analysis of the factors making up laboratory bioassays was needed in the hope that such an analysis would provide clearer and more useful directions in how to design more holistic or more relevant laboratory bioassay systems. The more holistic being a theoretical goal and the more relevant being a more pragmatic goal. More specifically this volume presents a detailed discussion of the underlying features, issues, and suppositions associated with seed and seedling laboratory bioassays and continues the retrospective analysis of such bioassays begun in the previous volume. It is, however, broader

in scope and substance in that the information provided is relevant to all water-soluble compounds released to soil by putative allelopathic living plants and their litter and residues. It is ultimately an attempt to update and expand the practical guidelines for designing seed and seedling laboratory bioassays that have previously been provided in the literature with the hope that the designs in the future will become more relevant to field systems. For a history of and previous guidelines for studying plant-plant allelopathic interactions, the reader may wish to read several of the following: Willis (1985, 1994, 2007), Inderjit and Dakshini (1995), Blum (1999, 2007, 2011), Foy (1999), Inderjit and Weston (2000), Romeo (2000), Wu et al. (2001), and Weidenhamer (2008).

1.2 Allelopathic Interactions

As presently used in the literature, plant-plant allelopathic interactions are due to the actions of active organic compounds (i.e., allelopathic compounds) released into the atmosphere and/or the soil by living plants or decomposing recognizable plant litter and residues where these organic compounds may influence associated plants in a positive or negative manner. The focus in this volume, however, will be on the soil where these released organic compounds can be oxidized or reduced by chemical, microbial, or faunal processes before influencing associated plants in a positive or negative manner (see Willis 1985, 1994, 2007; Blum et al. 1999; Molisch 2001; Blum 2011). The positive or negative influences on plants growing in soil may either be due to direct actions (e.g., impact on root membranes or cell processes) or indirect actions (e.g., impact on symbiotic relationships, i.e., formation, development, and function of nodules or mycorrhizae) of allelopathic compounds. The observed stimulation or inhibition of allelopathic compounds in soils will, however, likely be a product of the action of all free and active organic and inorganic compounds in the soil that act individually or in combination as promoters, modifiers, or inhibitors depending on their individual concentrations and environments and the sensitivity of the receiving seeds, seedlings, or older plants. This observation is based on the following premises:

a. Any organic or inorganic compound can theoretically be stimulatory, neutral, or inhibitory depending on its concentration and physicochemical state. The type and pattern of effect observed will vary with the sensitivity of the plant process being observed.
b. The actions of individual organic and inorganic compounds within a mixture of compounds can be due to independent actions, similar joint actions (synergistic, additive, or antagonistic), or modifying actions. Independent actions apply to individual compounds within a mixture of compounds which do not influence the actions of other compounds present. Compounds with independent actions produce their effects on seeds, seedlings or older plants in different ways (i.e., impact different sites and have different modes of action). The independent

actions of more than one compound within a mixture can be described by a multiplicative model. Similar joint actions (synergistic, additive, or antagonistic interactions) apply to individual compounds within mixtures of compounds that influence each other's actions and that produce their effects on seeds, seedlings or older plants in the same way (i.e., impact the same sites and have identical modes of action). Modifying actions apply to individual compounds within mixtures of compounds that do not function as promoters or inhibitors (i.e., are neutral) but increase or decrease the effective concentrations of the active compounds. Similar joint actions and modifying actions can be described by joint action and modification models, respectively (Morse 1978; Gerig and Blum 1993).

c. The sensitivity of seeds, seedlings, and older plants are determined by their genetic makeup, their stage of development, presence or absence of symbiotic relationships, and their past and present physicochemical and biotic environments (Blum and Rice 1969; Rice 1984, 1986; Blum et al. 1989, 1993; Gerig et al. 1989; Gerig and Blum 1991, 1993; Pandey 1994; Pue et al. 1995; Blum 1996, 2006, 2011; Fujita and Kubo 2003; Belz et al. 2005, 2007; Duke et al. 2006; Belz 2008; Gianinazzi et al. 2010). Please note that throughout this volume the term symbiotic relationship refers to the mutual beneficial relationships associated with functional *Rhizobium* or *Bradyrhizobium* nodulated legume roots or mycorrhizae. The term mycorrhizae as used in this volume will include the whole complex of root and fungal zones (i.e., the endorhizosphere, the rhizoplane, and the rhizosphere) and the hyphal zone beyond the rhizosphere (i.e., the hyphosphere). For additional details regarding symbiotic relationships see Sect. 3.6 and 3.7.4.

Thus from an experimental standpoint, laboratory bioassays investigating plant-plant allelopathic interactions should focus not only on the putative active organic allelopathic compounds but also on the organic and inorganic modifiers present in the soil. Furthermore since observed allelopathic effects due to active organic compounds (inhibitors or promoters) are not only modified by associated organic and inorganic modifiers but also by other physicochemical and biotic factors of the environment, the roles of these environmental factors in determining the ultimate observed effects must also be understood. In the broadest sense the term modifier should, thus, include all the functional organic compounds (excluding allelopathic compounds although these can also act as modifiers), inorganic compounds, and physical, chemical, and biotic (e.g., microflora and fauna) factors that can or will directly or indirectly modify the actions of organic allelopathic compounds (see Einhellig 1989; Lehman and Blum 1997; Gawronska and Golisz 2006; Pedrol et al. 2006; Blum 2011). There are, of course, limits to the number and types of modifiers that can be included in any given laboratory bioassay. Thus, for example if one is interested in the activities or modes of action of specific types of allelopathic compounds, then laboratory bioassays utilizing specific identified putative allelopathic compounds or their mixtures are appropriate as long as the bioassay system and its environment are clearly described and it is acknowledged that the resulting

observations for such bioassays are limited to the experimental and environmental conditions of the bioassay, which may or may not be directly relevant to field systems.

1.3 Nature of Allelopathic Compounds

The reader may have noticed some ambiguity in the way the term allelopathic compounds has been used up to this point. This stems from the fact that potentially any given organic compound can function as a promoter, a modifier, an inhibitor, or as a neutral compound depending on a variety of factors (see premises Sect. 1.2). Therefore, in an absolute sense there is no such thing as a compound that is always an allelopathic compound. There are only organic compounds that are promoters or inhibitors under the appropriate conditions. Restated, under the appropriate conditions and at the appropriate concentrations, organic compounds function as allelopathic compounds or agents. Theoretically this is true but from a practical perspective, there clearly are organic compounds in nature which at very low concentrations readily affect (stimulate or inhibit) plant processes and there are other organic compounds that require very high or unnatural concentrations to affect plant processes. However, the majority of the organic compounds will likely affect plant processes somewhere between these two extremes. Thus, the author will continue to use the term allelopathic compounds because of convention and the convenience of the term although the reader should be clear as to what is meant when the term is used.

A large number of putative allelopathic compounds have been identified. Rice (1984) for example has categorized these into fourteen chemical groups plus a miscellaneous group:

a. simple water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes, and ketones,
b. simple unsaturated lactones,
c. long-chain fatty acids and polyacetylenes,
d. naphthoquinones, anthraquinones, and complex quinones,
e. simple phenols, benzoic acid, and derivatives,
f. cinnamic acid derivatives,
g. coumarins,
h. flavonoids,
i. hydrolysable and condensed tannins,
j. terpenoids and steroids
k. amino acids and polypeptides,
l. alkaloids and cyanohydrins,
m. sulfides and mustard oil glycosides,
n. purines and nucleosides, and
o. a miscellaneous group of compounds.

Compounds within these chemical groups were identified by laboratory bioassays of individual or simple mixtures of compounds, a selection process that is consistent with the herbicide paradigm (see Sect. 1.5). For specific compounds within these groups and their range of structures, characteristics, and actions, the reader may wish to take at look at several of the following publications: Rice (1984), Mondava (1985), Macías (1995), Macías et al. (1999, 2004b), Duke et al. (2002), Duke and Dayan (2006), and Seigler (2006). The range of chemical and physical structures and characteristics of specific identified putative allelopathic compounds within these groups is extremely complex with no obvious consistent recognizable pattern. Compounds identified have a wide range of molecular weights and structures (e.g., straight chain compounds, aromatic compounds with a range of side chains, compounds composed of isoprene or iso-pentane units linked together in various ways, compounds with amino groups, compounds composed of heterocyclic rings, etc.) that are hydrophilic or hydrophobic, volatile (many of these are also hydrophobic) or nonvolatile, positively and/or negatively charged, neutral, and acidic or basic just to name a few of their structures and characteristics.

Once released from plant tissues, litter, and residues, organic compounds can be oxidized or reduced by chemical, microbial, or faunal processes to produce various types of degradation products. A number of these degradation products have also been identified as putative allelopathic compounds (see Macías et al. 2007a). In addition, microorganisms can utilize organic compounds released from plants to synthesize new and unique compounds that can also function as allelopathic compounds (see Duke 1986; Strobel et al. 1987; Barazani and Friedman 1999; Demain and Fang 2000; Cutler et al. 2004).

Although there are numerous examples of the effects of identified putative allelopathic compounds on cellular and plant processes (Rice 1984; Putnam and Tang 1986; Inderjit et al. 1995, 1999b; Rice 1995; Macías et al. 2004a; Reigosa et al. 2006; Fujii and Hiradate 2007), the information regarding primary sites of action, uptake by roots and subsequent distribution to the rest of the plant, and whether the actions of putative allelopathic compounds are local or systemic is extremely limited (see Duke and Dayan 2006; Field et al. 2006; Loi et al. 2008; Dayan et al. 2009; Blum 2011; Weston et al. 2012; Radwan et al. 2013). It appears the action of simple phenolic acids such as ferulic acid (a water-soluble compound; Blum 2011) and sorgoleone (a hydrophobic compound; Dayan et al. 2009) for example are local (not systemic) suggesting that this may not be uncommon for identified putative allelopathic (IPA) compounds. Known examples of systemic action of IPA compounds are presently difficult to find although phytotoxins from *Fusarium virguliforme* (a fungal pathogen; Fv toxin) and *Leptospermum scoparium* (plant in the Myrtaceae; leptospermone) may be examples of compounds with systemic actions (Chen et al. 2010; Owens et al. 2013; Radwan 2013).

Actually the diversity of allelopathic compounds involved in plant-plant interactions turn out to be much more extensive than described by Rice (1984). Present indications are that with the exception of a few highly toxic compounds, mixtures of a broad range of organic compounds at low concentrations and low activities acting as promoter/modifier/inhibitor complexes appear to be the primary driving force of

plant-plant allelopathic interactions within natural and managed ecosystems (also see Sect. 1.2).

Finally, since this volume highlights the actions of hydrophilic compounds, it seems appropriate here to distinguish hydrophilic compounds from hydrophobic and volatile compounds. A hydrophilic compound typically has an affinity for water, is usually charged or has polar side groups, and is capable of hydrogen bonding enabling it to more readily adsorb/absorb water and be dissolved in water or other polar solvents. Some hydrophilic compounds such as calloids do not dissolve. A hydrophobic compound typically is repelled by water, tends to be electrically neutral, and non-polar enabling it to more readily dissolve in neutral or non-polar solvents or molecular environments. Molecules can have both hydrophilic and hydrophobic sites. Fatty acids for example which are amphipathic have a hydrophilic head and a hydrophobic tail allowing them to dissolve in both polar (e.g., water) and non-polar solvents (e.g., oils). A volatile organic compound has a high vapor pressure, a low boiling point, tends to be hydrophobic, and evaporates or sublimates from liquids or solids at room temperature. The level of volatility for organic compounds varies from low to high. See the following references for additional details (Gauthier 1997; McNaught and Wilkinson 1997).

1.4 Sources of Allelopathic Compounds and Modifiers in Soils

Living plants are chemical factories that are surprisingly leaky (Börner 1960; Muller 1965; Tukey 1966, 1969; Kozel and Tukey 1968; del Moral and Muller 1969; Rovira 1969; Harley and Russell 1979; Tang and Young 1982; Rice 1984; Fisher et al. 1994; Bertin et al. 2003; Walker et al. 2003; Jones et al. 2004; Bais et al. 2006; Blum 2006; Toljander et al. 2007; Ens et al. 2009; Young and Bush 2009; Barto et al. 2011). Both organic and inorganic compounds are lost from living plants by active and passive processes to the soil (Fig. 1.1). Active processes include secretion by glands and hairs, secretion and exudation of high molecular compounds by cells particularly root cells, and the active transport of organic compounds across cell membranes (e.g., proton pumps, and cotransporters). Actually how and what types of organic compounds, particularly secondary plant products, are released from living cells is still not well understood. Recent findings, however, suggest that most secondary products which tend to be polar will require some type of transport mechanism (see Bertin et al. 2003; Grotewold 2004; Weston et al. 2012). Passive losses include exudation (e.g., diffusion) of low molecular weight compounds by cells, sloughing and lysis of cells, particularly root cells, abscission (i.e., shedding) of vegetative and reproductive tissues and organs, leaching of living, dying, or dead tissues such as leaves, stems, roots, flowers, and fruits by condensates or rain events, and loss of volatile compounds by living and dead vegetative and reproductive tissues. In addition for mycorrhizal roots active and passive uptake of organic and inorganic compounds by hyphal structures in the apoplast of the root cortex, the

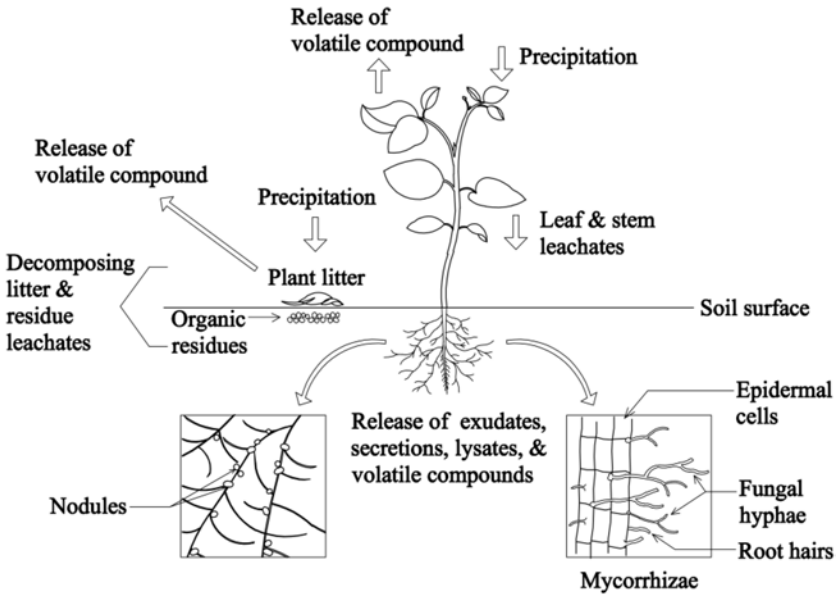

Fig. 1.1 Pathways by which organic and inorganic compounds are lost from plants. (Illustration by Amy Blum Grady, used with permission)

utilization, modification, and transport of some of these compounds by hyphae to the hyphosphere (see Sect. 3.7.4), and the subsequent active and passive losses of some of these compounds by hyphae within the hyphosphere to the soil represent additional pathways, although indirect, for plant roots. The organic and inorganic compounds lost from living plants, however, vary substantially depending on the source (e.g., species, ecotype, cultivar, health and acclimation of seedling or older plant, tissue type, presence or absence of symbiotic relationships, etc.), the solubility, volatility, and mobility of the compounds, the process (e.g., leaching, secretions, exudation, sloughing and lysis of cells, abscission, or volatilization), and the past and present physicochemical (e.g., rain events, soil type, nutrition, moisture, temperature, stress, etc.) and biotic (e.g., actions of soil organisms, presence or absence of symbiotic relationships, and pathogens) environments.

This appears to be a good place to stop and call attention to the fact that since the anatomy and morphology, chemistry, and functional behavior of roots with mycorrhizal fungi are more complex and diverse when compared to roots without mycorrhizal fungi the two (i.e., roots and mycorrhizae) should be recognized as functionally distinct entities, particularly when it comes to plant-plant allelopathic interactions (Lynch 1990; Jones et al. 2004, 2009; Blum 2006; Pinton et al. 2007; Smith and Read 2008; LF Grand 2012 Personal Communications; see Sect. 3.6 and 3.7.4). A similar argument can also be made for roots with and without nodules (Herridge and Pate 1977; see Sect. 3.6).

Finally the transfer of organic and inorganic compounds between different plants can also occur directly by way of mycorrhizal hyphae and root grafts. Transfer by mycorrhizal hyphae occurs in both herbaceous and woody species while transfer by root grafts occurs predominately in woody species (Graham and Bormann 1966; Brownlee et al. 1983; Francis and Read 1984). Such transfers would constitute either a loss or a gain of organic and inorganic compounds for a particular plant. For the potential implications of such transfers to plant-plant-allelopathic interactions see Sect. 3.6.

Volatilization, decomposition and leaching of plant litter and residues (litter particles) on and within the soil are another source of organic and inorganic compounds (Fig. 1.1; Börner 1960; Patrick 1971; Whitehead et al. 1979; Rice 1984; Buttery et al. 1985; Bradow and Connick 1990; Bradow 1991; Putnam 1994; Lavelle and Spain 2001; Blum 2004; Hadas et al. 2004; Blair et al. 2005; Xu et al. 2005; Xuan et al. 2005; Bonanomi et al. 2006; Young and Bush 2009). Losses from plant litter and residues will be determined by:

a. the nature of the species, ecotype, or cultivar,
b. the stage of development and type of living tissue at the time of mortality and abscission (e.g., immature vs. mature tissues, active vs. senescing tissues, woody vs. non-woody tissues, vegetative vs. reproductive tissues, or root vs. shoot tissues),
c. the state of the litter and residue (e.g., level of fragmentation, particle size, weathering, and decomposition),
d. the location of the litter and residues (e.g., standing upright, lying on the soil surface, or located within the soil),
e. the solubility, volatility, and mobility of compounds,
f. the past and present physicochemical environments (e.g., past and present rain events, soil moistures, soil pH, and temperatures), and
g. the past and present biotic environments (e.g., types of micro-, meso-, and macro-soil organisms that have acted on or are acting on the litter and residues).

Once in the soil, all the free (i.e., not sorbed or bound in the soil) organic and inorganic compounds released from living plants and plant litter and residues, in conjunction with the free organic and inorganic compounds already present in the soil and organic compounds produced by soil microorganisms can (Fig. 1.2):

a. be transported both vertically and horizontally by diffusion, capillary movement, and/or mass flow (e.g., gravitational flow or transpirational pull for solutions),
b. be reversibly and irreversibly sorbed to organic and inorganic soil particles,
c. be oxidized or reduced by physical and chemical processes,
d. be utilized, modified, and transported by a whole range of micro-, mesa-, and macro-soil organisms, and
e. interact with and be taken up by seeds, roots, and mycorrhizae (see Rice 1984; Hoagland and Williams 1985; Cheng 1989; Blum et al. 1999; Huang et al. 1999; Inderjit et al. 1999a; Lavelle and Spain 2001; Blum 2004, 2006; Jones et al.

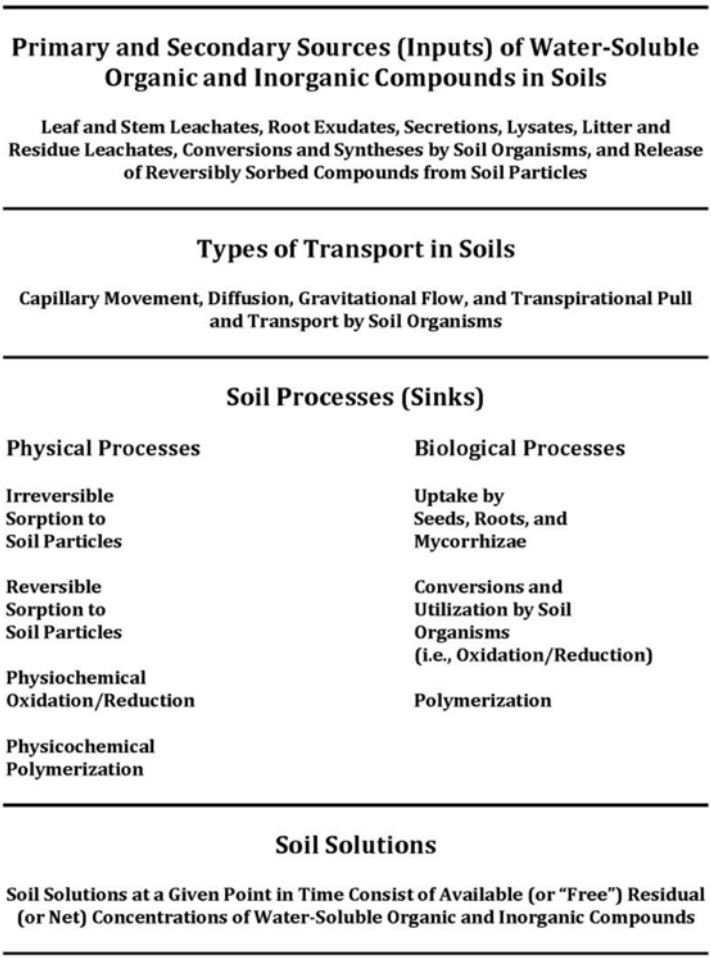

Fig. 1.2 Summary of sources, types of transport, and soil processes that determine the nature of soil solutions

2004, 2009; Tharayil et al. 2008). Uptake by roots and mycorrhizae can be an active or passive process.

The end result of all these processes (i.e., inputs, modifications, transport, and losses; Fig. 1.2) is that concentrations of free (e.g., in soil solution for water soluble or in soil air for volatile compounds) and reversibly sorbed allelopathic compounds and, for that matter, all free and reversibly sorbed organic and inorganic compounds within the soil are extremely dynamic. Restated, the quantitative and qualitative nature of organic and inorganic compounds in soils are not constant or static but are continually changing due to active plant, soil organism, and soil processes (e.g., inputs, transport, uptake, utilization, sorption, desorption, resorption, polymerization,

etc.). The dynamic nature of compounds and their interactions at the soil surface and within soil are, thus, an enormous challenge to the study of plant-plant allelopathic interactions since static or residual concentrations contained in leachates, root exudates, litter, residues, soil, and air samples are only part of the story. In fact, the turnover rates, fluxes, and flows of compounds (i.e., the source-sink relationships) into and within soil are very likely as important, or potentially much more important, to the final outcome of plant-plant allelopathic interactions as are the static or residual concentrations determined for living plant, litter, residue, and soil samples (Williamson and Weidenhamer 1990; Schmidt and Ley 1999; Blum 2011). Estimates of total available concentrations in soil will require data for both residual concentrations and source-sink transfers. However, ultimately what really matters is the total contact (or dose) of active promoters and inhibitors with sensitive seed, root, and mycorrhizal surfaces. Unfortunately determining absolute real-time doses in laboratory bioassays and in field systems are presently not possible and, thus, the past and present reliance of most researchers studying plant-plant allelopathic interactions on estimates (although frequently crude estimates) of identified putative available allelopathic compounds (free and reversibly sorbed compounds composed of active and inactive fractions).

The discussion and comments in this volume regarding laboratory bioassays will focus on water-soluble (hydrophilic) compounds that are released from living plants and plant litter and residues into soil. However, the actions of water-soluble compounds cannot be viewed or studied in isolation. Their actions must be placed into the proper context of their total physicochemical and biotic environments (see Blum 2011), and that may include, among others, the presence of hydrophobic compounds, volatile compounds (which frequently are also hydrophobic), and new and unique compounds synthesized by soil organisms. For example hydrophobic and volatile compounds will be or are very likely to be present when living tissues, litter, and residues are tested in laboratory bioassays (Bradow and Connick 1988a, b, 1990; Bradow 1991; Weidenhamer et al. 1994; Inderjit et al. 1997; Ens et al. 2009, 2010). Testing for the actions of hydrophobic and volatile compounds, however, requires a different set of tools and procedures than testing for the actions of water-soluble compounds and, thus, will not be covered in this volume.

1.5 Holism and Reductionism

Researchers studying plant-plant allelopathic interactions in the field are generally much more holistic or system oriented in their approaches than researchers working in the laboratory for obvious reasons. Researchers studying plant-plant allelopathic interactions in the laboratory are more reductionist or process oriented in their approaches also for obvious reasons, although the use of model systems in the laboratory is fairly common. The Merriam-Webster's Collegiate Dictionary (2013) defines holistic as:

> relating to or concerned with wholes or complete systems rather than the analysis of, treatment of, or dissection into parts,

holism as

> a theory that the universe and especially living nature is correctly seen in terms of interacting wholes (as living organisms) that are more than the mere sum of elementary particles,

and reductionism as:

> explanation of complex life-science processes and phenomena in terms of the laws of physics and chemistry; …. a procedure or theory that reduces complex data and phenomena to simple terms.

(Definitions used with permission of Merriam-Webster's Collegiate ® Dictionary, 11th Edition ©2013 by Merriam Webster, Inc. (www.Merriam-Webster.com)).

Both approaches to studying plant-plant allelopathic interactions, of course, have merit. Each side is merely addressing different aspects of the same problem. Unfortunately published laboratory research in the past and to some extent the present using a reductionist approach (i.e., cause and effect studies or dose-response studies; see Belz et al. 2005, 2007) consists to a large extent of one-factor bioassays. In essence this approach consists of studying the effects (y axis) of a range of concentrations (x axis) of putative individual allelopathic compounds or groups of similar compounds in the absence of other compounds found in leachates, root exudates, litter, etc. and in the absence of a whole range of physicochemical and biotic factors that directly or indirectly modify plant-plant allelopathic interactions. This approach to the study of plant-plant allelopathic interactions represents what has previously been referred to as the "herbicide paradigm" (Blum 2011). However, if we are to make progress in understanding when, where, and how plant-plant allelopathic interactions function in field systems a change in approach is necessary. We need to change from an approach where the emphasis is on the effects of individual or simple mixtures of putative allelopathic compounds under a given set of environmental conditions to a more systemic or more holistic approach where the effects and interactions of a range of physicochemical (e.g., pH, soil moisture, inorganic compounds, and organic compounds other than allelopathic compounds) and biotic (e.g., root and mycorrhizal surface area and soil and rhizosphere/rhizoplane microbes) factors are also included or accounted for in our laboratory studies. There are limits, of course, as to what can be included in laboratory bioassays. We cannot recreate holistic or complete field environments in the laboratory nor do we want to do that even if we could (see Sect. 1.5.1). What we can and need to do is to create simplified representative systems (i.e., model systems; Blum 2007) that hopefully function like field systems. To design such systems and make them more manageable, we need to know what must be included and what can be excluded from our model systems. That requires an understanding of how various physicochemical and biotic factors singly and in various combinations influence plant-plant allelopathic interactions. Fortunately we have made some progress in this area in recent years (see Einhellig 1987, 1989, 1999; Gawronska and Golisz 2006; Pedrol et al.

2006), particularly for one group of compounds, the phenolic acids. We know for example the following about phenolic acids.

a. Individual phenolic acids can be stimulatory, neutral, or inhibitory depending on their concentration and physicochemical state, the sensitivity of the species, and the process being observed (Blum et al. 1985b, 1989; Rice 1986; Pandey 1994; Lehman and Blum 1999b; Duke et al. 2006; Belz et al. 2005, 2007; Belz 2008; Blum 2011).
b. The effects of individual phenolic acids in mixtures of phenolic acids can be additive, partially antagonistic, or at times completely antagonistic depending on the concentrations of individual phenolic acids making up the mixture, the types of phenolic acids present, and the physicochemical environment (Blum et al. 1985a; Lyu et al. 1990; Li et al. 1993; Lehman et al. 1994; Blum 1996; Einhellig 1996; Inderjit et al. 2002). (Note: The present evidence for synergistic effects of phenolic acids within simple mixtures of phenolic acids is not very convincing (Inderjit et al. 2002; Belz et al. 2007)).
c. The presence of inhibiting and non-inhibiting organic compounds other than phenolic acids and inorganic compounds can influence the treatment concentrations required for a stimulatory or inhibitory effect (Hall et al. 1983; Blum et al. 1993; Pue et al. 1995; Inderjit et al. 1997).
d. Levels of inorganic compounds in the soil (e.g., anions such as nitrate and cations such as calcium and manganese) can modify the available free phenolic acids in the soil that can interact with seeds and roots (Blum et al. 1993; Dalton 1999; Ohno et al. 2002).
e. The inhibitory effects of phenolic acids in plant tissues appear to be influenced by the inorganic compounds (e.g., N, P, and K) within plant tissues (Hall et al. 1983).
f. Phenolic acid concentrations required for an effect on sensitive species are directly or indirectly determined by both past and present physicochemical and biotic environments (Glass 1976; Duke et al. 1983; Einhellig and Eckrich 1984; Einhellig 1987, 1996, 1999; Lehman and Blum 1999a; Blum 2004, 2006; Blum and Gerig 2005, 2006).
g. The effects of active phenolic acids are determined by the health, acclimation, and stage of development of a species (Waters and Blum 1987; Holappa and Blum 1991; Lehman and Blum 1999a).
h. The effects of phenolic acids are not systemic and as a result the effects increase or decrease depending on the proportion of the root system in contact with phenolic acids (Klein and Blum 1990; Lyu and Blum 1990; Lehman et al. 1994; Lehman and Blum 1999b; Blum 2006).
i. The source of and the amount of phenolic acids released to or produced within soil depends on a variety of factors including plant tissue, litter, and residue content, soil microbial conversion, utilization and synthesis, rates of decomposition, leaching, soil moisture, soil nutrition, etc. (see Sect. 1.4, 2.1.3.1, and 3.7). Production and content of phenolic acids within plants varies with species, tissue type and maturity, and the environment (see Rice 1984).

j. Phenolic acids in soils are readily utilized by soil microbes, fixed into recalcitrant organic matter or onto clays, transported in the soil by capillary movement and mass flow, and taken up by roots (see Blum 2004, 2006, 2011).
k. The effects of phenolic acids on plant processes are lost (i.e., seed, seedling, and older plant processes recover) as contact with phenolic acids is reduced due to removal, microbial utilization, soil fixation, root uptake, etc. (Blum and Dalton 1985; Blum et al. 1985a; Blum and Rebbeck 1989; Bergmark et al. 1992; Blum and Gerig 2005, 2006).

So what do these observations about phenolic acids tell us about plant-plant allelopathic interactions and laboratory bioassays in general? First, it should be evident that plant-plant allelopathic interactions are extremely complex, i.e., multifactor and multidimensional. Second, that one-factor laboratory bioassays (e.g., simple dose-response bioassays) provide only a very thin slice of the potential responses of sensitive seeds, seedlings, and older plants. Third, that more holistic or multifactorial bioassays increase the observed portion of seed, seedling, and older plant responses ranging from response surfaces to response clouds (multidimensional space) depending on the complexity of the experimental design. The use of factorial bioassays instead of one-factor bioassays is, thus, clearly an improvement that can/will lead to a better understanding of plant-plant allelopathic interactions in the field. Researchers should, therefore, be encouraged to be more holistic or, stated in another way, to implement relevant factorial bioassays wherever and whenever feasible. More specifically, bioassays should include a number of environmental factors, each factor being represented by several levels forming a treatment grid (Steel and Torrie 1997). In addition to providing greater insight about plant-plant allelopathic interactions in the field, such factorial bioassays are also particularly valuable in exploratory work where little is known concerning the optimum levels of factors or even which ones are important.

1.5.1 Why is It Important to Design Laboratory Bioassays that are More Holistic?

To a certain extent this question was answered in the previous section but restated in a more precise manner, more holistic bioassays (e.g., designing and using multifactorial bioassays instead of one-factor bioassays) provide researchers with a broader or more comprehensive perspective of the degree of response that may occur under a range of environmental conditions in the field. Depending on the physicochemical and biotic environments chosen, the response of sensitive species to individual or simple mixtures of putative allelopathic compounds, leachates, root exudates, litter, and residues in the laboratory will very likely range from stimulation to inhibition. In other words researchers actually have considerable control over the final outcome of laboratory bioassays whether they recognize it or not since they determine the physiochemical and biotic environments of their bioassays to a large extent. Thus, if any conclusions or generalizations about field systems are to be made from

the results of laboratory bioassays, those bioassays must more closely approximate the physicochemical and biotic environments of the field (i.e., be more holistic or more realistic).

On a more practical side, what does it really mean to make laboratory bioassays more holistic? It does not mean more holistic in just any random way but in a way that makes bioassays more relevant and consistent with the field environmental conditions under study. What we need to do is to create physicochemical and biotic environments in the laboratory that are better approximations of field environments. Secondly, we clearly do not wish to make laboratory bioassays completely holistic since that would mean we would have the same problems as for field bioassays in determining cause and effect (Blum 2011). The object is to make them more holistic than they presently are! Some may say that more holistic is simply a euphemism for making laboratory bioassays more complex. That is not the intent. Indeed making laboratory bioassays more holistic will make them more complex. However if the reader prefers, there is nothing wrong with substituting the words more complex for more holistic or more relevant since to be forthright when it comes to identifying and characterizing processes, responses, and functions of living plants in the field, an understanding of the complex nature of plants and the range of environments under which they exist is essential. In other words the answers to our questions regarding field plant-plant allelopathic interactions lie somewhere among the complex actions and interactions that occur within plant-microflora-fauna-soil systems.

1.5.2 What can be Done to Make Laboratory Bioassays More Relevant to Field Environments?

Since we cannot recreate field systems and their environments in the laboratory, our only option is to make laboratory bioassay systems as relevant as possible to field systems. We can do that by:

a. minimizing, or better yet eliminating, the problematic factors, and features of laboratory bioassays described in Chap. 3 (e.g., absence of key modifiers, absence of symbiotic relationships, atypical microbial populations and densities of bioassay species, etc.),
b. avoiding the common pitfalls described in Sect. 1.7,
c. identifying and using relevant functional laboratory model systems,
d. developing better tools and procedures to identify, characterize, and quantify crucial sources, pathways, processes, and environmental factors,
e. determining when, how, and at what rates and concentrations organic compounds (e.g., promoters, modifiers, and inhibitors) are released from putative allelopathic living plants, litter, and residues,
f. determining how the actions of promoters and inhibitors are influenced by each other and by other modifiers (see Sect. 1.2) present in soils. and

g. determining under what types of physicochemical and biotic environments organic compounds contact and/or are taken up by seeds, roots, and mycorrhizae and function as promoters or inhibitors.

1.5.3 Is It Always Necessary to Make Laboratory Bioassays Relevant to Field Environments?

Not really, since laboratory bioassays have a number of other functions besides providing potential evidence for or against plant-plant allelopathic interactions in the field (see Sect. 1.6). However no matter their function, bioassays should whenever and wherever possible be designed to be as relevant as possible to field systems.

1.6 Benefits and Limits of Laboratory Bioassay

Natural and managed field systems are extremely complex and, thus, the action of individual factors on plant growth and development in the field are difficult, if not impossible, to identify and characterize. Stimulation or inhibition of plant growth for example could be due to any number of physicochemical (e.g., temperature, water, pH, soil type, and organic and inorganic compounds) and biotic (e.g., pathogens, symbionts, soil organisms, and competitors) factors. Thus, researchers studying plant-plant interactions in the field long ago recognized that insight regarding causation required the isolation and testing of individual potential causative factors. This can best be done under laboratory conditions where individual factors can be studied and where some factors can be held constant while others are modified. This approach, however, comes at a cost. The cost, an incomplete or limited understanding of how interactions between all the factors present in the field can/will modify the action of any targeted factor under study in the laboratory. This also means that any conclusions regarding causation derived from laboratory bioassays are at best tentative and very limited in scope and scale. Restated, laboratory bioassays suggest potential modes of action occurring in the field, not the actual day to day modes of action that are operating in the field. In fact, the simpler (e.g., more artificial) and the more managed or manipulated the test species, substrate and environment of a bioassay system in the laboratory, the more tentative the conclusions that can be drawn about causation in field systems. Unfortunately there are few, if any, other options. Thus laboratory bioassays continue to be one important way of identifying and characterizing the stimulation or inhibition of plant products from putative allelopathic plants and determining how this stimulation or inhibition may be modified by the action of various physicochemical and biotic factors (Einhellig 1989; Blum 1995, 1999, 2011; Inderjit and Dakshini 1995; Romeo and Weidenhamer 1998; Wu et al. 2001; Inderjit and Nilsen 2003).

A quick perusal of the literature will indicate that many, if not most, laboratory bioassays can be classified as dose-response studies. They are an attempt to determine the effective concentrations of identified putative allelopathic (IPA) compounds, leachates, root exudates, and plant tissues, litter, and residue (i.e., test materials). On occasion one finds single concentration studies (i.e., plus or minus) but for all intents and purposes these are of very limited value. After all, most researchers are looking not only for the biologically effective ranges of their test materials but also for their minimum effective concentrations and that cannot be achieved unless an appropriate range of concentrations and environments are tested.

When appropriate ranges of concentrations and environments are tested, dose-response bioassays can provide considerable insight regarding:

a. the potential activities (e.g., stimulation [facilitation] or inhibition [phytotoxicity]) of IPA compounds, leachates, root exudates, and plant tissues, litter, and residues under laboratory conditions,
b. the modes of action for individual IPA compounds or mixtures of IPA compounds under laboratory conditions,
c. how potential stimulation or inhibition of IPA compounds or mixtures of IPA compounds at various concentrations may vary with species, plant health, acclimation, presence or absence of symbiotic relationships, and growth environment, etc. under laboratory conditions or more specifically the range of variation, response times, and change in magnitude as modified by previous, present, and potential future environments, and
d. the roles and interactions of various physicochemical and biotic processes in the soil and atmosphere on the action of IPA compounds, leachates, root exudates, and plant tissues, litter, and residues.

Therefore, laboratory bioassays are simply one form of hypothesis testing (Blum 2007). Their predictive powers are restricted to the range of experimental and environmental conditions of a given bioassay. Thus, causation (i.e., modes of action) and processes operating in field soils can only be inferred or hypothesized from laboratory bioassays. In other words in an absolute sense laboratory bioassays, no matter how sophisticated, can never prove that a particular organic compound or group of compounds is/are functional allelopathic agents under field conditions. Field systems are much too complex for that. What laboratory bioassays can do, if done correctly, is to test alternative hypotheses, suggest modifications of old hypotheses, and suggest new hypotheses for testing. In the process considerable insight can be gained about potential effects (i.e., stimulation or inhibition), interactions and modes of action of environmental factors including IPA compounds. More importantly, laboratory bioassays can provide insight about how effects of putative allelopathic compounds can be modified by the actions and interactions of chemical and physical processes associated with plants, microbes, and soils. Having such insight will help us:

a. to place the data from our bioassays of IPA compounds, leachates, root exudates, tissues, litter, and residues in the proper context of the field environment,

b. to understand how ecosystem factors and processes influence plant-plant allelopathic interactions, and
c. to determine how ecosystem factors and processes may be manipulated and managed to maximize or minimize plant-plant allelopathic interactions.

The bottom line is that the most useful function of laboratory bioassays is to identify and characterize modes of action and processes that potentially determine and regulate plant-plant allelopathic interactions in the field. They are not useful in determining the actual day to day presence, magnitude, or absence of plant-plant allelopathic interaction in the field.

1.7 False Assumptions and Misconceptions for Laboratory Bioassays

Common pitfalls can lead to false assumptions and misconceptions regarding the insight provided by laboratory bioassays. Pitfalls are defined as unapparent (not easily recognized) sources of trouble, danger or hidden hazards (Merriam-Webster's Collegiate Dictionary 2013). In this context the author would define them as unapparent or unrecognized flaws or deficiencies in experimental designs and procedures and false underlying assumptions and misconceptions about causes and effects that have lead inadvertently to erroneous conclusions and generalizations about plant-plant allelopathic interactions. Such unintentional erroneous conclusions and generalizations have not been uncommon in the literature. Unfortunately false assumptions and misconceptions are not always clearly evident but frequently buried in the experimental designs and procedures chosen. What follows are some examples of false assumptions and misconceptions. Some may seem trivial or intuitively obvious but even those will aid researchers in designing more relevant bioassays in the future.

a. That specific organic compounds can be definitively designated as either promoters, neutrals, or inhibitors when, in fact, all organic compounds can theoretically be stimulatory, neutral, or inhibitory depending on their concentrations, physicochemical states, and the sensitivity of the species process being monitored (Belz et al. 2005, 2007; Duke and Dayan 2006; Zanardo et al. 2009; Ghareib et al. 2010; also see Sect. 5.2.1 and 5.2.2).
b. That allelopathic interactions are purely the result of a single or a small subset of organic compounds in the soil (i.e., the herbicide perspective; Blum (2011)) when, in fact, they are a product of the action of a complex set of organic and inorganic promoters, modifiers, and inhibitors as well as a range of environmental factors (Einhellig 1989, 1999; Blum 1996, 2011; Gawronska and Golisz 2006; Pedrol et al. 2006). For additional references see Sect. 3.2.2 and 5.2.3 for the roles of modifying factors and Sect. 5.3 for the roles of individual compounds in organic mixtures.

c. That the stimulation and inhibition of germination or early seedling growth observed in laboratory bioassays for leachates, root exudates, tissues, litter, or residues are primarily due to organic compounds when, in fact, they can be due to actions and interactions of other factors (e.g., effects of pH, water potentials, nutrition, microbial activity, anaerobic conditions, etc.) when leachates, root exudates, tissues, litter, or residues are added to bioassay systems (see Hall et al. 1983; Blum et al. 1992; Lehman and Blum 1997; Blum 1999, 2011; also see Sect. 5.2.3, 5.3, 5.4, and 6.3).
d. That the responses of seeds or seedlings to identified putative allelopathic (IPA) compounds (or any other stressor) are somehow indicative of responses of mature or reproductive stages of plants. They are not (Blum and Heck 1980; Waters and Blum 1987).
e. That the anatomy and morphology of roots and the morphology of root systems grown in different media will be identical and have identical sensitivities to IPA compounds when in fact they are very likely to be different (Carson 1974; Taylor 1974; Bennie 1996; Waisel et al. 1996; Blum 2006).
f. That seedlings or plants with or without root nodules or mycorrhizae behave in an identical manner to individual IPA compounds, simple mixtures of IPA compounds or IPA compounds imbedded in complex mixtures. This is unlikely to be the case (Lynch 1990; Jones et al. 2004, 2009; Blum 2006; Pinton et al. 2007; Smith and Read 2008; see Sect. 3.6).
g. That the organic and inorganic compounds in extracts of living tissues, litter, and residues are representative of what is released from living tissues, litter, and residues in the field when, in fact, organic and inorganic compounds lost from living tissues, litter, and residues over time are very different from those obtained by extraction of living tissues, litter, and residues (see Wilson and Rice 1968; Murthy and Shihora 1977; Gallardo-Williams et al. 2002; Andreasson et al. 2009; Sect. 2.1.3.2).
h. That the free and reversibly bound organic and inorganic compounds recovered/extracted from field soils represent the available and active concentrations in field soils when they constitute only the residual or net concentrations within the soil, not the total that is potentially available to interact with seeds, roots, and mycorrhizae (see Blum et al. 1992; Dalton 1999; Blum 2004, 2006, 2011; Sect. 2.1.3.3).
i. That plants grown under light banks, in growth chambers, in greenhouses, or in the out-of-doors have similar organic and inorganic content, active and passive losses of organic and inorganic compounds, morphology (e.g., leaf shape, thickness, and size, cuticle thickness, root/shoot ratios, etc.), growth rates, development, and sensitivity to IPA compounds. They do not (Koeppe et al. 1969, 1970; Bates 1971; Raper and Downs 1976; Hall et al. 1982, 1983; Rice 1984; Drossopoulos et al. 1996; Chaves et al. 1997; Chaves and Escudero 1999; Zhao and Oosterhuis 1999; also see e. and f. above and Sect. 7.2).
j. That treating whole root or mycorrhizal systems with IPA compounds is all that is necessary to demonstrate the effectiveness of such compounds. This may be true for systemic compounds but clearly is not true for non-systemic compounds

(Lyu and Blum 1990; Klein and Blum 1990; Lehman et al. 1994; see Sect. 6.4 and 7.2.4).

k. That there is considerable lee-way as to how and when plant leachates, root exudates, tissues, litter, residues, and soil solutions are collected, processed, and stored when, in fact, it makes an enormous difference as to what is recovered and the subsequent composition of what has been recovered (see Sect. 2.1.3.2).

l. That there is considerable lee-way as to how IPA compounds, leachates, root exudates, tissues, litter, residues, and soil solutions are applied to bioassay systems when, in fact, it makes enormous difference in the end result (see Sect. 2.1.3.4 and 3.3).

m. That there is considerable lee-way in the density of sensitive species that can be used in bioassays and that the presence or absence of non-sensitive species in bioassays is not all that important. This is not true. For example, total root density of sensitive and non-sensitive species determines the number of molecules that can interact with a given segment of root and non-sensitive species release organic compounds that modify the root environment of the sensitive species (see Sect. 2.1.1.5, 3.2.2.6, 6.5, 7.2.4, and 7.2.7).

n. That there is considerable lee-way in when and what is measured to determine primary and secondary allelopathic effects. Not true. Timing is everything because immediately after primary effects occur there is rapid development of secondary, tertiary, etc. effects. In addition most plants can acclimate fairly rapidly to and partially or wholly compensate for changing environmental conditions (see Sect. 2.1.4 and 3.11).

o. That bioassay systems without the addition of IPA compounds, leachates, root exudates, tissues, litter, or residues represent legitimate controls for bioassay systems treated with such test materials. Non-treated systems cannot function as true controls unless additions of IPA compounds, leachates, root exudates, tissues, litter, and residues do not modify the overall physicochemical and biotic environments of the bioassay system. The likelihood of that is extremely small since the addition of such test materials will not only modify organic and inorganic compounds but will also modify a range of other factors (e.g., pH, aeration, water potential, microbial populations, etc.). Non-treated systems, however, can provide artificial baselines or references for comparisons (see Sect. 3.10).

p. That physicochemical and biotic environments created under laboratory conditions resemble or function as field environments when, in fact, they are highly simplified and modified environments (Blum 2007, 2011; Sect. 7.2).

q. That effective concentrations ranging from minimum to maximum for stimulation or inhibition by individual organic IPA compounds or by simple mixtures of organic IPA compounds determined in laboratory bioassays are directly relevant to field environments. Effective concentrations for a susceptible species actually increase or decrease as the physicochemical and biotic environments and the development, acclimation, and health of susceptible species change. Thus, actual effective concentrations ranging from minimum to maximum are moving targets (see Sect. 3.2.1). (Note: Effective concentrations of individual or simple mixtures of IPA compounds will also change with the increasing or decreasing

chemical and physical complexity of the treatment solutions or materials (see b. and c. above)).

r. That direct parallels can be drawn between results from laboratory bioassays and the field (see Sect. 7.2). Laboratory bioassays can assist in identifying and characterizing potential modes of action and processes but cannot predict what is happening on a day to day basis in the field (see Sect. 1.5 and 1.6).

s. That the role of microorganisms in bioassays can be disregarded or marginalized (see Sect. 3.6 and 3.7).

How to avoid some of these common pitfalls when designing laboratory bioassays is a primary focus of the remaining chapters.

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Chapter 2
Introduction to the Fundamentals of Laboratory Bioassays

Abstract This chapter describes and provides comments on the following basic features of laboratory bioassays: (a) biotic and physicochemical factors, (b) test materials, (c) measurements, hypotheses, experimental designs, and data analyses, and (d) basic information that should be provided by researchers for all bioassays.

2.1 Factors of Bioassay Systems

2.1.1 Biotic Factors

2.1.1.1 Bioassay Species

The species to be used in a bioassay really depends on the objective of the study. For example, if one is interested in understanding basic modes of action and processes of plant-plant allelopathic interactions, choosing sensitive species such as lettuce, tomato, clover, or cucumber as model plants may be quite appropriate. However, if the primary objective is to determine if seedlings of species A are stimulated or inhibited by allelopathic compounds released from mature plants of species B (putative promoters or aggressors) then the use of lettuce, tomato, clover, or cucumber seedlings by themselves as bioassay species is not appropriate. In this instance seedlings of species A should be the primary bioassay species being tested. The inclusion of frequently used sensitive species in addition to species A, however, is acceptable and in fact useful since these sensitive species can serve as a baseline for determining the relative sensitivity/resistance of species A and as a means of comparing results from the present bioassay to those of previously published bioassays as well as those that may be published in the future.

2.1.1.2 Stage of Life Cycle for Bioassay Species

The most vulnerable parts of a higher plant life cycle to environmental stressors, including allelopathic compounds individually or imbedded in complex mixtures of organic and inorganic compounds, (e.g., leachates, root exudates, litter, etc.) are likely to be germination, seedling emergence, early seedling development, and plant

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reproduction (Blum and Heck 1980; Waters and Blum 1987). However, since plant species adjust (i.e., acclimate) to stresses differently due to genetic variation, stage of development, presence or absence of symbiotic relationships, and past and present environments, broad generalizations about sensitive life cycle stages (i.e., the most or least sensitive) must be viewed with some caution and skepticism (Levitt 1972; Cox and Conran 1996; Kozlowski and Pallardy 2002; Marshall et al. 2005). Thus, the responses of all the appropriate stages of the life cycle of a plant to allelopathic compounds must be studied. However, since growing seedlings to mature or reproductive stages is frequently time consuming and unwieldy under laboratory conditions, most researchers studying allelopathic interactions in the laboratory have chosen to utilize changes in germination or seedling physiology and growth as their main response indicators. In spite of the convenience of using seeds and seedlings in bioassays, it is important to recognize that responses of seeds and seedlings to allelopathic compounds are not necessarily (in fact unlikely to be) representative of (i.e., cannot be extrapolated to) the responses of mature or reproductive stages of plants.

The essential point is that the stage of the life cycle of a species used in a bioassay depends on the experimental objectives. For example, if the primary interest is germination or seedling emergence, then there is no need to study other parts of the life cycle. However, using germination or seedling emergence as an indicator of seedling or older plant responses is not appropriate.

2.1.1.3 Symbiotic Relationships

There are two structures resulting from microbial-plant symbiotic relationships that are of particular interest when it comes to laboratory bioassays, legume nodules and mycorrhizae (Fig. 2.1). Clearly when appropriate, roots should be inoculated with *Rhizobium*, *Bradyrhizobium* or mycorrhizal fungi. The presence of nodules and mycorrhizae will in most instances dramatically change the response of seedlings or older plants to allelopathic compounds (see Sects. 3.6 and 3.7). However, since:

a. it takes approximately a week or more to develop functioning nodules and mycorrhizae and then an additional 4–9 weeks, depending on species and environment, for the extensive formation of nodules and the colonization of roots by mycorrhizal fungi (see Sect. 3.6),
b. the target organisms are frequently seeds or very young seedlings, and
c. most laboratory bioassays studying the effects of identified putative allelopathic (IPA) compounds last no more than a week or so, it could be argued that the inclusion of inoculums for the induction and development of nodules and mycorrhizae is not necessary.

In fact from a reductionist perspective, the inclusion of inoculums for the formation of nodules and mycorrhizae can at times make it much more difficult to sort out cause and effect. From a holistic perspective such inoculums should be included even when full development of the symbiotic relationships will not occur during

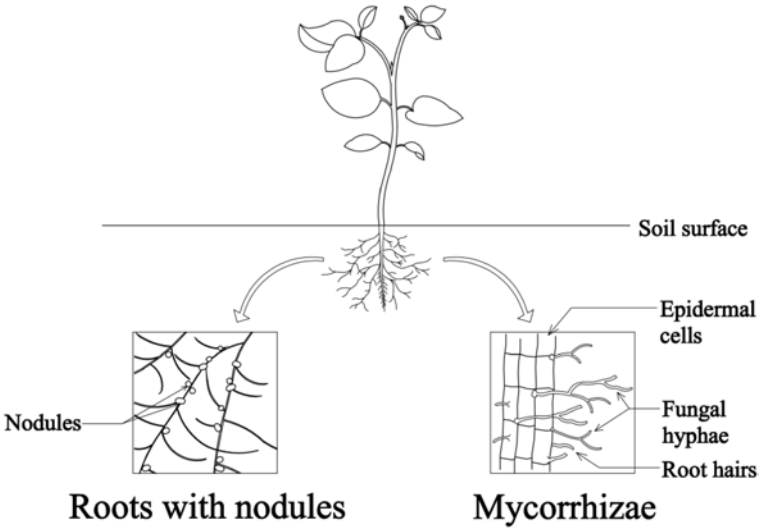

Fig. 2.1 Two symbiotic relationships, nodules and mycorrhizae. (Illustration by Amy Blum Grady, used with permission)

the time of the bioassay. After all there is considerable evidence that the presence of microbes, no matter their source, can and will modify and regulate, sometimes very dramatically, the observed effects of individual IPA compounds or IPA compounds imbedded in complex mixtures of organic and inorganic compounds (see Sect. 3.7). Furthermore, the absence of microorganisms involved in the formation of nodules and mycorrhizae will also prevent the detection of effects of allelopathic compounds on the induction, formation, and development of symbiotic relationships, important relationships that influence and regulate how seedlings or older plants respond to allelopathic compounds (see Sects. 3.6 and 3.7).

2.1.1.4 Treatment Surface Areas

The total or potential treatment surface area within a bioassay system is determined by seed, seedling, or older plant density, root initiation, rates of root growth, and the formation and development of mycorrhizae. The effective treatment surface area of a species is the proportion of the total surface area that actual interacts with allelopathic compounds. The effective surface area within a medium is determined by the distribution of the total surface area in relationship to the distribution of allelopathic compounds. In solution culture the total and effective surface areas are identical. In soil culture total and effective surface areas are not necessarily, in fact unlikely, to be identical.

Seed, seedling, or older plant effective treatment surface areas in bioassays may not be a major concern for systemic allelopathic compounds since the whole organism will be impacted even if only a small proportion of the surface area is contacted

by such compounds. However, one might expect that as the effective treatment surface area is increased, the speed of the response by a seed, seedling, or older plant to allelopathic compounds would increase at least initially.

For non-systemic allelopathic compounds the effects are local and, thus, the proportion of the total surface area in contact with such compounds will determine the ultimate observed effects both in speed and magnitude of response. Since most seeds and their initial radicles have such small surface areas, the proportion of their surface area contacted by allelopathic compounds are unlikely to be a major concern for even non-systemic allelopathic compounds. That, however, is not the case for seedlings and older plants (see Sect. 6.5) since a link between effective treatment surface area and seedling responses has been observed by Lyu and Blum (1990); Klein and Blum (1990); and Lehman et al. (1994). These authors found that for a given treatment concentration, inhibition increased as the proportion of the effective root treatment surface area was increased (see Sects. 5.2.3.1 and 6.5 for details).

Unfortunately most laboratory bioassays of seedlings or older plants ignore the potential variation in contact between allelopathic compounds and surface areas of roots and mycorrhizae. In fact, bioassays are frequently designed so that there is a high probability that the entire surface area of the roots and mycorrhizae are in direct contact with IPA compounds or sources. Data from such bioassays represent a worst-case scenario, a scenario that may or may not be consistent with what has occurred or is occurring in the field. The author suspects that in most instances, particularly for older seedlings and older plants, treating whole root and mycorrhizal systems is at odds with what occurs in the field. See Sects. 5.2.3.1 and 6.5 for ways to deal with this issue in laboratory bioassays.

2.1.1.5 Density

A quick perusal of the early literature will indicate that the densities of seeds and seedlings used in bioassays were extremely variable and frequently arbitrary; not until recently have researchers paid much attention to this factor of bioassays. The density used in a bioassay will, of course, depend on the experimental objectives but every effort should be made to use relevant densities or the artificial nature of the density used should be accounted for in the interpretation of the resulting data. The role of density in seed or seedling bioassays should not be underestimated since density can influences/determine the magnitude of effects of allelopathic compounds, the level of autotoxicity in some species, the total and effective treatment surface area of a bioassay species, and the levels of resource competition (e.g., Wilson and Rice 1968; Weidenhamer et al. 1987, 1989; Lyu and Blum 1990; Sinkkonen 2001, 2003, 2007; Huang et al. 2013; see Sects. 2.1.1.4, 3.2.2.6, and 3.5).

2.1.1.6 Microorganisms

The fact is that the populations of microorganisms present in laboratory bioassay systems will not be representative of the populations of microorganisms found in field systems. In spite of that the roles of microorganisms in laboratory bioassay

systems should not be underestimated (see Sparling and Vaughan 1981; Vaughan et al. 1983; Vaughan and Malcolm 1985; Heisey et al. 1985; Hoagland and Williams 1985; Siqueira et al. 1991; Schmidt 1991; Schmidt and Ley 1999; Barazani and Friedman 1999; Inderjit 2005; Blum 2006; Zhang et al. 2009). Their roles within bioassay systems must be identified, characterized, quantified, and understood, and when possible, controlled or regulated. Unfortunately, researchers have only limited options to control or regulate microbial populations within bioassay systems. The following are the only options:

a. Eliminate the microbial populations for part or all of the components of a bioassay system by sterilization (Shay and Hale 1973; Přikryl and Vančura 1980; Dalton et al. 1983; Vaughan et al. 1983; Vaughan and Malcolm 1985; Blum et al. 1994, 1999; Pue et al. 1995; Wu et al. 2000a, b; Blum 2004; Kaur and Singh 2007).
b. Inoculate bioassay systems that have been sterilized with cultures of individual microbial species, subsets of microbial species, or complex mixtures of microbial species such as soil extracts (Vaughan et al. 1983; Kosslak and Bohlool 1984; Vaughan and Malcolm 1985; Pue et al. 1995; Blum 2004; Kaur and Singh 2007; Zhang et al. 2009).
c. Do nothing and live with whatever microbial populations are present within a bioassay system.
d. The use of antibiotics, inhibitors, etc. to control or regulate microorganisms are usually not recommended unless their use is a specific part of an experimental design since such substances will not only lead to differential selection, amplification, and suppression of specific microbial species but can also function as promoters, modifiers, or inhibitors of bioassay species. However, the use of such compounds in selective media to enumerate populations of specific types of microorganisms (e.g., actinomyces, fungi, phosphatase-positive bacteria, gram-negative bacteria, phenolic acid utilizing microorganisms, etc.) can be very useful (see Blum and Shafer 1988; Shafer and Blum 1991).

When microorganisms are present in bioassay systems, the disparity between laboratory bioassay systems and field systems becomes even greater when laboratory bioassay systems are treated with IPA compounds, leachates, root exudates, extracts, or litter (see Sect. 3.2.2.8). Such treatments lead to intense modifications of microbial populations by differential selection, amplification, and suppression of specific microbial species (Blum and Shafer 1988; Shafer and Blum 1991; Blum et al. 1993; Pue et al. 1995; Blum 2006, 2011; see Sects. 3.6 and 3.7). The initial and induced differences in the nature and associated functions of microbial populations for laboratory systems are thus a major conundrum that desperately needs resolution.

2.1.2 Physicochemical Factors

2.1.2.1 Containers

There are a whole range of options ranging from Petri dishes to open or closed containers of various sizes constructed from a variety of materials (e.g., glass, plastic,

clay, metal), each with its own benefits and limitations. Ultimately the type and size of container used will be determined by the experimental objectives. The following are some of the factors that should be considered when choosing a container:

a. the life-form characteristics, age, and density of the species to be used,
b. root characteristics and their normal distribution with or without their symbiotic relationships,
c. the desired type of growth medium (e.g., nutrient solution, sand, soil, levels of aeration, nutrition, and moisture retention),
d. the desired control over environmental factors to be manipulated,
e. the plant processes of interest (e.g., cellular processes, germination, seedling emergence and growth, nutrient uptake, water utilization, and transpiration),
f. the duration of the bioassay (e.g., hours, days, weeks, or months),
g. the actual growth environment (e.g., available space [e.g., container size and shape], aeration [e.g., open or closed container], light bank, growth chamber, or greenhouse),
h. the potential release of chemical residues from container walls and the affinity of the container walls to the compounds being tested, and
i. the normal growth environment and perceived requirements of the species in the field.

When the right type and size of container is chosen, day to day management (e.g., frequency of watering and fertilization) and the creation of inappropriate environments (e.g., pot-bound roots and mycorrhizae, or anaerobic media) will be minimized. Clearly if the containers used create a completely unrealistic or unrepresentative environment for the bioassay species, then the resulting data may have limited or little value to field systems recognizing that bioassays, particularly in the laboratory, represent simplified models of real systems and not the real thing (Blum 2007). However, the type and size of container chosen will not only affect the nature of the physicochemical growth environment of seeds, roots, and mycorrhizae but can also determine the dose of allelopathic compounds experienced by them. For example as the volume of a container and the associated medium containing a specific concentration of an IPA compound (mM/liter or μmol/g of media) are increased the total number of molecules of that IPA compound that can potentially interact with seeds, roots, and mycorrhizae at a given density will also increase (see Weidenhamer et al. 1987; Hoffman and Lavy 1978).

2.1.2.2 Media

A variety of media have been used in bioassays. They include solutions (e.g., water, nutrient solutions, extracts, leachates, root exudates, etc.), filter paper, agar, sand, gravel, vermiculite, soil, and other substrates. What is used depends on the experimental objectives. Each has benefits and limitations that must be kept in mind when choosing a particular medium (see sect. 3.2.2.1). The choice of medium, however, should not be underestimated since the choice of media will directly influence the effective treatment concentrations required for observed allelopathic effects on cel-

lular processes, germination, growth, development, and reproduction of bioassay species (also see Sect. 3.2.2).

a. Solutions: Solutions containing IPA compounds, extracts, leachates, and root exudates provide direct and continuous contact with seeds, roots, and mycorrhizae. For solutions, concentrations of both organic and inorganic compounds, pH, water potential, and aeration will change fairly rapidly over time. Changes will likely be due to microbial utilization and synthesis, uptake or losses from seeds, roots, and mycorrhizae, water loss due to transpiration and evaporation, and oxidation/reduction. Unless accounted for, these changes will make it difficult to identify cause and effect since such changes can also directly modify cellular processes, germination, and seedling growth and development of the bioassay species. However if experimental time intervals are short enough, then most of these changes will have only a limited impact. In addition the regulation by external means and inclusion of appropriate references/controls can potentially help to identify and quantify the impact of such changes. For example containers can be sterilized, solutions can be aerated, buffers can be added to slow pH changes, solutions can be filter sterilized, seeds and roots can be surface sterilized, water can be added back to compensate for transpiration and evaporation, chemically inactive osmotic compounds can be added to regulate water potential, and organic and inorganic compounds can be supplemented to minimize concentration changes. Ultimately, however, it may be simpler to include appropriate references/controls (e.g., a range of aeration, pH values, inorganic compounds [e.g., nutrients], and water potentials, etc.) in factorial designs that will allow for the identification of causes and effects or replace solutions completely at given time intervals to maintain a narrow range of solution changes. If done correctly, solution culture can provide an estimate of the potential stimulation (i.e., facilitation) or inhibition (i.e., phytotoxicity) of IPA compounds, extracts, leachates, and root exudates for the experimental conditions of the bioassay. Producing functional nodules and mycorrhizae in solution culture is possible but special conditions are required (Hawkins and George 1997; Mosse and Thompson 1984; Bollman and Vessey 2006; Mortimer et al. 2008).
b. Filter paper. Filter paper is generally included in Petri dishes to provide for an even distribution and contact of treatment or reference/control solutions for seeds or seedling radicles in Petri dishes. Filter paper is generally assumed to be inert and, thus, have minimal effect on the solutions added to Petri dishes. Some adsorption and absorption, however, may be expected. Concerns regarding changes in pH, solute potential, inorganic and organic compounds, etc. for solution cultures apply here as well. Regulating these factors, however, is more cumbersome than for solution cultures.
c. Agar: Very similar to solution culture (see a. and b.), with the exception that agar substrates (pure or with inclusions) tend to have more buffering capacity, provide support for seeds or seedlings, and adsorb and absorb organic and inorganic compounds on or within their matrix. The initial magnitude of sorption and fixation of IPA compounds, extracts, leachates, etc., and the pH and water potential of an agar matrix will vary with agar concentration, the presence of

other inclusions (e.g., inorganic and organic compounds, etc.), and the IPA compounds, leachates, etc. added to the matrix. Concerns regarding changes in pH, solute potential, etc. for solution cultures apply here as well. Regulating these factors, however, is much more cumbersome than for solution cultures. With proper care and conditions functional symbiotic relationships can be obtained in agar medium (Bago et al. 2004; Zhu et al. 2010; Shukla et al. 2012).

d. Sand, gravel, etc.: These represent another form of solution culture and, thus, have very similar concerns since clean quartz sand and gravel are essentially inert (e.g., minimal adsorption or absorption). They do provide support for seeds and seedlings and aeration is generally much better than solution and agar cultures as long as these systems are open or flow-through systems. However, these media generate a resistance to root and mycorrhizal growth. The smaller the pore sizes, the greater the resistance. In addition, available water will very likely be much more limiting than for solution and agar media. Since such systems can be readily flushed, some of the concerns listed for solution and agar media can be minimized or controlled. Similar observations can be made for vermiculite and perlite which are, however, not inert. The use of peat moss or pine bark is generally not recommended because of their acidic nature and the high content of tannins, phenolic acids, etc. Finally inert materials such as sand and gravel, etc. have an additional advantage over solution cultures. They provide a support structure on and in which plant tissues, litter, and residues can be tested. Functional symbiotic relationships can readily be obtained in sand, gravel, etc. media as long as conditions are appropriate for their formation (Francis and Read 1984; Lynch et al. 1990; Medeiros et al. 1993; Gryndler et al. 2005; Zhu et al. 2010; Pang et al. 2011).

e. Soil: The concerns associated with solution systems are more extensive and much more difficult to regulate or control in soils. Processes such as sorption, desorption, resorption, utilization by soil organisms (flora and fauna), movement, distribution, and contact of organic and inorganic compounds by seeds, roots and mycorrhizae, as well as water potential, nutrition, and the growth of roots and mycorrhizae are generally much more variable, dynamic, and unpredictable in soil systems than in solution, agar, sand, gravel, etc. systems. The physicochemical and biotic environments of field soils are even more complex in that every field soil sample is very likely to be different from every other field soil sample. (Note: Such difference in soil samples can be a particular concern for soil samples taken at various distances from a plant or row of plants.) To get around this problem soil samples are frequently sieved, mixed, air-dried, etc. (i.e., processed). Homogenizing soil samples by sieving, mixing, and air-drying leads to some soil uniformity for experimental units but this uniformity comes at a cost. The cost, structure, hydration, aeration, compaction, soil organism populations, and organic matter distribution of the resultant soil substrate are all modified in the process. The addition of clean quartz sand (or other inert substances with very low absorptive and adsorptive properties) or materials such as vermiculite and perlite to soil to reduce compaction, increase soil aeration and percolation, nodulation, formation of mycorrhizae, or reduce resistance to

growth of roots and mycorrhizae is another common practice. Homogenizing soils and adding such materials should only be made after carefully considering the experimental implications of such practices since these practices can modify soil processes and dramatically alter the responses of bioassay species to IPA compounds, leachates, root exudates, tissues, litter, and residues. Functional symbiotic relationships can readily be obtained in most soils as long as conditions are appropriate (Fitter et al. 1998; Arias et al. 1999; Vázquez et al. 2001; García et al. 2008; Jalonen et al. 2009; Hasbullah et al. 2011).

2.1.2.3 Environments

The first things that should be determined are the environmental limits (e.g., minimum and maximum) for growth and development of the chosen bioassay species. However if that is not possible or practical, then at least a normal-growth range for the bioassay species should be determined. Environmental conditions of interest within this range should then be used. The investigator could also just choose to use average or representative environmental conditions of the field in which the species is growing. However, since the field environment is constantly changing, it is important to remember that the effects observed for a laboratory bioassay at a given temperature, light intensity, humidity, pH, soil moisture content, etc. represent only a very small slice of the potential range of effects and physicochemical and biotic conditions that occur within the field. But no matter what environmental conditions are chosen, the actual environmental conditions of a laboratory bioassay must be clearly characterized or described.

Standard dose-response bioassays frequently are designed to have a single treatment factor, i.e., increasing or decreasing concentrations of allelopathic compounds. Researchers tend to ignore that these presumable single-treatment factor bioassays in reality turn out to be multi-treatment factor bioassays since the physicochemical (e.g., presence of organic and inorganic compounds other than those being tested, soil moisture, pH, compaction, aeration, solute potential, and soil temperature to name a few) and biotic (e.g., microbial populations) environments change, sometimes dramatically, as concentrations of IPA compounds, extracts, leachates, root exudates, litter, and residues are increased or decreased within bioassay systems. For them to function as single factor systems, requires that bioassay systems treated with increasing or decreasing concentrations of IPA compounds etc. be adjusted so that with the exception of the compounds of interest they are environmentally equivalent. That turns out to be an extremely tedious job particularly for complex mixtures of organic and inorganic compounds found in extracts, leachates, root exudates, litter, and residues since each system would have to be monitored for a variety of environmental factors and adjusted accordingly. But unless that is done, it will be impossible to characterize the effects of increasing concentrations of specific allelopathic compounds independently of other active (i.e., regulating and modifying) environmental factors.

Unfortunately it is not always possible to know and identify the entire range of environmental factors that will influence the results of a bioassay. However, that should not prevent researchers from characterizing the obvious factors (e.g., light intensity, temperature, moisture, nutrition, pH, aeration, water potential, composition and volume of substrate, presence or absence of microorganisms and other organisms, and symbiotic relationships). The importance of adequate monitoring and characterization of environmental factors for laboratory bioassays cannot be overemphasized since environmental factors will not only influence the bioassay species directly but also modify the actions and effects of the IPA compounds individually or imbedded in complex mixtures of organic and inorganic compounds (see Einhellig 1989; Lehman and Blum 1997; Gawronska and Golisz 2006; Pedrol et al. 2006; Blum 2011).

Most of the physical factors (e.g., light, temperature, moisture, nutrition, and soil compaction) can be readily regulated or controlled in laboratory bioassays. However the biotic factors, particularly the microorganisms, are another story. Soil microbial populations are a particular pernicious problem in laboratory bioassay systems since the enrichment or treatment of environmentally rich bioassay systems (e.g., adequate nutrients, moisture, and temperature) with carbon sources, such as IPA compounds, extracts, leachates, root exudates, litter, and residues can dramatically alter the qualitative and quantitative nature of microbial populations within the soil. Such changes are a concern because microorganisms not only utilize and modify organic compounds and in the process increase or decrease their potential allelopathic effects but also produce their own and sometimes unique allelopathic compounds. Furthermore, such enrichments or treatments can also modify potential symbiotic relationships (e.g., formation, development, and function of nodules and mycorrhizae) adding another level of complexity to our ability to identify causation in seedling or older plant bioassays. One could, of course, argue that microbial populations in field soils will also change with increasing and decreasing inputs of organic and inorganic compounds of leachates, root exudates, litter, and residues. However, the present evidence suggests that changes in microbial populations in laboratory bioassays are much more dramatic than in field soils. For a more detailed discussion of this problem see Sects 3.6 and 3.7. Clearly this is a subject that deserves much more attention.

2.1.3 Test Materials

2.1.3.1 Nature of Test Materials

Compounds tested in laboratory bioassays include identified putative allelopathic (IPA) compounds isolated and identified from living plant tissues (e.g., extracts of leaves, roots, mycorrhizae, and whole plants, leachates, root exudates, etc.), litter and residues (e.g., extracts or leachates of dead plant materials), soils (e.g., soil extracts, solutions or leachates), and complex unknown mixtures of compounds associated with the testing of extracts, leachates, and root exudates of living tissues,

litter, residues, and soils. IPA compounds can be purchased from a vender or isolated, purified, and collected from living tissues, litter, residues, and soils. However, locating, identifying, and collecting representative source materials (i.e., plants, tissues, residues, litter and soils) for testing or for obtaining extracts, leachates, or root exudates are another matter since for any given species, ecotype, cultivar, or soil these materials can be highly variable in time and with location.

Chemical Composition Since the chemical compositions of living plants, plant tissues, litter, and residues, and soils are highly variable their chemical composition should/must be characterized before they are used in laboratory bioassays. The reasons for this variability in chemical composition are as follows:

a. Living plants or their tissues: The organic and inorganic composition of plants or their tissues at a particular time and location will vary with species, ecotype, cultivar, stage of plant development, stage of life cycle, time of the growing season, tissue type, presence or absence of symbiotic relationships, and past and present growth environments (Koeppe et al. 1969, 1970; Bates 1971; Hall et al. 1982, 1983; Rice 1984; Hocking 1994; Drossopoulos et al. 1996; Chaves et al. 1997; Lambers et al. 1998; Chaves and Escudero 1999; Zhao and Oosterhuis 1999; Bertran et al. 2010; also see Sects. 3.6 and 3.7). Since the allelopathic potential of living plants and their tissues varies with their inorganic and organic compositions, collecting them at the right time of the growing season, the right place (e.g., site where putative interactions are occurring), and under the right conditions (e.g., before or after major rain events or other disturbances) is essential.
For example, Hall et al. (1982) found that total phenolic acid content of sunflower (*Helianthus annuus* L.) plants grown in sand culture varied with the nutrition and growth environment. As the concentrations of Hoagland's nutrient solution (Hoagland and Arnon 1950) supplied to sunflower plants growing in the greenhouse or in the out-of-doors were increased, the total phenolic acid content in chlorogenic acid equivalents of the plants declined. Total phenolic acid content ranged from 40 (1/4 strength) to 32 (full strength) mg/g dry weight for plants grown in the greenhouse and from 79 to 52 mg/g dry weight for plants grown outdoors. When sunflower plants were grown in a local garden, the total phenolic acid content was 19 mg/g dry weight. For additional environmentally induced variation and for developmental and tissue variation of phenolic acids in sunflower plants, see Koeppe et al. (1969, 1970) and Rice (1984).
Hall et al. (1982) found that total phenolic acid content of freeze-dried and coarsely ground sunflower plant tissues obtained from sunflower plants grown under a range of environmental conditions (see above) was directly related to the inhibition of pigweed (*Amaranthus retroflexus*) seed germination when these sunflower tissues were mixed into soil. However, inhibition of pigweed seedling dry weight was not significantly related to total phenolic acid content unless NPK (total N, P and K determined on dry-ashed plant samples) content of the sunflower tissues was also included in the regression model (Hall et al. 1983). Total phenolic acid and N were directly related and P and K were inversely related to the inhibition of pigweed seedling dry weight. Inhibition

of germination was reduced and inhibition of seedling biomass was eliminated when NPK solution (in the form of NO_3^-, KNO_3, and HPO_3) roughly equivalent to NPK content of sunflower tissue was also added to the sunflower tissue-soil bioassay system. These observations lead the authors to suggest that nutrients within tissues and nutrients within soils appear to act differently in modifying allelopathic interactions. The fact that increasing soil nutrients tends to reduce or eliminate the action of inhibitory allelopathic compounds by way of microbial utilization would tend to support this observation (Vaughan et al. 1983; Blum et al. 1993, 1999; Schmidt and Ley 1999).

Variations in organic and inorganic content of living plants and plant tissues are actually very common and can occur over a range of time intervals. For example, alkaloid content of hemlock fruits (*Conium maculatum* L.) changed not only weekly but also hourly during fruit development (Fairbairn and Wassel 1964). Hourly changes were particularly dramatic during times of pericarp development of fruits but were not obvious during early seedling development in the vegetative tissues. For 2 year old hemlock plants the alkaloid γ-coniceine (slightly water soluble) was found in the aerial parts and roots during March (spring growth) but only in the aerial parts during vigorous growth in June. Seasonal variation of organic compounds in plant tissue is actually a common occurrence (Witzell et al. 2003; Muscolo and Sidari 2006; Solar et al. 2006; Kotilainen et al. 2010). For examples of variations in inorganic compounds in plant tissues see Bates (1971); Hocking (1994); Drossopoulos et al. (1996); and Zhao and Oosterhuis (1999).

b. Plant litter and residues: The organic and inorganic composition of plant litter and residues will vary with the:

1. species, ecotype, and cultivar,
2. tissue type (e.g., stems, leaves, or roots),
3. stage of development and stage of life cycle,
4. time of growing season,
5. physicochemical and biotic growth environments of living plants and their tissues prior to senescence,
6. timing and rates of senescence, mortality, and abscission (shedding),
7. location of litter and residues (e.g., standing upright, lying on the soil surface, or located within the soil),
8. length of time the litter and residues have been in the field (e.g., amount of fragmentation, weathering and decomposition), and
9. precipitation events (e.g., rates, volume, frequency, and timing), and management (e.g., tillage and chemical applications).

That allelopathic potential of plant litter and residues will vary with their composition of organic compounds has been well established (Guenzi et al. 1967; Patrick 1971; Vaughan and Malcolm 1985; Coleman et al. 1989; Koch et al. 1992; Blum et al. 1997; Lehman and Blum 1997; Bonanomi et al. 2006). Circumstantial evidence would suggest that this is also the case for the composition of inorganic compounds within litter and residues (see a. Hall et al. 1983 above). Thus, collecting litter and residues for bioassays at the right time of the year, the

right place (e.g., site where putative interactions are occurring), and under the right conditions (e.g., before or after major rain events or other disturbances) is essential.

For example, total phenolic acid content in ferulic acid equivalents of crimson clover (Trifolium incarnatum L.), rye (Secale cereale L.), subterranean clover (Trifolium subterraneum L.), and wheat (Triticum aestivum L.) cover-crop residues taken at monthly intervals from a field after glyphosate desiccation, declined over time (Lehman 1993). However, the decline was not evident until 2 months after the glyphosate desiccation for wheat and rye residues. After 4 months the total phenolic acid content of the cover crop residues had declined by 31, 36, 38, and 56 % for wheat, crimson clover, rye, and subterranean clover, respectively. Similar observations have been made for DIBOA (2,4 dihydroxy-1,4-benzoxazin-3(4H)-one) and two related compounds in rye (Yenish et al. 1995). For this study DIBOA and the related compounds, DIBOA glucoside and BOA (benzoxaolin-2-one), declined by 50 % 10–12 days after clipping. Both phenolic acids and DIBOA and related compounds have been shown to have inhibitory properties under appropriate conditions at the appropriate concentrations (see Barnes and Putnam 1987; Blum 2011). For variation of inorganic compounds in litter and residues over time see Buchanan and King (1993); Schomberg and Steiner (1999); Lupwayi et al. (2006); and Ventura et al. (2010).

c. Soils: The organic and inorganic content of soils and, thus, their allelopathic potential will vary with:

1. soil type,
2. the absence, or presence and nature of plant roots, mycorrhizae, and soil flora and fauna,
3. the nature, state, and horizontal and vertical distribution of tissues, litter, residues, organic matter, and soil flora and fauna,
4. the physicochemical and biotic environments of the soil and associated types and magnitudes of soil processes, and
5. management. (Börner 1960; Buckman and Brady 1965; Patrick 1971; Alexander 1977; Brady 1984; Vaughan and Malcolm 1985; Coleman et al. 1989; Foth 1990; Blum et al. 1991, 1992; Hartel 1998; Lavelle and Spain 2001; Blum 2004, 2006, 2011).

Finally with the exception of pure IPA compounds, all other test materials (e.g., extracts, leachates, root exudates, tissues, litter, residues, and soil) are composed of complex mixtures of organic and inorganic compounds. In the past it was assumed, or at least implied by the experimental approaches taken, that plant-plant allelopathic interactions were primarily determined by specific organic compounds within such test materials. That was unfortunate since all active organic and inorganic compounds within a bioassay system, not just identified putative allelopathic organic compounds within the test materials, ultimately determine the stimulation or inhibition of sensitive species (see Sect. 1.2). Thus a few additional comments about allelopathic compounds, organic compounds, inorganic compounds, and the physical state of the test materials are warranted at this point.

Allelopathic Compounds As stated previously (see Sect. 1.3) the reader may have noticed some ambiguity in the way the term allelopathic compounds has been used in this volume. That stems from the fact that potentially any given organic compound can function as a promoter, a modifier, an inhibitor, or as a neutral compound depending on a variety of factors. Some determining factors are the sensitivity of the species and the process being studied, the concentrations of the compounds, the nature of other organic (and inorganic) compounds present, and the biotic and physicochemical environments (Blum and Rice 1969; Rice 1984, 1986; Blum et al. 1989, 1993; Gerig et al. 1989; Gerig and Blum 1991, 1993; Pandey 1994; Pue et al. 1995; Blum 1996, 2006, 2011; Belz et al. 2005, 2007; Duke et al. 2006; Belz 2008; Gianinazzi et al. 2010; also see Sects. 3.6 and 3.7). Therefore, in an absolute sense there is no such thing as a compound that is always an allelopathic compound. There are only organic compounds that are promoters or inhibitors under the appropriate conditions. Restated, under the appropriate conditions and at the appropriate concentrations, organic compounds function as allelopathic compounds or agents. Theoretically this is true but from a practical perspective, there clearly are organic compounds in nature which at very low concentrations readily affect (stimulate or inhibit) plant processes and there are other organic compounds that require very high or unnatural concentrations, to affect plant processes. Additional justification for the continued use of the term allelopathic compounds given this ambiguity can be found in Sect. 1.3. The reader, however, should be clear as to what is meant when the term is used.

Organic Compounds What is important to remember about organic compounds in the environment is the following:

1. That organic compounds can be reversibly or irreversibly sorbed to soil particles.
2. That organic compounds can be taken up by roots and mycorrhizae and detoxified, sequestered, and/or metabolized.
3. That microorganisms (flora and fauna) can modify and/or utilize organic compounds as a carbon and energy source.
4. That all active organic compounds can directly or indirectly promote or inhibit germination, growth, development, and reproduction of seeds, seedlings, and older plants, and promote or inhibit growth and reproduction of microorganism.
5. That the actions of individual active organic compounds within mixtures of organic compounds on seeds, seedlings, older plants, and microorganisms can be independent or similar joint action (synergistic, additive, or antagonistic) depending on the composition of the mixture, the bioassay system, and the physicochemical and biotic environments of the system (see Sects. 1.2, 1.4, 3.2, 5.3, and 6.2). That individual organic compounds within mixtures of organic compounds can also function as modifying agents of active organic compounds.
6. That the composition of organic (and inorganic) compounds within a bioassay system will be determined by the background levels of the system, the treatments added to the system, and the actions of the bioassay species, microorganisms, and medium present within the system (Sects. 1.3, 2.1.1, 2.1.2, and 2.1.3).

7. That the composition of the treatment materials will be determined by how and when the treatment materials were collected, processed, and applied to the bioassay systems (see Sects. 2.1.3.2 and 2.1.3.4).
8. That the concentrations required for specific effects of individual organic compounds (or even simple mixtures) in laboratory bioassays are not consistent with the concentrations required for individual compounds imbedded in complex mixtures of organic (and inorganic) compounds (see Sect. 3.2.2.3).

Inorganic Compounds As a general rule, the exception being nutrient and nutrient stress studies, researchers studying plant-plant allelopathic interactions have simply ignored or downplayed the importance of inorganic compounds. In fact, in some solution culture bioassays nutrients have been deliberately omitted. The general tendency has been to simply make sure that nutrition is adequate or more than adequate for seedlings and older plants. The initial presence of inorganic compounds in the media (e.g., solutions and substrates such as soil) and the subsequent inputs of inorganic compounds by extracts, leachates, root exudates, litter, residues, and nutrient solutions (e.g., Hoagland's solution; Hoagland and Arnon 1950), however, can have considerable impact on when and how potential allelopathic organic compounds will affect seeds, seedlings, older plants, and microorganisms (see Hall et al. 1982, 1983; Blum and Shafer 1988; Blum et al. 1993, 1999; Blum 2004, 2006, 2011). The types and concentrations of inorganic ions present will not only determine the availability of nutrients for plants and microorganisms but also determine pH, buffering capacity, and solute potential of test solutions, substrate sorption of organic compounds (e.g., multivalent-cation bridging and formation of polymers), salinity of substrates, and substrate structure (e.g., formation of aggregates), to name a few (see Blum 2006, 2011). Care should, therefore, be taken when designing bioassay systems to provide and maintain inorganic compounds (e.g., nutrients) at consistent and relevant levels (see Sect. 3.2.2.4).

Physical State of Test Materials Let us assume that the test materials have been properly collected, processes, stored, and applied to laboratory bioassay systems (see Sects. 2.1.3.2–2.1.3.4). Since the physical states of individual organic and inorganic compounds in solutions such as leachates and root exudates are ultimately determined by the physical and biotic environments of a bioassay systems creating and maintaining field-relevant bioassay systems and/or adequately characterizing other types of bioassay system are/is essential for interpreting and evaluating the relevance of the resulting data (see Sect. 3.2.2). For tissues, litter and residues the outcome of a bioassay can be, and likely will be, determined by the type, composition, and particle size (physical state) of tissue, litter, and residue added to the system (Ells and McSay 1991; Blum 1999). (Note: Effects are also determined by their location (see Sect. 6.5).) The smaller the size of the particles of a treatment sample, the faster the water soluble compounds will be released and decomposed. The larger the size of the particles of a treatment sample, the slower the water soluble compounds will be released and decomposed. The former will provide a pulse dose (higher concentration over a short time interval) and the latter a more phased or

chronic dose (lower concentration over an extended time interval although it is very likely that an initial pulse will also occur). Clearly actual particle size of the samples collected in the field should be maintained wherever and whenever possible.

Final Comments Given this range of variability for test materials, the importance of adequate and representative sampling is absolutely essential if meaningful (i.e., field relevant) information is to be obtained from bioassays. Otherwise the stimulation or inhibition observed in bioassays for the collected test materials may not be representative for the time period and location of interest. The bottom line, one of the important decisions a researcher studying plant-plant allelopathic interactions makes is when and where he or she collects test materials (i.e., the source) and then how those samples are processed and used in bioassays.

2.1.3.2 Collecting and Storing of Test Materials

Identified Putative Allelopathic (IPA) Organic Compounds For purchased, isolated, or synthesized pure compounds this is fairly straight forward since different concentrations of compounds can be mixed directly into a substrate, added in solution form to a substrate, (e.g., soil, sand, or gravel; substrate culture; Blum and Rice 1969; Blum et al. 1993) or used in solution form without a substrate (solution culture; Einhellig et al. 1970; Einhellig and Kuan 1971; Blum et al. 1984). A description of the synthesis, isolation, purification, and collection of sufficient quantities of pure putative allelopathic compounds is beyond the scope of this volume. For procedures regarding isolation, identification, purification, and collection of allelopathic compounds, the reader may wish to consult Waller et al. (1999); Yu and Dahlgren (2000); and Sampietro et al. (2009). Stocks of most pure liquids or dry compounds can be readily stored in their containers in the laboratory. Replacing air in storage containers with nitrogen may also provide a benefit for some compounds by reducing or eliminating oxidation. However, if liquid dilution series are created, they should be properly adjusted for pH and water potential and used immediately in bioassays. If there is a delay in their use, they should be filter sterilized or refrigerated for short delays and frozen for longer delays. However, freezing of such dilution series is generally not recommended since freezing and subsequent thawing are likely to alter the nature of the solutions.

Extracts For the extractions of tissue, litter, residue and soil samples, the following aspects should be considered before choosing and implementing a particular type of extraction procedure. Although tissues, litter, and residues extracts have frequently been tested in bioassays in the past, such bioassays are no longer recommended since they are not representative of what is lost from tissues, litter, and residues (for details see Sect. 2.1.3.3). For crude initial surveys of allelopathic potential they may, however, be useful as long confounding factors such as water potentials, pH, and potential anaerobic conditions are accounted for in the experimental design.

a. Tissues: Organic solvent extracts of tissues cannot be used directly in bioassays for obvious reasons. Organic solvents must first be replaced by water. However, removal of organic solvents by freeze-drying, flash evaporation, or air-drying alters the nature of extracts as the organic and inorganic compounds in extracts are concentrated and precipitated. The bottom line, it is best to avoid organic solvent extracts altogether in bioassays. Water extractions are much more appropriate.
   Cold, ambient or boiled distilled water extractions of ground or chopped living tissues have frequently been used in the past (Abdul-Wahab and Rice 1967; Wilson and Rice 1968; Rice 1984; Quayyum et al. 2000; Kumar et al. 2009). Boiled extractions are probably better than ambient or cold water extractions because the boiling reduces or eliminates degradation by enzymes associated with released by cold extraction procedures and reduces or eliminates microbial utilization and synthesis after extraction. However, since solubility of both organic and inorganic compounds is modified by temperature, the quantitative and qualitative composition of cold, ambient, and hot extracts will be different. Extraction of uncut, cut or ground freeze-dried, air-dried, or oven-dried tissues has also been a common practice (Guenzi et al. 1967; Leather 1983a; Liebl and Worsham 1983; Wardle et al. 1992; An et al. 2000; Wu et al. 2007). Freeze-drying maintains the chemistry of the living tissue. Air- or oven-drying changes the chemistry of the tissue during the drying process. The chemistry of air-dried tissue may, in fact, be more closely related to early stages of senescing tissues than living tissues. Oven-dried tissues have a highly modified chemistry, something that is to be avoided. The actual physical and chemical nature of extracts are determined by the source and type of the tissue (e.g., species, tissue type: leaves, roots, etc., health, acclimation, and growth environment of the plants from which the samples were taken, presence or absence of symbiotic relationships), tissue processing prior to extraction (e.g., freeze-drying, oven-drying, chopping, grinding, etc.), and the extraction procedure used (e.g., extraction time, temperature, solvent, solvent/tissue ratio, etc.).
b. Residues: Most of the above comments about living tissue extracts are directly applicable to plant litter and residue extracts. However, extracts of litter and residues have generally not been tested. More commonly leachates of litter and residues or litter and residues have been tested directly in bioassays (see Sect. 2.1.3.3).
c. Soil: A variety of extractants and extraction procedures have been utilized to recover a range of potential allelopathic compounds present in soil (see Whitehead 1964; Flaig 1971; Kaminsky and Muller 1977; Whitehead et al. 1981, 1982; Hartley and Whitehead 1985; Dalton et al. 1987; Cheng 1990; Blum 1997, 2011; Blum et al. 1991, 1992, 1994; An et al. 2000; Ohno et al. 2000). Extractants have ranged from water to organic (e.g., methanol, ethylenediamine tetraacetic acid [EDTA], sodium acetate) and inorganic (e.g., NaOH) solutions. Extraction procedures have ranged from soaking for various time periods to autoclaving with subsequent filtering or centrifugation. What is recovered (i.e., free, reversibly sorbed, or irreversibly sorbed) depends on soil type, the physicochemical nature

of the extractant (e.g., pH, temperature, and chemical composition), and extraction procedure (e.g., soaking, shaking, time, pressure, and solvent/soil ratio). However, only extraction procedures that recover compounds that are available to seeds, roots, mycorrhizae, and soil organisms (e.g., free and reversibly bound) are appropriate for field-relevant bioassays (Blum 2011). Furthermore, to test soil extracts in bioassays requires that the extracts be water based since both organic and inorganic extractants will/may be inhibitory/toxic to seeds, roots, and mycorrhizae and their associated microorganisms.

In general distilled water, rain water (real or simulated (see Sect. 4.3.3)), or irrigation water (when fields are irrigated) are recommended for the extraction of available (i.e., free and some reversibly sorbed) organic compounds from soil. However, the use of distilled water can be disruptive to algal, microbial, and plant cells and, thus, release contents of such cells into the soil during soil extractions. The length of time for water extractions, particularly at ambient temperatures, should be fairly short, since longer extraction times will likely be associated with the utilization and synthesis by microorganisms, sorption, desorption, and resorption of compounds to soil particles, oxidation/reduction, and polymerization. Utilization and synthesis by microorganisms can be eliminated by first sterilizing the soil (Dalton et al. 1983) or by using a water-autoclave extraction procedure (see Blum et al. 1991, 1992; Blum 2011). However, sterilizing soils directly or indirectly by the water-autoclave extraction procedure will modify the physicochemical nature of soils (e.g., modify sorption, desorption, and fixation of both organic and inorganic compounds present in the soil) and soil extracts (e.g., modify organic and inorganic composition).

Once collected, plant tissue, litter, residue, and soil extracts should be filtered or centrifuged to remove insoluble particles before testing and used immediately. As stated previously plant tissue, litter, and residue extracts are no longer recommended for use in bioassays (see Sect. 2.1.3.3). If plant tissue, litter, and residue extracts are to be used for crude initial surveys of allelopathic potential, they and soil extracts should be filter sterilized or refrigerated for short delays and frozen for longer delays. Extended refrigeration or freezing, however, may modify the overall chemical composition of extracts due to the precipitation of various components within the extracts. For those not filter sterilized, utilization and synthesis by microorganisms will modify those extracts over time.

Leachates A variety of approaches has been used to collect leachates from plants, litter, residues, and soils. Among them are the following procedures:

a. Leaves: Throughfall and fog drip under vegetation canopies are the most representative of actual leachates in nature (del Moral and Muller 1969; Gallet and Pellissier 1997; Nilsen et al. 1999). However, the chemical and physical properties of throughfall and fog drip do vary with the type and nature of the canopy, sample location in and under the canopy, when and how samples are collected, and the physical (e.g., frequency, volume, and rates) and chemical nature of rain and fog events. Representative sampling locations and proper collecting and handling of samples are, therefore, essential.

Leachates of leaves (also petioles, stems, bark, flowers, and fruit leachates) have been collected by spraying plants with distilled water, rain water, simulated-rain water, or other types of solutions (Long et al. 1956; Tukey et al. 1957; Tukey 1966; Wilson and Rice 1968; Al-Naib and Rice 1971; Chou and Muller 1972; Cogbill and Likens 1974; Scherbatskoy and Klein 1983; Percy 1986; DuBay and Heagle 1987; Shafer et al. 1985; Shafer 1988, 1992). The physicochemical nature (e.g., composition, pH, etc.) of leachates collected will not only vary with species, leaf shapes and surface areas, amounts and types of leaf hairs and glands, nature of cuticles (e.g., thickness, cracks and crevices), angles and positions of leaves on plants and in the canopy, stages of maturity of the leaves, types and amounts of leaf injury, and past and present growth environments of plants and their leaves but also with the composition, temperature, pH, pressure, droplet size, volume, rate, frequency, retention time of the solution applied and time of day when samples are collected.

Leaf leachates have also been collected by immersing leaves in distilled water (Tukey and Mecklenburg 1964). Leaf leachates obtained with distilled water by either immersion or spraying are the least natural in their physicochemical nature (e.g., composition and pH, etc.) because the gradient generated between leaf content and distilled water is greater than what would be found with rain water. Distilled water can also be very disruptive to leaf cells because of its water potential (i.e., approximately zero).

Finally, no matter how collected, the composition of both organic and inorganic compounds in leaf leachates (also petiole, stem, bark, flower, and fruit leachates) will also be determined by the solubility (e.g., the hydrophobic or hydrophilic nature) of compounds being leached, the mobility of the compounds within and outside of leaves (e.g., bound or free, membrane and cuticle permeability), and the existing gradient between internal concentrations of leaves and the external concentrations of the leachate solution. For a given solution, however, the longer the droplets are on leaves (or the leaves are immersed in a solution) the greater will be the concentration of the compounds leached from those leaves. The highest concentrations will be recovered the first time leaves are leached and will decline with subsequent leaching events unless adequate time is provided for leaves attached to plants to recover. The first leachate will include materials on the surface of the leaves (e.g., dust, soil particles, microbe, insect remnants, etc.). A series of plants (leaves, etc.) should be used in rotation if bioassays are to be run over extended periods.

b. Litter and Residues: In theory surface litter and residue leachates collected by properly preconditioned lysimeters (e.g., acid washed, aged in soil; see Dalton 1999) just underneath plant litter and residue layers in the field are the most representative of actual surface litter and residue leachates in nature. The chemical and physical properties of surface litter and residue leachates do, of course, vary with the type and nature of the litter and residue layer, the chemical and physical properties of rain, the frequency and volume of each rain event, the location of the lysimeter, the time samples are collected (e.g., during or after a rain event; length of time solutions were in lysimeter), and time of year. Simulated-rain

events may also be used with lysimeters in the field (see Sect. 4.3.3). Another option would be to bring undisturbed or minimally disturbed surface litter and residue samples (i.e., maintaining the profile of the litter and residue layer) into the laboratory. For these samples simulated-rain events could be used to collect leachates directly without lysimeters. Surface plant litter and residue leachates have also been collected by soaking plant litter or residues in water solutions for various periods of time at temperatures from below ambient to boiling (Nilsen et al. 1999; An et al. 2000; Bonanomi et al. 2006; Nakano et al. 2006). Ambient temperatures are probably the most realistic but degradation by microorganisms can be a problem if leachate times are too long. Placing residues in boiling water to obtain leachates is the least realistic particularly when the residues have been chopped or ground.

The author is not aware of any studies on belowground litter and residue leachates independent of soil and living roots and mycorrhizae. Studies of belowground root litter and residue leachates independent of living roots, mycorrhizae, and soils, however, could prove to be very useful in distinguishing their role from the role of surface litter and residue leachates in allelopathic interactions. Once collected and cleaned (i.e., soil is gently removed by brushing), simulated-rain events (see Sects. 2.1.3.2, 4.3.3, and 4.3.5.1) may be used to collect leachates from root and mycorrhizal litter and residues in the laboratory. When cleaning belowground plant samples, water should not be used unless absolutely necessary and then washing should be minimized as much as possible to limit the loss of both organic and inorganic compounds from the samples. Finally, litter and residue samples should never be chopped, ground or oven-dried if they are to be used to collect realistic leachates.

c. Soils: Soil leachates and soil solutions have been collected with lysimeters (Grover and Lamborn 1970; Neary and Tomassini 1985; Debyle et al. 1988; Hughes and Reynolds 1988; Dalton 1993, 1999; Zabowski and Ugolini 1990) in soils with adequate moisture or after sufficient rain or irrigation events (e.g., gravitational water). Lysimeters need to be properly preconditioned (e.g., acid washed, aged in soil; see Dalton 1999) before they are used to collect leachates and soil solutions. What is recovered from the lysimeters is determined by their location in the soil, physical and chemical nature of the soil, rate and extent of soil solution movement in the soil, volume of the soil solution, activity of microorganisms, tissues, litter, and residues present, etc. Soil solutions have also been collected from moist soils by centrifugation of soil samples. However, the volume collected is generally not sufficient for seed and seedling bioassays (Zabowski and Ugolini 1990).

Leachates, no matter their source, should be collected and filtered to remove insoluble particles and used immediately. If there is to be a delay in their use they should be filter sterilized or refrigerated for short delays and frozen for longer delays. Extended refrigeration or freezing, however, may modify the overall chemical composition of the leachates due to degradation, activity of microorganisms, and the precipitation of various components within the leachates.

Plant Tissues, Litter, and Residues Plant tissues, litter, and residues may be collected from plants grown in growth chambers, greenhouses, or field plots. Growth chamber and greenhouse environments can provide ample sources of living tissues while field environments are an excellent source of both living tissue and dead tissues (i.e., litter and residues). However, due to the differences in morphological, anatomical, and chemical characteristics of plant materials from these locations and, thus, their allelopathic potentials, it is generally best to collect these materials from field plots. This can be fairly easily done for aboveground shoot tissue, litter, and residues. Root and mycorrhizal tissues, litter, and residues are somewhat more difficult to obtain and are usually obtained with soil cores or from soil pits. The soil associated with root and mycorrhizal tissues, litter, and residues may then be removed by sorting, screening, or washing (see next paragraph). At that point recognizable root and mycorrhizal litter and residues may be separated from living roots and mycorrhizae. However, identifying and isolating root and mycorrhizal litter and residues from living roots and mycorrhizae is an onerous task since identifying belowground living and dead tissues is in most cases extremely difficult and time consuming (Böhm 1979; Schenk 1982). After plant tissues, litter, and residues are collected, cleaned, and sorted they may be used directly in bioassays, air-, oven-, or freeze-dried before being used in bioassays, or stored for future use (Abdul-Wahab and Rice 1967; Wilson and Rice 1968; Al-Naib and Rice 1971; Leather 1983a, b; Putnam et al. 1983; Weston et al. 1989; Lehman and Blum 1997; Kamara et al. 1999; Staman et al. 2001; Moonen and Bàrberi 2006; Kumar et al. 2009).

The chemical composition of plant tissues, litter, and residues collected will, of course, vary with species, tissue type, stage of development, stage of the life cycle, and growth environment. For litter and residues chemical composition will also vary with the cause of mortality (e.g., natural causes, herbicide desiccations, etc.), time of abscission or loss, weathering, and decomposition. Tissues, litter, and residues should not be washed unless absolutely necessary and then washing should be minimized as much as possible to limit the loss of both organic and inorganic compounds. Oven-drying of plant tissues, litter, and residues is not recommended since that will modify their chemistry excessively. Air-drying will also change the chemistry but one can argue that these changes in chemistry are somewhat similar to changes that occur in nature. This may be the case for aboveground litter and residues but is unlikely to be the case for root and mycorrhizal litter and residues except under extended drought conditions. Freeze-drying will maintain the chemistry at the time of collection. Dried plant tissues, litter, and residues can be stored for short periods in a cool dry place in the dark before they are used in bioassays but extended storage is not recommended. Dried plant tissues, litter, and residues should never be chopped or ground before storage because the increased surface area will expedite oxidation and associated chemical changes. Furthermore, chopped and ground living or dead tissue should not be mixed into soil for bioassays unless the researcher wishes to determine a worst-case scenario or doing so would be consistent with field management practices (Blum 1999).

Root Exudates The use of the term root exudates in root-root allelopathic interactions is somewhat misleading since exudates actually constitute only a part of the loss of organic compounds from roots (Rovira 1969; Rovira et al. 1979; Smith 1970, 1976; Moody et al. 1988; Bertin et al. 2003; Bais et al. 2006). Exudates according to Rovira et al. (1979) are composed of organic compounds of low molecular weight which leak (a passive process) from root cells. Roots also lose organic compounds by way of secretions, lysates and the production and release of mucilages. Secretions are composed of low and high molecular weight compounds which are released from root cells mediated by metabolic processes. Lysates are composed of low and high molecular weight compounds which are released by autolysis of epidermal and cortical cells. Mucilages are composed of a complex of high molecular weight compounds secreted by living epidermal cells, root hairs, and root cap cells. They are also a product of hydrolysis of primary cell walls and bacterial degradation of primary cell walls of old and dead epidermal cells. The resulting gelatinous matrix, a product of the root-microbial-soil complex, is referred to as mucigel. Roots also loose inorganic compounds to the soil and take up both organic and inorganic compounds from the soil. All of the above processes (e.g., exudation, secretion, uptake, etc.) will be determined and regulated by the species involved, their state of development, presence or absence of symbiotic relationships, and their biotic and physicochemical environments (Rovira 1969; Shay and Hale 1973; Barber and Gunn 1974; Barber and Martin 1976; Smith 1976; Přikryl and Vančura 1980; Bertin et al. 2003; Jones et al. 2004, 2009; Neumann and Römheld 2007; Carvalhais et al. 2011). Plants with mycorrhizae have additional pathways of uptake and of loss for both organic and inorganic compounds by the mycorrhizal hyphae in the rhizosphere and hyphosphere (Jones et al. 2004, 2009; Toljander et al. 2007; see Sect. 3.7.4). Given the actual multifaceted nature of what is lost from roots and mycorrhizae, root exudates from here on out will be referred to as "root exudates plus". Furthermore, given:

a. the complex chemistry of "root exudates plus",
b. the close association of microorganisms (e.g., actinomycetes, bacteria, fungi, etc.; see Sect. 3.7) and fauna with the rhizoplane and rhizosphere of roots, and the rhizoplane and mycorrhizosphere (i.e., rhizosphere, and hyphosphere, i.e., see Sect. 3.7.4) of mycorrhizae,
c. the continuous turnover of organic and inorganic compounds released from roots and mycorrhizae (e.g., uptake, utilization, transformation, transport, sorption, desorption, oxidation/reduction, polymerization, etc.), and
d. the variable and complex nature of soils (e.g., texture, structure, clay, organic, litter and residue content, etc.) surrounding roots and mycorrhizae of both producers and receivers, the collection of realistic "root exudates plus" samples from roots and mycorrhizae, as well as soils surrounding roots, will be extremely difficult under field conditions although some have tried (e.g., Smith 1970, 1976).

To get around this problem researchers studying allelopathic plant-plant interactions in the past have primarily used two approaches. "Root exudates plus" leachates have been tested by direct transfer from producing plants to receiving plants

(see leachate bioassays of Sects. 4.2.3 and 6.3) or "root exudates plus" samples have been extracted from media and tested in bioassays. However, the rhizosphere and mycorrhizosphere, per say, have been probed by much more sophisticated methods (see Narasimhan et al. 2003; Newmann et al. 2009; Badri et al. 2013).

Direct transfers of "root exudate plus" leachates between plants have been carried out in solution-sand/gravel culture (two or more container or stair-step systems; Bonner and Galston 1944; Martin and Rademacher 1960; Abdul-Wahab and Rice 1967; Wilson and Rice 1968; Bell and Koeppe 1972; Newman and Miller 1977; Kochhar et al. 1980) without and with XAD-4 resin columns (Tang and Young 1982; Tang 1986; Tang et al. 1989; Yu and Matsui 1994, 1997), and sterile agar culture (Wu et al. 2000a, b, 2007; Huang et al. 2003). In the absence of microorganisms, leachates consist primarily of the water-soluble (hydrophilic) compounds released by roots. (Note: Some chemical oxidation/reductions may occur after compounds are released from roots.) In the presence of microorganisms, leachates consist of water-soluble compounds released by roots and mycorrhizae, compounds modified by microorganisms, compounds synthesized by microorganisms, and compounds that have been oxidized or reduced during the transfer process. Few, if any, water insoluble compounds would be expected to be transferred between plants by "root exudates plus" leachates. The question of whether non-polar (hydrophobic) compounds may be partially solubilized or carried in water solution by mixtures with other amphiprotic compounds (having characteristics of both an acid and a base and acting as either) has really not been adequately determined. There is also the issue that some volatile compounds such as cineole which have been considered to be "insoluble" (hydrophobic) may in fact be fairly water-soluble. For example, oxygenated monoterpenes are soluble in the hundreds of ppm and which is much higher than the solubility of phenolic compounds like juglone (Weidenhamer, Personal Communications; also see Martin and Weidenhamer 1995). In addition since the rates and amounts of "root exudates plus" released from roots and mycorrhizae are to a large extent regulated by rates of leaching, activity of microorganisms, growth rates of shoots and roots, presence or absence of symbiotic relationships, type of substrate, nature of the substrate solution surrounding the roots and mycorrhizae, rates of substrate sorption/desorption, and the growth environment, the "root exudates plus" produced and tested by direct transfer in sand/gravel culture or that produced under sterile conditions in agar or solution culture will not be qualitatively or quantitatively the same as "root exudates plus" produced in soils in the laboratory and in the field (Shay and Hale 1973; Barber and Gunn 1974; Barber and Martin 1976; Přikryl and Vancura 1980; Hodge et al. 1996). The direct-transfer technique(s) can, however, provide some insight into potential plant-plant allelopathic interactions and how such interactions may be modified by various physicochemical and biotic factors that regulate the production of water-soluble components of "root exudates plus" by roots and mycorrhizae.

Samples from direct-transfer systems, sterile or non-sterile solution culture, or sterile or non-sterile agar culture have also been collected and tested for allelopathic activity. Frequently organic compounds from these samples have been isolated by a range of techniques (e.g., chromatographic techniques) or captured on and then

extracted from various types of sorption materials such as XAD resins (see Sect. 6.3 for other potential sorption materials; Börner 1960; Rovira 1969; Shay and Hale 1973; Smith 1970, 1976; Přikryl and Vančura 1980; Tang and Young 1982; Tang 1986; Tang et al. 1989; Moody et al. 1988; Pérez and Ormeño-Nuñez 1991; Yu and Matsui 1997; Pramanik et al. 2000; Wu et al. 2001a, b; Hao et al. 2007; Yang et al. 2010; Zhang et al. 2010). Capillary mats and in situ tube microextractions have also been used to recover a range of organic compounds on root surfaces (i.e., rhizoplane) and within the rhizosphere and mycorrhizosphere and identified and quantified (Czarnota et al. 2001, 2003; Mohney et al. 2009; Weidenhamer et al. 2009; Barto et al. 2012). In case of delay in testing collected "root exudate plus" leachates or IPA compounds collected from "root exudate plus" they should be processed and stored the same way as leachates, solutions, and IPA compounds described in previous subsections.

2.1.3.3 Uses for Test Materials Collected

Identified Putative Allelopathic (IPA) Organic Compounds Purchased compounds or compounds that have been isolated from plant and soil extracts, leachates, and "root exudates plus" when used in solution culture (see Sect. 6.2), can provide considerable insight regarding the potential stimulation (facilitation) or inhibition (phytotoxicity) of individual compounds or mixtures of compounds and how such stimulation or inhibition may be modified by various environmental factors (e.g., pH, water potential, inorganic compounds, other organic compounds, temperature, light intensity, populations of microorganisms, and with or without the presence of symbiotic relationships; see Sect. 5.2.3). This can readily be achieved by manipulating these factors for solution cultures. Factors, however, change over time for solution cultures due to physical and biotic processes. The ability to completely replace solutions at various time intervals for solution-culture bioassays can help in maintaining the desired range of minimum and maximum concentrations of IPA compounds, pH, solute potential, etc. over time and to some extent controlling microbial populations. Solution-culture bioassays also provide an opportunity to gain insight into the modes of action by which IPA compounds affect bioassay species. The primary down side of using solution culture bioassays is the atypical environment (e.g., aeration, total immersion of seeds, root, and mycorrhizae, distribution of compounds being tested, and absence of modifying compounds, etc.) of solution cultures for seeds, roots, and mycorrhizae (see Sect. 7.2).

When added to substrate culture in solution form (see Sect. 6.2), they provide an opportunity to determine how substrate characteristics (e.g., pH, organic matter and clay content, inorganic compounds, microbial populations, moisture content, aeration, and compaction) modify the stimulation or inhibition of such compounds. Benefits of using treatment solutions containing IPA compounds in substrate bioassays are:

a. the ability to modify, adjust, and maintain the composition and concentrations of identified putative allelopathic (IPA) compounds for the treatment solution,
b. the ability to readily adjust treatment solutions for such things as pH, content of inorganic compounds, solute potential, etc. so that these remain within desired limits for all treatments,
c. the ability to readily alter the nature of treatment solutions in order to determine how pH, inorganic compounds, other organic compounds, etc. can modify the effects of IPA compounds, and
d. the ability to add treatment solutions repeatedly over time at predetermined intervals or continuously by drip irrigation to replenish and regulate maximum and minimum concentrations of IPA compounds under study.

The down side of using treatment solutions containing IPA compounds is:

a. there is little control over the distribution patterns within a substrate system,
b. the amount of solution that can be added per treatment to substrates can be limiting particularly for small volumes of substrate,
c. depending on solubility, there are limits to the concentrations that can be added per treatment or over time,
d. that for repeated additions, if substrate losses are slower than inputs, concentrations of the compounds of interest will increase in the soil with time, and
e. the potential creation of atypical factors (e.g., intense modification of microbial populations by differential selection, amplification, and suppression of specific microbial species) by frequent additions of treatment solutions to environmentally enriched (e.g., adequate to excessive nutrition, soil moisture, etc.) substrate cultures.

IPA compounds in dry form can be directly mixed into a substrate (e.g., soil; Blum et al. 1993). The initial desired distribution pattern within the substrate for such additions can readily be achieved except for very low concentrations of IPA compounds. However, multiple additions to substrate once the bioassay is in progress are not possible without considerable disturbance of the bioassay system. They can be applied to the substrate surface at that point but, depending on the solubility of the compounds being tested, toxicity at or near the substrate surface and the resulting steep vertical gradients within the substrate will be a problem. Adjusting pH, solute potential, etc. of the substrate solutions for the different IPA concentrations added to a substrate in dry form will also be extremely cumbersome. In addition the amount of dry compounds added to the surface or mixed into substrate that is dissolved in the substrate solution is not predictable and, thus, available concentrations within a system are an unknown.

Organic Solvent Extracts of Plant Tissues, Litter, and Residues Organic solvents are best used to isolate, identify, and collect solvent-soluble organic compounds that may have promoter, inhibitor, or herbicidal properties. Organic solvent extracts cannot be used in bioassays unless the organic solvents are first removed and replaced with water. In the process of replacing organic solvents with water the physical and chemical properties of the extract will be modified.

Water Extracts of Plant Tissues, Litter, and Residues Water extracts are best used to isolate, identify, and collect water-soluble organic compounds that may have promoter, inhibitor, or herbicidal properties. They could be used in bioassays but their use is not recommended for even preliminary tests of potential allelopathic activity because composition and concentrations of both organic and inorganic compounds in tissue, litter, and residue extracts are totally inconsistent with what is released from living plants, plant litter, and plant residues under field conditions. In addition, effects of water potential, pH, potential anaerobic properties, and enzymatic degradation of extract are confounding factors that make the identification of cause and effect extremely challenging.

Organic and Inorganic Solvent/Extractant Extracts of Soils Some examples of solvents or extractants that have been used are methanol, sodium acetate, citrate, Melich III, ethylenediamine tetraacetic acid (EDTA), NaOH, etc. (see Dalton et al. 1987; Blum 1997; Dalton 1999). Such extracts are best used to isolate, identify, and collect compounds that may have promoter, inhibitor, or herbicidal properties. They are also excellent when used in combination with water extracts of soil for determining the states of the compounds in soil (free, reversibly or irreversibly sorbed) as well as for determining how various physicochemical and biotic factors may modify reversible and irreversible sorption to soil particles, uptake by seeds and roots (with or without symbiotic relationships), and utilization by soil organisms (see Blum 2011). These types of extracts cannot be used in bioassays unless the organic solvents (e.g., methanol) or the organic (e.g., EDTA, citrate, acetate) and the inorganic compounds (e.g., Na) of the extractants are first removed and replaced with water. Replacement of organic solvents may be achieved by evaporating or flash-evaporating extracts to dryness before dissolving the remaining organic and inorganic precipitates in water. Removal of organic and inorganic compounds (e.g., EDTA, citrate, acetate, Na) of extractants may be achieved with dialysis, anion and cation resin columns, or molecular sieves but these are very cumbersome and uncertain processes particularly for large volumes of extracts. In both instances the resulting solutions will be very different from the original soil solutions in pH, content of organic and inorganic compounds, water potential, etc. An additional reason for not using these extracts in bioassays is that in some instances they contain organic compounds that are not available to interact with seeds, roots, mycorrhizae, and soil organisms (e.g., compounds that are irreversibly sorbed to soil particles and the recalcitrant organic matter of the soil).

Water Extracts of Soils At ambient temperature and pressure water extracts recover what is in the soil solution and probably a small portion of what is reversibly sorbed to soil particles. This fraction has frequently been referred to as the free or soil solution fraction in the soil (compared to the reversible and the irreversible fractions) that is available to interact with seeds, roots, mycorrhizae, and soil organisms (Note: Both free and reversibly bound organic compounds are available to interact with seeds, roots, mycorrhizae, and soil organisms, thus ambient water extractions recover only part of what is available.). However, extraction times for these must be fairly short. For longer extraction times extracts will be modified

by the utilization, conversion, and synthesis of organic compounds by soil organisms, sorption, desorption, and resorption to soil particles, and oxidation/reduction of organic compounds.

Extractions using higher temperatures and pressures, assuming temperature and pressure are sufficiently high enough, will eliminate activity of soil organisms but will increase the probability that compounds that are normally not available to seeds, roots, mycorrhizae, and soil organisms will be recovered (e.g., compounds that are irreversibly sorbed to soil particles and the recalcitrant organic matter of the soil; see Blum 2011). When utilizing water extracts obtained with ambient or higher temperatures in bioassays, extract properties such as level of dilution, water potential, pH, and inorganic compounds must be accounted for in the experimental design (see Sects. 4.4.3 and 6.4.2). However with an appropriate experimental design, potential allelopathic effects of soil-water extracts for a given set of soil samples can be determined in the presence of other causative and modifying environmental factors (e.g., see Sect. 6.4.2). Water extracts of soil may also be used to isolate, identify, and collect compounds which may have promoter, inhibitor, or herbicidal properties although the author suspects that other more rigorous extraction procedures would be more effective in isolating and collecting such compounds.

When all is said and done, water extracts of soil at a given point or given points in time will provide only minimal, if any, information on turnover, flux, or flow rates of organic and inorganic compounds in soil systems and only a portion of the reversible sorbed fraction in the soil. Quantities of both organic and inorganic compounds recovered by water extraction of soil, if done properly, actually represent water extractable residuals or net concentrations left in the soil (i.e., what is left after reversible and irreversible sorption, microbial utilization, uptake by seeds, roots and mycorrhizae, etc. have taken place; see Sect. 1.4). If quantities of free organic and inorganic compounds recovered are expressed as units/unit of actual moisture in the soil [e.g., μM], then the resulting concentrations are approximately equivalent to soil solution concentrations at a given point in time. Estimating total available concentrations in soil will require not only data for the water extractable fraction but also data for the reversibly sorbed fraction not recovered by water extraction, and the flows, fluxes, or flow rates to seeds, roots, mycorrhizae, microbes, soil particles, etc. (i.e., source-sink relationships).

Throughfall and Leaf Leachates If carried out and collected properly, throughfall leachates contain what was actually lost from leaves, stems, fruits, etc. within the canopy and what was present in the initial rain water during a specific rain event in the field. Leachates generated by artificial or simulated-rain events in the field or laboratory using collected rain water, simulated-rain water (see Sect. 4.3.3), or irrigation water are an attempt to simulate throughfall leachates under more controlled conditions. The use of irrigation water is, however, not recommended unless overhead irrigation is part of the normal system management. Unfortunately the composition of leachates no matter how they are collected are highly variable both in the field and the laboratory for obvious reasons (e.g., composition and nature of canopy or plants, frequency of rain events, time between rain events, rates and length of rain events, etc.). The bottom line is that the chemical compositions of

throughfall and simulated leachates for any given event or location represent only a small fraction of all the possible chemical compositions of leachates that can occur in the field or in the laboratory. Therefore, the best that either can demonstrate is the potential for allelopathic effects (e.g., the difference in effects between rain water above the canopy and throughfall or leachate below the canopy) for the conditions under which the throughfall or leachates were collected and the nature and environment of the bioassay system. An upside for using collected rain water, simulated-rain water, or irrigation water to obtain leachates is that their physicochemical nature (e.g., composition, pH, etc.) can be readily manipulated prior to application to leaves, plants, or canopies to determine how different physical and chemical factors of rain water, simulated-rain water, or irrigation water can modify the leachates recovered and subsequently how such modifications could potentially affect plant-plant allelopathic interactions. No matter the approach taken, with an appropriate experimental design, potential allelopathic effects of throughfall and simulated leachates for given points in time and for given places and systems can be determined in the presence of other causative and modifying environmental factors (see Sect. 4.2.2).

Throughfall and simulated leachates can also be used to isolate, identify, and collect compounds that may have promoter, inhibitor, or herbicidal properties. Finally, immersing leaves, plants, etc. in distilled water, rain water, simulated-rain water, or irrigation water results in poor imitations of actual leachates under field conditions and, thus, are of limited value. However, they may be useful as an initial screening tool.

Litter and Residue Leachates The comments for throughfall and simulated-leaf leachates are directly applicable to natural and simulated-surface litter and residue leachates. The primary differences are location, i.e., canopy vs. litter layer, and the ease with which they are collected. Collecting representative leachates from litter layers is much more difficult (e.g., defining what constitutes a litter layer, the need for lysimeters or similar tools, the proximity and potential interference of soil materials, etc.). As with throughfall and simulated leachates from canopies, the litter and residue leachates are also highly variable due to composition and nature of litter layer, frequency of rain events, time between rain events, rates and length of rain events, etc.

Immersions and soaking of or simulated-rain events for root and mycorrhizal litter and residues separated from living roots and mycorrhizae and soil are artificial but that may be the only way to obtain and test root and mycorrhizal litter and residue leachates independent of living roots, mycorrhizae, and substrates such as soil. The use of distilled water to collect leachates from root and mycorrhizal litter and residues should be avoided. Even the use of rain water or simulated-rain water (see Sect. 4.3.3) is questionable since the physical states and chemical compositions of simulated-rain solutions are very likely to be different from soil solutions or gravitational water.

No matter the approach taken, with an appropriate experimental design, potential allelopathic effects of litter and residue leachates for given points in time and for

given places and systems can be determined in the presence of other causative and modifying environmental factors (see Sect. 4.3.5). Litter and residue leachates can also be used to isolate, identify, and collect compounds that may have promoter, inhibitor, or herbicidal properties. However given the difficulties in obtaining representative litter and residue leachates, it is not surprising to find that most researchers have chosen to use litter and residues directly in soil bioassays instead of litter and residue leachates.

Soil Leachates and Soil Solutions For all intents and purposes soil leachates collected with lysimeters will most likely be a combination of gravitational water and soil solutions. The level of dilution, and thus the potential allelopathic effects, will be determined by the volume of rain water or simulated-rain water added to the soil system during natural or simulated-rain events, respectively. Those collected by centrifugation will likely represent soil solutions. Just like soil extracts (another potential way of collecting organic and inorganic compounds in soil and soil solutions), soil leachates and soil solutions represent residual concentrations and not total available concentrations. With an appropriate experimental design, potential allelopathic effects of soil leachates and soil solutions (assuming enough can be collected) for given points in time and for given places and systems can be determined in the presence of other causative and modifying environmental factors (see Sects. 4.4.3 and 6.4.2). Soil leachates and soil solutions can also be used to isolate, identify, and collect compounds that may have promoter, inhibitor, or herbicidal properties.

Plant Tissues, Litter, and Residues If tissues, litter, and residues are collected at the correct time, if litter and residues are collected at the right state of weathering and decomposition, if the collected materials are not altered dramatically by processing, and if they are added to field soil samples at the appropriate concentration (i.e., quantity, amount, or level) and location, then plant tissue, litter, and residue bioassays can provide reasonable insight regarding their potential stimulation or inhibition of bioassay species. Changes in soil moisture, temperature, pH, aeration, structure, inorganic compounds, and microbial populations with increasing levels of plant tissues, litter, and residues on the surface or within the soil will, however, make identification of the actual cause and effect challenging. Thus, when utilizing increasing or decreasing amounts of plant tissue, litter, and residues in bioassays, linked changes in soil pH, moisture, inorganic compounds, organisms, soil aeration, etc. must be accounted for in the experimental design. However, given an appropriate experimental design, it is possible to determine allelopathic effects of plant tissues, litter and residues for given points in time and for given places and systems in the presence of other causative and modifying environmental factors (see Sects. 4.3.4 and 6.4.1).

Soil cores with and without plant tissue, litter, and residues can also be used to determine effects of tissue, litter, and residues. However, actual causation will be extremely difficult, if not impossible, to determine for soil cores unless differences in the physiochemical and biotic environments for the soil cores can also be determined.

"Root Exudates Plus" "Root exudates plus" (see Sect. 2.1.3.2) collected with sterilized solution (e.g., in culture flasks) and from agar cultures are adequate for the isolation and identification of compounds released (active and passive processes) from roots. However, since the rates and amounts of "root exudates plus" lost or released from roots are to a large extent regulated by rates of leaching, activity of microorganisms, presence or absence of symbiotic relationships, the growth of shoots and roots, type of media, the nature of the solutions surrounding the roots and mycorrhizae, the rates of sorption and desorption, the rates of uptake of roots and mycorrhizae, etc., the "root exudates plus" produced in such closed sterile systems are not qualitatively and quantitatively representative of "root exudates plus" found under field conditions (Shay and Hale 1973; Barber and Gunn 1974; Barber and Martin 1976; Prikryl and Vančura 1980; Hodge et al. 1996; Neumann and Römheld 2007). Bioassays using such closed sterile system, thus, provide at best only limited insight regarding the actual production, modifications, and effects of allelopathic compounds released into natural soil systems.

The direct transfer in larger semi-closed or open systems with or without XAD resins or other sorbents (two container or stair step systems; see Sects. 2.1.3.2 and 4.2.3) should theoretically be more natural in the production of "root exudates plus" but the use of sand or a substrate (essentially porous inert materials), the frequent absence of symbiotic relationships, and the constant recycling of leachates within semi-closed systems or the collection of multiple leachates from the same plant or even different plants in open systems will generate different total and available concentrations than would be expected in field soils. Furthermore particularly for semi-closed systems used over extended periods, unless inorganic compounds, pH, and water levels of the solution re-circulating throughout the systems are carefully monitored and frequently adjusted, competition for inorganic compounds (i.e., nutrient), pH and solute potentials may be confounding factors. These systems also tend to be highly aerobic and, therefore, activities of microorganisms and oxidation/reduction are unlikely to be similar to those of soil environments. Finally, only the water-soluble compounds for "root exudates plus" are tested in these multi-pot systems since only those are leached and transferred from pot to pot. However, even with these limitations and if systems are properly managed, a potential role, if any, of "root exudates plus" leachates in allelopathic interactions may be determined (e.g., Sect. 4.2.3.1).

2.1.3.4 Application of Test Materials

The common procedure for solutions containing water soluble compounds (e.g., IPA compounds, extracts, and leachates) has been to apply these solutions directly to a bioassay species (e.g., Petri dish or solution culture) or to apply them to the surface of a medium containing a bioassay species (e.g., sand or soil). The application of test materials in recirculating systems (e.g., a "root exudates plus" stair step system) is, thus, unique since the source of the treatment materials comes directly from a putative allelopathic plant within the system. Fresh or dried tissues have

frequently been chopped or ground and placed on the surface of a medium or mixed throughout the medium. Unfortunately, in many instances such applications of test materials are not consistent (i.e., atypical) with what occurs under field conditions. Every effort should be made so that test or treatment materials applied to laboratory systems are consistent with the nature, location, and distribution of materials in the field. For more details regarding how such atypical applications may impact the behavior of bioassay species see Sects. 3.3 and 7.2.1.

2.1.4 Measurements

Both destructive and non-destructive measurements have been utilized to quantify how IPA compounds, extracts, leachates, litter, residues, etc. modify the behavior of bioassay species and system components and processes (e.g., physical, chemical, and biotic [predominately microbial] components and processes). Measurements range from the molecular level to the whole system level. What is measured and when, where, and how measurements are to be taken really depends on the experimental objectives of the research. On the surface it would appear that determining what to measure and when, where, and how to take measurements should be a simple matter. However, making these choices for a bioassay species and system should not be taken lightly since the measurements chosen can/will for example:

a. determine the nature and complexity of the experimental design and the type of model system chosen for a study (e.g., solution culture, substrate culture, etc.),
b. determine the level (e.g., molecular, cellular, tissue, organ, whole plant, or system) at which the effects of treatment materials are observed,
c. determine the types of questions that can reasonably be answered, and
d. determine how well measurements and observations made and conclusions reached for a chosen laboratory bioassay system will relate to the behavior of seeds, seedlings, plants, microorganisms, etc. in natural or field (managed) systems.

Most plant-plant allelopathic bioassays are designed to study how the behavior of a bioassay species (e.g., seeds, seedlings or older plants) is modified by promoters or inhibitors released from putative allelopathic plants or how various system components (e.g., media, biotic and physicochemical environment, etc.) and processes (e.g., sorption, leaching, uptake, and microbial utilization and transformation) may modify this behavior. When we add that the behavior of bioassay species and their associated systems are highly variable, at times ephemeral and extremely complex, it should not be surprising to find that most researchers have chosen measurements that integrate, summarize, or average all this behavior (i.e., represent some final end product). Thus, measurements such as rates of germination, radicle length, or changes in biomass of seedlings (actual size, weight, or growth rates) dominate the literature. In many ways this is logical since the processes of germination, seedling emergence, and growth, as well as tolerance/resistance, competitiveness, reproduction, and seed dispersal are or can be excellent indicators of the

success or failure of species in nature. For agricultural or horticultural species, the bottom line varies somewhat with the commercial and aesthetic interests of the producer and consumer. However, the processes of germination, seedling emergence, competition, survival, and vegetative and sexual reproduction (yield) are just as important as in natural systems. A lack of seed dispersal at maturity is a desirable characteristic for most agricultural and horticultural plants. In spite of their usefulness these measurements are far removed from the primary effects of stressors including allelopathic compounds. They have arisen from primary effects by way of a cascade of secondary, tertiary, etc. effects. Thus, these types of measurements are excellent for surveying or screening types of bioassays (i.e., are treatment materials effective as promoters or inhibitors) but not all that helpful for identifying primary sites and effects and modes of action (see Chaps. 3, 4, and 6).

For determining primary sites, effects, and modes of action lower levels (i.e., at cellular level and below) and frequently more ephemeral and elusive processes must be measured. For example, determining primary effects of phenolic acids would appear to require measurements of root membrane depolarization or alteration of membrane permeability since root membranes are a likely site of initial contact (i.e., primary sites; see Sect. 5.2.2). The other initial site of contact would be root cell walls. Changes in net water uptake or net nutrient uptake, nutrient efflux, metabolites, energy flow (i.e., carbon fixation, respiration, and translocation), cell wall lignifications, etc. resulting from the phenolic acid induced changes in membrane depolarization/permeability and cell walls already represent secondary, tertiary, etc. effects (i.e., the cascade of effects) that may or may not eventually lead to reductions in growth, yield, etc. Plants after all have considerable buffering capacity, the ability to acclimate, and a broad range of responses to biotic and physiochemical environments, (see Chaps. 3 and 7), thus, cellular-level measurements in laboratory bioassays may or may not be directly relevant to the final product of interest, the rates of germination, or seedling emergence, growth, and reproduction under natural or field conditions. For additional details see Sect. 3.11 and Chaps. 5 and 6.

To identify and characterize the primary sites and modes of action by which allelopathic compounds initiate and ultimately produce plant effects, however, requires much more comprehensive molecular and cellular methods than the type of measurements just described. A Group of tools that presently show considerable promise are the omics methods (e.g., genomics, proteomics, metabolomics, and physionomics). What omics methods can do is provide detailed information on genetic and biochemical impacts of allelopathic compounds (e.g., Gidman et al. 2003; Macías et al. 2007; Duke et al. 2008, 2013; Chen et al. 2009; Leão et al. 2009). For details regarding the potential use of omics methods in this way see Sect. 6.6.

There are three primary reasons for measuring other aspects of laboratory bioassay systems besides the behavior of the bioassay species.

a. To characterize the biotic and physicochemcial environment of the bioassay system.
b. To determine how a range of biotic and physicochemical factors (i.e., modifiers) found in nature may influence or modify the behaviors of bioassay species to promoters and inhibitors (see Sect. 5.2.3).

c. To determine cause and effect. Determining cause and effect requires that all other environmental factors (other than the treatment factor of interest) are initially constant, stay constant, or that changes occurring during the experimental period are measured and accounted for adequately before the final interpretations of the results are made by the researcher (see Sect. 6.4).

Measurements taken initially and over time could among other things include the following for solution and substrate bioassays.

Solution Bioassays

a. types and concentrations of treatment compounds,
b. types and concentrations of organic compounds other than treatment compounds present,
c. types and concentrations of inorganic compounds present,
d. types and population levels of microorganisms present,
e. presence or absence of mycorrhizae,
f. solute potentials, pH, temperature, and aeration of solutions, and
g photoperiod, light quality and quantity, temperature, and relative humidity of the growth environment.

Substrate Bioassays

a. types, concentrations, and distribution of treatment compounds,
b. types, concentrations, and distributions of organic compounds within the substrate other than treatment compounds,
c. types, concentrations, and distributions of inorganic compounds within the substrate,
d. types, population levels, and distribution (bulk soil, rhizosphere, rhizoplane, etc.) of microorganisms present,
e. presence or absence of mycorrhizae,
f. composition, texture, and structure of substrate,
g. temperature, pH, moisture status, compaction, and aeration of substrate, and
h. photoperiod, light quality and quantity, temperature, and relative humidity of the growth environment.

2.1.5 Hypotheses, Experimental Designs, and Data Analyses

Initial formulation of testable hypotheses before designing bioassays is essential. Once these have been formulated, experimental designs to test these hypotheses can be constructed. Afterwards hypotheses can be modified as appropriate and necessary or new hypotheses can be formulated. Hypotheses, thus, have a major role in determining the design of bioassay systems that will be used, the types of data that will be collected, when and how data will be collected, and how the resulting data will be analyzed. Hypotheses are tentative experimentally testable statements that provide potential explanations for phenomena, processes, or events and that

frequently include predictions as to the outcome if the hypotheses are true. Recall, however, that Science progresses not by trying to confirm hypotheses but by attempting to falsify hypotheses (Blum 2011). Examples of testable hypotheses can be found in Chaps. 4 and 6.

Examples of experimental designs for laboratory bioassays, methods and types of data to be collected, and general comments regarding data analyses can be found throughout the volume and in particular in Chaps. 4 and 6. In addition the benefits and limits of laboratory bioassays (Sect. 1.6), false assumptions and misconceptions of laboratory bioassays (Sect. 1.7), the fundamentals of laboratory bioassay systems (Chap. 2), issues and challenges when designing laboratory bioassays (Chap. 3), comparisons of field and laboratory systems (Sect. 7.2), and how to make laboratory bioassays more relevant to field systems (Sects. 1.5.2 and 7.4) are also provided in various sections and chapters of this volume.

Finally, it is beyond the scope of this volume to provide an in-depth description of statistical analytical procedures and techniques. Thus, the reader is encouraged to consult with a statistician and books on statistics for identifying and implementing appropriate experimental designs and to use the appropriate statistical techniques and tools to analyze and interpret their data (Steel and Torrie 1997; Summer et al. 2003; Cox and Donnelly 2011; Sokal and Rohlf 2012).

2.2 Basic Information Required for All Bioassay Systems

The following information should always be provided for the following types of bioassays:

a. Germination and early seedling development in Petri dishes with filter paper or other substrates:
   1. size and type (e.g., plastic or glass) of Petri dish,
   2. size and type of filter paper or type (e.g. agar, sand, and soil), composition, amount, and volume of other substrates,
   3. presence or absence and source of microorganisms; in the absence of microorganisms, the type of sterilization procedure used,
   4. source and type of species being tested, number of seeds or seedlings per Petri dish, and location of seeds in reference to substrate (i.e., on surface or the depth below surface),
   5. type (e.g., IPA compounds, extracts, leachates [e.g., tissues, litter, residues, or "root exudates plus"], tissues, litter, or residues) and source of the materials being tested (e.g., How, when, and where was it collected? How was it processed and stored prior to use?),
   6. volume, concentration, pH, and solute potential of IPA compound solutions, extracts, or leachates added to Petri dish, or concentration, particle size, nature, and location (e.g., on top of substrate or mixed into substrate) of tissues, litter, or residues added to substrate in Petri dish,

7. volume, concentration, composition (e.g., nutrient solution, water, etc.) and nature (e.g., pH, solute potential, etc.) of reference or control solutions, or substrates,
8. type and frequency of treatments and other additions (e.g., nutrients),
9. incubation temperature, relative humidity if Petri dishes are not sealed; if sealed, type of seal, length of incubation, levels and type of lighting, and photoperiod,
10. numbers of times solutions (e.g., water, treatments solutions) were added to compensate for evaporation if Petri dishes were unsealed and range of maximum and minimum volume of solution or moisture content of the substrate,
11. method and frequency of measurements and length of experiment, and
12. other relevant treatment and environmental factors.

b. Seedling or older plant solution bioassays:
   1. size (diameter and volume) and type of solution container (e.g., shape, plastic, glass, etc.)
   2. presence or absence and source of microorganisms; in the absence of microorganisms, the sterilization procedure used,
   3. presence and source of microorganisms for the formation of symbiotic relationships,
   4. source and type of species being tested, age of seedlings or plants, stage of development, and number of seedlings or plants per container,
   5. type (e.g., IPA compounds, extracts, or leachates [e.g., tissues, litter, residues, or "root exudates plus"]) and source of materials being tested (e.g., How, when, where was it collected or obtained? How was it processed and stored prior to use?),
   6. volume, concentration, pH, and solute potential of IPA compound(s) solutions, extracts, or leachates added to container
   7. volume, concentration, composition (e.g., nutrient solution, water, etc.) and nature (e.g., pH, solute potential, etc.) of reference or control solutions,
   8. frequency and timing of solution changes or additions and frequency and timing of water additions to compensate for evapotranspiration,
   9. type of growth environment before, during and after treatments (e.g., level of aeration, levels and type of lighting, photoperiod, and temperature),
   10. method and frequency of measurements and length of experiment, and
   11. other relevant treatment and environmental factors.

c. Seed, seedling or older plant substrate bioassays other than Petri dishes:
   1. size (diameter and volume) and type of container (e.g., shape, plastic, glass, etc.),
   2. composition, type, nature, amount, and volume of substrate,
   3. presence or absence and source of microorganisms; in the absence of microorganisms, the sterilization procedure used,
   4. presence and source of microorganisms for the formation of symbiotic relationships,

5. source and type of species being tested, age of seedlings or plants, number of seeds, seedlings or plants per container, and location of seeds in reference to substrate (i.e., on surface or the depth below surface),
6. type (e.g., IPA compound solutions, extracts, leachates [e.g., tissues, litter, residues, or "root exudates plus"], tissues, litter, or residues) and source of materials being tested (e.g., How, when, and where was it collected or obtained? How was it processed and stored prior to use?),
7. volume, concentration, pH, and solute potential of IPA compound(s) solutions, extracts or leachates added to substrate, or amount, particle size, nature, and location (e.g., on top of the substrate or mixed into the substrate) of tissue, litter or residues added to substrate,
8. controls, reference or baseline: volume, concentration, composition (e.g., nutrient solution, water, etc.) and nature (e.g., pH, solute potential, etc.) of reference or control solutions added to substrate or amount, particle size, nature, and location (e.g., on top of the substrate or mixed into the substrate) of inert material added to substrate,
9. if no nutrients are added to the substrate, then inorganic compound content particularly nutrient content of the substrate should be provided,
10. timing and frequency of solution or material additions to substrate, frequency and timing of water additions to compensate for evapotranspiration, and range of maximum and minimum moisture content of the substrate,
11. type of growth environment before, during and after treatments (e.g., levels and type of lighting, photoperiod, and temperature),
12. method and frequency of measurements and length of experiment, and
13. other relevant treatment and environmental factors.

In addition to the materials and methods, clear and precise information regarding location of the bioassay (e.g., light banks, growth chambers, or greenhouses), the distribution of the experimental units within a location (e.g., completely randomized, blocked, randomized and blocked, etc.), experimental design, and the tools and procedures used to analyze the resulting data collected must be provided. Background of the research topic, justification for the research, objectives of the research, and hypotheses tested should also be provided in clear and precise language.

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Chapter 3
Some Issues and Challenges When Designing Laboratory Bioassays

Abstract This chapter discusses a number of issues and challenges associated with creating more relevant model laboratory bioassays including the following: treatment concentrations, mobility and transport, species density, symbiotic relationships, microorganisms, controls and references, and measurements among others.

3.1 Introduction

If we are going to make progress in understanding the role of plant-plant allelopathic interactions in the field, then we need to shift our present approach in the laboratory from single-factor bioassays (e.g., simple dose-response studies; see Belz et al. 2005, 2007) to more holistic bioassays (Note: Not completely holistic since that is not possible but more holistic; See Sect. 1.5). That requires that we have a clear understanding of the criteria for identifying and characterizing plant-plant allelopathic interactions and the limits and flaws of our present laboratory bioassay systems and experimental designs.

For a complete listing of original and updated (i.e., more holistic) criteria for establishing plant-plant allelopathic interactions in the field, see Blum (2011, p. 3 and 180). Among the updated criteria customized here to fit laboratory bioassays, are the following:

a. Treatment effects cannot be solely explained by physicochemical modifications (the exception being promoters and inhibitors, i.e., allelopathic compounds) of bioassay systems, the utilization of applied treatments as sources of nutrients, carbon, and energy, and by competition, herbivory, or disease. (Note: The physicochemical and biotic environments are still central to the observed allelopathic effects but cannot be solely responsible for the observed effects. They are in fact modifiers of active allelopathic compounds [i.e., promoters and inhibitors] since pH, available water, inorganic compounds and organic compounds other than promoters and inhibitors [although promoters and inhibitors can also act as modifiers], aeration, density of bioassay species, microorganisms, presence or absence of symbiotic relationships, etc. all will influence the available and active concentrations and the actions of promoters and inhibitors that contact and interact with bioassay species.)

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b. Treatment solutions (i.e., identified putative allelopathic [IPA] compounds, leachates [e.g., tissues, litter, residues, or "root exudates plus"], and soil solutions [e.g., extracts]), tissues, litter, and residues added to bioassay systems must contain and/or release organic compounds (i.e., promoters or inhibitors) that will stimulate or inhibit cellular and plant processes of bioassay species under the prevailing conditions of a bioassay and/or contain and release organic compounds that will ultimately lead to the production of organic compounds (i.e., promoters or inhibitors) that will stimulate or inhibit the cellular and plant processes of bioassay species under the prevailing conditions of a bioassay. (Note: The inclusion of "lead to the production of" is an acknowledgment of the substantial roles oxidation, reduction, microflora, and microfauna have in modifying and producing new allelopathic compounds from organic compounds including allelopathic compounds in the soil.)
c. The affected bioassay seeds, seedlings, and plants must come in contact with and interact with organic promoters or inhibitors. The surface area contacted by promoters and inhibitors must be sufficient enough to have an effect. (Note: The affected seeds, roots, and mycorrhizae, with the possible exception when testing pure IPA compounds in solution culture [e.g., Petri dish germination], are actually in contact with a complex of both organic and inorganic compounds including promoters, modifiers, and inhibitors. Stimulation requires that promoters dominate within these complexes and inhibition requires that inhibitors dominate. This assumes that any organic compound can function as a promoter, modifier, inhibitor, or neutral compound depending on its concentration and physicochemical state and the sensitivity of the process being monitored [see Sect. 2.1.3.1].)
d. The organic promoters and inhibitors must be at sufficient concentrations and be present for sufficient length of time to modify the behavior of the bioassay species directly by way of membranes and cell processes or indirectly by impacting the formation, development, and function of nodules or mycorrhizae (i.e., symbiotic relationships).

So how do we design laboratory bioassays that are relevant to field systems (e.g., not atypical) and meet these criteria? Achieving that goal will require addressing and solving a number of challenging design issues and an appropriate appreciation and understanding of the processes and the behaviors of laboratory and field ecosystems. Although a number of issues and challenges regarding experimental design have already been alluded to in Chap. 2, in this chapter we take another more in-depth look at those and add a number of others. What follows is a sampling of some of the major issues and challenges related to the design of more relevant bioassay systems that need resolution.

3.2 Treatment Concentrations

3.2.1 Minimum Concentrations

Some initial efforts have been made to identify the range of effective concentrations for stimulatory effects (primarily hormesis) of individual and simple mixtures of identified putative allelopathic (IPA) compounds (Rice 1986; An et al. 1993; Belz et al. 2005, 2007; Duke et al. 2006; Belz 2008). However, current data available for such stimulatory concentrations are very limited. Data for stimulatory effects of complex mixtures of compounds (e.g., extracts, leachates, "root exudates plus", tissues, litter, and residues) are also very limited (Rice 1986; Mallik and Williams 2005; Wang et al. 2009; Li et al. 2010; Lorenzo et al. 2010; Song 2012). Efforts to determine the range of effective concentrations for inhibitory effects of individual and simple mixtures of IPA compounds and complex mixtures of compounds have been much more frequent and extensive and, thus, the focus here on inhibitory concentrations. (Note: The focus for inhibitory compounds has been primarily on the lower end of the range [i.e., minimum concentrations required].) Finally, for simplicity and clarity comments will be limited primarily to bioassays of IPA compounds recognizing that most of the comments will also be pertinent to some extent for complex mixtures of compounds.

In the laboratory the range of inhibitory concentrations for individual or simple mixtures of IPA compounds has frequently been determined by dose-response studies (see Rice 1984; Blum 1996, 2011; Belz et al. 2005, 2007). Based on such dose-response studies, rough approximations of minimum effective concentrations for inhibitory effects have been identified. Since in many instances these minimum effective concentrations for inhibitory effects have been greater than found in soil extracts and soil solutions, detractors have used these high minimum effective concentrations of IPA compounds as one piece of evidence that plant-plant allelopathic interactions are not important in nature (for additional critical comments see Harper 1975, 1977; Stowe 1979; Sect. 7.3). Although from a theoretical standpoint minimum effective concentrations for laboratory bioassays can be useful (e.g., determining how modifiers impact inhibitory effects of IPA compounds), from a practical standpoint they are not that helpful in determining the presence or absence of inhibitory plant-plant allelopathic interactions in the field. It seems that both proponents and detractors of inhibitory plant-plant allelopathic interactions have neglected to integrate the following elements in their thinking:

a. Soils contain complex heterogeneous mixtures of available (free) and active organic and inorganic compounds (Flaig 1971; Brady 1984; Paul and Clark 2007; Lavelle and Spain 2001; Blum 2011).
b. The effect of any individual organic and inorganic compound on any given plant process can theoretically be stimulatory, neutral, or inhibitory depending on its concentration and its physicochemical environment (Rice 1986; An et al. 1993; Belz et al. 2005, 2007; Duke et al. 2006; Belz 2008).

c. The actions of individual compounds in a mixture can be independent, similar joint action (synergistic, additive, or antagonistic), or modifying to the actions of the other compounds present in a mixture (see Sect. 1.2; Rasmussen and Einhellig 1977; Einhellig and Rasmussen 1978; Einhellig et al. 1982; Williams and Hoagland 1982; Blum et al. 1984, 1985a, 1989, 1993; Bradow and Connick 1988a, b; Lyu et al. 1990; Gerig and Blum 1991; Li et al. 1993; Lehman et al. 1994; Weidenhamer et al. 1994; Pue et al. 1995; Blum 1996; Reigosa et al. 1999; Inderjit et al. 1997, 2002; Fujita and Kubo 2003).
d. For a given percent inhibition, as the number of promoters, modifiers, and inhibitors are increased in a treatment mixture, the concentrations of the individual compounds making up that mixture must be adjusted (increased or decreased) to maintain the same percent inhibition (Blum et al. 1993; Pue et al. 1995; Blum 1996).
e. Nature of the medium (e.g., type of solution or substrate), pH, solute potential, temperature, organic compounds (other than promoters and inhibitors; although promoters and inhibitors can also act as modifiers as well), inorganic compounds, utilization and synthesis by microorganisms, bioassay species density, presence or absence of symbiotic relationships, etc. all can dramatically modify the minimum concentrations required for inhibition (see Sparling et al. 1981; Weidenhamer et al. 1987, 1989; Blum et al. 1985b, 1989, 1993; Einhellig 1989; Pue et al. 1995; Lehman and Blum 1997; Blum 2004, 2006, 2011; Gawronska and Golisz 2006; Pedrol et al. 2006).
f. The minimum concentration required for inhibition (or stimulation) also depends on the process or end product being monitored.

The essential points here are: a. that minimum concentrations required for inhibitory effects of IPA compounds in field and laboratory systems are a product of the actions and interactions of all the active organic and inorganic compounds present (i.e., all the promoters, modifiers, and inhibitors present not just IPA compounds) and the physicochemical and biotic environments of their respective systems and b. that the nature and complexity of active organic and inorganic compounds and environments of field and laboratory systems are very different (see Sect. 7.2). If we accept this as true, then the following statements are also true:

a. Our past dependence on minimum effective concentrations of individual or simple mixtures of IPA compounds in laboratory bioassays as a means of demonstrating the presence of inhibitory plant-plant allelopathic interactions in the field was and is not appropriate.
b. It is also not appropriate for skeptics to argue that inhibitory plant-plant allelopathic interactions are not important in the field because minimum concentrations of individual or simple mixtures of IPA compounds used in laboratory bioassays to demonstrate inhibition are completely unrealistic (i.e., much too high) and that such high concentrations are very seldom if ever found in field soils.

Actually, the minimum concentrations (inputs) required for an observable effect (stimulation or inhibition) in laboratory bioassays by putative allelopathic com-

pounds or complex mixtures containing promoters, modifiers, and inhibitors are determined by the bioassay species chosen, the bioassay system chosen, the process measured, and the management of that system by the researcher. Minimum concentrations required for an effect in laboratory bioassays are, thus, a moving target. Data on minimum concentrations required for an effect in field systems are not available. Meaningful comparisons of minimum concentrations for laboratory and field systems will require such data. In essence, we do not have the data to make a fair comparison of laboratory and field systems (also see Sect. 7.3).

If minimum effective concentrations of individual IPA compounds or even simple mixtures of IPA compounds in laboratory bioassays are presently not that helpful in establishing the presence of plant-plant allelopathic interactions in the field, then what is needed to establish such interactions in the field? That turns out to be the $ 64,000 question to which we presently have no clear answer. What is clearly evident, however, is that to answer this question we urgently need to characterize the promoter/modifier/inhibitor complexes in field systems of interest and determine the concentrations by which IPA compounds individually or within complex mixtures stimulate or inhibit sensitive species. Recent advances in genomics, proteomics, metabolomics, and physionomics (Gidman et al. 2003; Dunn and Ellis 2005; Macías et al. 2007, Wallenstein and Weintraub 2008; Duke et al. 2008, 2013; Chen et al. 2009; Fan et al. 2009; Leão et al. 2009; Liebeke et al. 2009; Metz 2011) and uptake and collection techniques (Mohney et al. 2009; Weidenhamer et al. 2009) in conjunction with appropriate analytical tools (Summer et al. 2003; Dunn and Ellis 2005; Xi et al. 2008; Corte et al. 2010; Kim et al. 2011) could no doubt greatly assist in doing that in the future (see Sect. 6.6).

3.2.2 Modifiers of Active/Effective Concentrations

Since the minimum and range of treatment concentrations for inhibition of a given sensitive species depends on the process being monitored and the nature of a given laboratory bioassay system, a clear understanding of the functions of modifiers (and the nature of the promoter/modifier/inhibitor complex) within a given bioassay system is essential. Furthermore if concentrations for inhibition in laboratory bioassays are to be linked with concentrations for inhibition in field systems, we will need answers to the following questions:

a. What modifiers are present and absent for a specific laboratory bioassay system compared to the field systems of interest?
b. Is the function (e.g., rate or action) of a specific modifier within a given laboratory bioassay system typical or atypical when compared to its function within a field system of interest? If the function of a modifier is atypical, how can it be adjusted or modified to make it “more typical”?
c. How is the function of a given modifier influenced by the presence of other modifiers?

d. How do the presence or absence of modifiers influence the treatment concentrations required for inhibition (or stimulation)?
e. What modifiers are essential for laboratory bioassay systems if inhibitory (or stimulatory) concentrations of allelopathic compounds in laboratory bioassay systems are to be linked with inhibitory (or stimulatory) concentrations of allelopathic compounds in the field?

What follows are some general comments about the potential roles of some of the most obvious modifiers in laboratory bioassay systems. Some examples of the ways factors and processes may actually modify treatment and solution concentrations and the activity and effectiveness of IPA compounds are summarized in Tables 3.1 and 3.2.

3.2.2.1 Media

The choice of media greatly influences the range of concentrations at which putative allelopathic compounds are effective in stimulating or inhibiting germination, growth, development, and reproduction of sensitive bioassay species. This has already been alluded to in various ways in previous sections (e.g., see Sects. 1.4 and 2.1.2.2). It is, however, important to reemphasize this point here. Different media ranging from solutions to substrates will have different:

a. physical composition (makeup and structure),
b. pore sizes, compaction, and moisture retention for substrates,
c. levels of aeration and oxidation/reduction,
d. physical resistances to growth of radicles, roots and mycorrhizae,
e. amounts of organic and inorganic modifiers present within the media,
f. levels of nutrition, pH and solute potentials,
g. types and amounts of surface area for sorption and thus different rates and amounts of fixation of organic compounds including IPA compounds,
h. populations and activities of micro-, mesa-, and macro-organisms, and
i. transport rates of and distribution patterns for organic and inorganic compounds including promoters, modifiers, and inhibitors.

All of these factors interact in various ways depending on the medium and the order of their importance will change with environments. If, however, we simply base our conclusions on media structure and chemistry and assume that everything else is roughly equivalent, then the minimum effective concentrations required for an effect from lowest to highest might theoretically look like this: solution media < sand media <soil media. The author is not aware of any attempt to actually quantify these relationships for a broad range of media but Blum (2011) estimated that a 20% inhibition of cucumber seedling leaf expansion required three treatments of 0.14 mM of *p*-coumaric acid in nutrient culture (Blum and Gerig 2005) and three treatments of 0.55 µmole/g soil in a Cecil A-horizon soil system (Blum and Gerig 2006). Treatments were given on alternate days. Each treatment of 0.55 µmole/g

Table 3.1 Some examples of potential modifiers of active/effective concentrations of water-soluble identified putative allelopathic (IPA) compounds in the absence of microorganisms

Modifier	Factors and processes that can modify the concentration, activity, or effectiveness of IPA compounds
Media	
Solution	Oxidation/reduction of IPA compounds depending on aeration; little if any resistance to movement of IPA compounds in solutions; even distribution of IPA compounds in solutions, maximum contact of IPA compounds with seeds, roots, and mycorrhizae
Quartz sand	Generally minor sorption of IPA compounds; considerable potential for oxidation of IPA compounds; resistance to movement of IPA compounds; distribution of IPA compounds fairly consistent in sand although gradients are possible; reduced contact and uptake of IPA compounds with seeds, roots and mycorrhizae
Soil	Reversible and irreversible sorption and polymerization of IPA compounds; oxidation/reduction of IPA compounds depending on aeration; considerable resistance to movement of IPA compounds; variable distribution of IPA compounds in soil; gradients of IPA compounds in soil are common; much reduced contact with seeds, roots, and mycorrhizae
Solution volume or soil moisture	Determines the concentration of IPA compounds in solutions and the residual concentrations of IPA compounds in solid media
pH	Determines the solubility and charges (i.e., neutral, positive, or negative) of IPA compounds
Organic compounds other than identified putative allelopathic (IPA) compounds	Modifies observed effects of IPA compounds; actions of other organic compounds can be independent, synergistic, additive, or antagonistic
Inorganic compounds	Can enhance polymerization and sorption of IPA compounds to substrate particles; particularly true for multivalent cations such as Mn^{++} (e.g., polymerization) and Ca^{++} (e.g., cation bridges)
Distribution and mobility	Determines the frequency of contact and uptake by seeds, roots, and mycorrhizae
Frequency of treatments	Determines the concentrations of IPA compounds in bioassay systems over time
Density of seeds and roots and growth of roots	Determines size of surface area for contact and uptake of IPA compounds; the contact and potential uptake concentrations (e.g., molecules per unit surface area) of IPA compounds; the frequency of contact of IPA compounds in solid media

soil was roughly equivalent to 4.12 mM in the soil solution immediately after addition (i.e., before sorption, utilization by microbes, etc.). Based on these observations, the mM of *p*-coumaric acid added for a 20 % inhibition in this soil system was 29 times greater than in the nutrient culture system.

Table 3.2 Some examples of potential modifying actions of microorganisms on active/effective concentrations of water-soluble identified putative allelopathic (IPA) compounds

Modifiers	Factors and processes which can modify the concentration, activity, or effectiveness of IPA compounds
Organic compounds other than identified putative alleopathic (IPA) compounds	Preferential utilization of organic compounds over IPA compounds by microorganisms can prolong the presence of IPA compounds, i.e., can maintain higher concentrations for longer periods of time; preferential utilization of IPA compounds over other compounds will do the opposite
Inorganic compounds (nutrients)	When nutrients are limiting, increasing nutrient concentrations can stimulate microbial conversion and utilization of IPA compounds and the synthesis of new and potentially unique organic compounds
Symbiotic relationships	Presence or absence of symbiotic relationships can directly or indirectly influence the sensitivity/susceptibility of plant species to IPA compounds
Mycorrhizal fungi	Presence or absence of mycorrhizae will determine the surface area for contact of IPA compounds, the uptake of IPA compounds by seedlings or older plants, and the types and activities (e.g., utilization of IPA compounds) of associated microorganisms present in the rhizoplane, rhizosphere, and hyphosphere of mycorrhizae
Rhizobium or *Bradyrhizobium*	Presence or absence of root nodules can determine the types of organic (e.g., compounds containing nitrogen) and inorganic compounds (e.g., ammonia and nitrate) immediately associated with the rhizoplane and rhizosphere of roots and mycorrhizae and the types and activities (e.g., utilization of IPA compounds) of associated microbes present
Rhizoplane, rhizosphere, and mycorrhizosphere microorganisms	Determine the utilization and conversion of IPA compounds, turnover rates of IPA compounds and their breakdown products, and the synthesis of new compounds from IPA
General environment (pH, temperature, moisture, etc.)	Influences or determines the species of microorganisms that are active in the bulk soil, on the rhizoplane, and in the rhizosphere or mycorrhizosphere and the types and rates of IPA compounds utilized and synthesized; may also modify the sensitivity/resistance of bioassay species
IPA compounds	Application(s) of IPA compounds to bioassay systems will frequently lead to induction and selection of microorganisms that can utilize IPA compounds as a carbon and energy source

3.2.2.2 Soil Moisture

Concentrations of IPA compounds in soils are frequently expressed as µg/g soil or µmol/g soil. For purposes of comparing a range of different compounds, µmol/g soil is better because of the differences in molecular weights of IPA compounds. Unfortunately the actual concentrations in soil solutions (µM or mM) that are available to interact with seeds, roots, mycorrhizae, and soil organisms are not adequately represented by the per gram soil values since soil moisture changes constantly over time due to inputs (e.g., rain or irrigation events) and losses (e.g., evapotrans-

piration and gravitational flow). For example, Blum and Gerig (2006) found that a 1 µmol/g soil *p*-coumaric acid treatment was initially equivalent to 7.21 mM in their cucumber seedling-microbe-soil bioassay system (150 g soil-sand mixture plus 20 ml solution; before sorption, utilization by soil organisms, etc.). Subsequently, the 7.21 mM of *p*-coumaric acid declined to 3.44 mM (a 52 % reduction) over the first 16 h and then slowly increased to 4.34 mM (a 26 % increase) over the next 8 h. The decline was due to a combination of soil processes (e.g., sorption, utilization by soil organisms, and root uptake [no mycorrhizae present]) and the increase was primarily due to the loss of water by evapotranspiration. Meaningful values for concentrations of available allelopathic compounds for plant tissues, litter, and residues added to soil (on top or within) are even more elusive when we add that a large portion of what is added to the soils is not available to enter the soil solution (e.g., bound organic compounds, cellulose, and lignin). Furthermore, what enters the soil solution changes unpredictably with time as tissues, litter, and residues are leached by watering and are acted upon by soil organisms (i.e., decomposed).

Ultimately the best that can be done is to minimize this problem by maintaining a minimum and a maximum solution volume for solution cultures or moisture content for substrate cultures (e.g., soil). This can be achieved by adding water back to the culture systems at regular intervals to compensate for evapotranspiration and other losses (e.g., gravitational loss from containers). The central point is that the concentrations of water-soluble compounds added and the concentrations experienced by seeds, root, mycorrhizae, and soil organisms are, among other things, highly dependent on solution volume or soil moisture.

3.2.2.3 Organic Compounds

The modes of action or mechanisms by which available (free) complex mixtures of organic compounds present in media interact to modify observed inhibition (or stimulation) has not been adequately explored. There are sufficient data, however, to suggest that they do modify the magnitude of inhibition of plant processes by IPA compounds. For example, Blum et al. (1993) and Pue et al. (1995) observed that the concentration of *p*-coumaric acid required for a given percent inhibition of morning glory biomass decreased as non-inhibitory concentrations of glucose were added. The increased inhibitory activity of *p*-coumaric acid in the presence of glucose appeared to be due to a reduced microbial utilization of *p*-coumaric acid by soil microorganisms (Pue et al. 1995). Non-inhibitory concentrations of phenylalanine and *p*-hydroxybenzoic acid also slowed the utilization of *p*-coumaric acid by soil microorganisms. This, however, was not the case for methionine. Such differential utilization of organic compounds by microorganisms should not be surprising since it is fairly common (Martin and Haider 1979; Harder and Dijkhuizen 1982; Papanastasiou 1982; Sugi and Schimel 1993).

It has also been demonstrated that to maintain a given percent inhibition, the concentrations of individual inhibitory organic compounds within a treatment mixture had to be reduced as the number of compounds was increased. For example

increasing addition of organic compounds such as glucose and methionine at inhibitory concentrations reduced the concentrations required for a given percent inhibition of *p*-coumaric acid (Blum et al. 1993). Effects of compounds on each other, when not independent, were either additive or partially antagonistic. Similar observations have been made for phenolic acid mixtures (Blum et al. 1989; Lyu et al. 1990; Li et al. 1993; Lehman et al. 1994; Blum 1996). Synergistic effects for phenolic acid mixtures have also been reported but the evidence for synergistic effects is not very convincing (see Sect. 5.3). The author is not aware of any study that demonstrates how organic compounds that function as promoters and inhibitors may act or interact to bring about effects for sensitive species.

It should be evident from these examples that the types and combinations of available organic compounds present in bioassay systems will influence the magnitude of effects for allelopathic compounds. How such observations may specifically relate to the effects of complex organic mixtures such as leachates, soil solutions, litter, and residues has not been determined.

3.2.2.4 Inorganic Compounds

When nutrients are limiting in the soil, microbial utilization of organic compounds such as phenolic acids are also limited (Blum et al. 1999a). As nutrient supply is increased, microbial utilization increases until nutrient supply is no longer limiting. (Note: This assumes that no other environmental requirements are limiting microbial utilization.) As already stated in the previous section, the rate of utilization by microorganisms can or will determine the concentrations required for the inhibition of phenolic acids (Stowe and Osborn 1980; Hall et al. 1982, 1983; Blum et al. 1993, 1999a).

The roles of nutrients are, however, much more complex (see Sect. 2.1.3.1). For example, increasing nitrate in the soil decreases the inhibition of phenolic acids such as *p*-coumaric acid but increases the inhibition of methionine (Blum et al. 1993). There is also circumstantial evidence that nutrients such as NPK within plant tissues, and potentially within litter and residues, can influence inhibition more directly. Hall et al. (1983) observed that regression models for dry weight of pigweed seedlings versus total phenolic acid content and the variation of N, P, and K content in sunflower tissue were significant. Total phenolic acid alone did not account for a significant amount of the variability found for seedling dry weight unless N, P, and K content of the tissue (total N, P and K determined on dry-ashed plant samples) was included in the model. The relationships were negative for total phenolic acid and N and positive for P and K.

Soil nutrition will also influence the physiology, chemical composition, growth, and development of seedlings and older plants and, thus, their potential responsiveness to promoters and inhibitors. Finally, the types and concentrations of inorganic compounds in the media will not only provide the nutrients available to plants and microorganisms but also influence:

a. pH, buffering capacity, and solute potentials of test and soil solutions,
b. pH, salinity, and structure of substrates, and
c. substrate sorption and polymerization of organic compounds such as IPA compounds (see Blum 2006, 2011).

It should be evident at this point that the modifying influence of inorganic compounds in the media and within tissues, litter, and residues is substantial in nature and that these modifying influences will change the active/effective concentrations of the organic molecules (including IPA compounds) required for an effect.

3.2.2.5 pH

The role of pH as a modifying factor has been well established particularly when it comes to phenolic acids (Harper and Balke 1981; Blum et al. 1985b, 1989; Shann and Blum 1987; Lehman and Blum 1999). In general inhibitory effects of phenolic acids increase as acidity is increased since pH appears to determine the contact and uptake of phenolic acids by seeds, roots, and mycorrhizae (see Sect. 5.2.3.1). Among other things the pH of the media also determines solubility and availability of organic and inorganic compounds, sorption of organic and inorganic compounds to soil particles, and physiology, growth, and development of plants and microorganisms.

3.2.2.6 Density

There is sufficient evidence in the literature to suggest that as density of seeds, roots, and mycorrhizae (also microorganisms) increase, the concentrations required for a given percent inhibition must also be increased. Increasing density is somewhat analogous to increasing the dilutions of treatment solutions (e.g., increasing density of seeds and seedlings increases the treatment surface area and, thus, there are fewer molecules per unit surface area of seeds, roots, and mycorrhizae). There are also additional confounding factors for dose-response bioassays with increasing density of seedlings and older plants. As seedling and plant density increase, so do competition for resources and the potential for autotoxicity. See Sects. 2.1.1.5 and 3.5 for additional comments regarding seeds and seedling densities.

Density effects of microorganisms (see Sect. 3.7 for details) on promoter and inhibitor concentrations in a media depend on whether the microorganisms are producers or utilizers of promoters and inhibitors. For utilizers an increase or decrease in density will decrease and increase, respectfully, the effects observed. For example, Blum et al. (2000) using regression analysis observed that a 500 % increase in the colony-forming units of phenolic acid utilizing bacteria lead to a 5 % decrease in the inhibition of absolute rates of leaf expansion for cucumber seedlings treated with a 0.6 µmol/g equal-molar phenolic acid mixture of 4 phenolic acids.

3.2.2.7 Symbiotic Relationships

The absence or presence of nodules and mycorrhizae dramatically influences the nature of bioassay species as well as modifies their response (increase or decrease stimulation or inhibition) to a given concentration of IPA compounds, leachates, soil solutions (e.g., extracts), tissues, litter, and residues (for details see Sect. 3.6). The change in response to a given treatment concentration can be due to differences in the sensitivity of seedlings or older plants, differences in treatment surface areas of roots and mycorrhizae (see Sect. 3.7.4), and differences in the actions (e.g., synthesis, conversion, and utilization of organic compounds) of associated microorganisms (see below).

3.2.2.8 Microorganisms

Microorganisms are one of the key factors of bioassay systems that determine the minimum concentrations of IPA compounds, leachates, soil extracts, litter, or residues required for allelopathic effects, i.e., stimulations or inhibitions of sensitive species. They do that by modifying and utilizing organic compounds including allelopathic compounds and synthesizing new compounds, some of which may function as modifiers or allelopathic compounds (i.e., promoters or inhibitors). The activities of microorganisms in bioassay systems, thus, regulate the types and concentrations of modifiers and allelopathic compounds that reach seeds, roots, and mycorrhizae. Unfortunately our options for regulating and maintaining realistic microbial populations in bioassay systems are extremely limited. There are essentially three options (see Sect. 2.1.1.6). We can sterilize systems, inoculate sterilized systems with single microbial species or species mixtures, or live with the microbial populations inherent within bioassay systems. In addition for systems with microorganisms, we can to a very limited extent regulate their actions by environmental manipulations (e.g., type of media, pH, nutrition, input concentrations, rates, and frequency of organic compounds). The use of antibiotics, inhibitors, etc. to control or regulate microorganisms are usually not recommended unless their use is a specific part of an experimental design since such substances will not only lead to differential selection, amplification, and suppression of specific microbial species but can also function as promoters, modifiers, or inhibitors of bioassay species. We can, however, use them to monitor the actions and numbers of some types of microbial species (e.g., see Blum and Shafer 1988; Shafer and Blum 1991). For monitoring microbial species, antibiotics, inhibitors, etc. can be used in selective media to enumerate populations of specific types of microorganisms (e.g., actinomyces, fungi, phosphatase-positive bacteria, gram-negative bacteria, phenolic acid utilizing microorganisms, etc.; see Blum and Shafer 1988; Shafer and Blum 1991). For details regarding the types, locations, roles, and numbers of microorganisms in soil systems see Sects. 3.6 and 3.7.

The data that are presently available suggest that the actions and possible active populations of microorganisms in laboratory bioassay systems turn out to be considerably different and potentially greater than in field soil systems (e.g., see

Blum 2004). In field systems the residual or net concentrations (input-loss) of available phenolic acids for example turn out to be fairly constant over the growing season (Whitehead et al. 1981, 1982, 1983; Blum et al. 1991; Blum 2011). In laboratory bioassay systems that is not the case. For example phenolic acids applied to laboratory bioassay systems decline very rapidly and frequently are no longer detectable within media after 24–48 h (see Blum and Shafer 1988; Blum et al. 1994, 1999a, b; Blum and Gerig 2006). The primary processes that bring about this decline are conversions and utilization by microorganisms, although soil sorption, oxidation/reduction, and seed, root, and mycorrhizal uptake also have a role in this decline (see Blum et al. 1994; Blum and Gerig 2005; Blum 2006). The effects of phenolic acids on seed, seedling, and plant processes decline as contact with seed, root, and mycorrhizae decline (see Blum and Dalton 1985; Blum et al. 1985a; Blum and Gerig 2006). Thus, to maintain a desired effective concentration range over time, dose response studies require that phenolic acids be supplied continuously or at specific intervals. The frequency of addition is determined by the nature of the bioassay system and the narrowness of the concentration range desired by the researcher. Such multiple additions, however, can potentially lead to misidentification of cause and effect since the continuous production of breakdown products and synthesis of new compounds by microorganisms could turn out to be just as important if not more important than the original phenolic acids added to the system.

3.2.2.9 Distribution

The way in which the distribution of promoters, modifiers, and inhibitors influence the effects observed for sensitive species has not been adequately determined since the majority of the experimental designs used for allelopathic studies have completely ignored this important modifying factor. What has been demonstrated is that some minimum level of contact between IPA compounds and seed, root, and mycorrhizal surface area is required before an effect is observed. For example, the effects of IPA compounds with local actions (i.e., not systemic in action) such as phenolic acids increased as the contact surface area of seedlings was increased (Klein and Blum 1990; Lyu and Blum 1990; Lehman et al. 1994). The latter behavior has also been observed for seed surface area (see Sect. 3.2.2.6). It is also known that both horizontal and vertical distributions of organic and inorganic compounds including allelopathic compounds are very irregular and unpredictable in field soils (see Blum 2006). In addition it is known that IPA compounds added to solution-bioassay systems (complete contact with roots and high mobility), soil-bioassay systems treated with solutions (variable root contact partly due to soil gradients and reduced mobility), and soil-bioassay systems treated with residues (variable root contact partly due to variable distribution, input, and reduced mobility) require different minimum concentrations for an effect (e.g., Sects. 3.2.2.1, 5.2.3.1, and 6.5). It is fairly certain that some part of the differences in observed effects for these systems stems from their differences in distribution of allelopathic compounds and treatment surface areas. How much of this difference is actually due to distribution patterns has not been determined.

3.2.2.10 Final Comment

There are clearly substantial numbers of modifying factors in field systems that ultimately influence the observed stimulation or inhibition of plant species. Unfortunately for most laboratory bioassay systems many of these modifiers are either atypical or absent. A renewed effort is required to adjust atypical factors so that they are more typical, to incorporate modifiers wherever possible, and to define and quantify the roles of modifying factors so that laboratory bioassay systems can become more relevant to field systems.

3.2.3 Frequency of Treatments and/or Adjustments

Single, multiple, and continuous treatments have been used in laboratory bioassay. Single treatments have frequently been used for short term Petri dish germination bioassays and for longer term litter and residue bioassays, multiple treatments, for solution and soil-culture bioassays, and continuous treatments, for "root exudates plus" leachate and some soil bioassays (see Chaps. 4 and 6 for specific examples). Unfortunately, present data indicate that treatment frequencies and/or adjustments necessary for maintaining persistent effects in many laboratory bioassays are not consistent with the frequencies of inputs and turnover rates in field systems. The reasons for this (see below) are largely due to the rapid atypical turnover rates of organic compounds within laboratory bioassay systems. What is needed are ways to adjust these turnover rates in laboratory systems so that the inputs are more typical to that of field soils. To simplify this discussion we will assume that all test solutions have initially been adjusted to have similar pH values, solute potentials, inorganic content, etc. and that appropriate inert materials have been added to compensate for the physical differences generated by the addition of tissues, litter, and residues (see Putnam and DeFrank 1983; Putnam et al. 1983).

Addition of Treatment Solutions In solution-culture bioassay systems initial or starting concentrations of IPA compounds and other organic and inorganic compounds decline at different rates over time due to different rates of uptake by seeds, roots and mycorrhizae, modification and utilization by microorganisms, and oxidation/reduction. In some instances concentrations may actually increase due to evapotranspiration. Associated with these changes are also shifts in pH and solute potential of the treatment solution. In order to maintain a specific range of concentrations, pH values, and solute potentials of treatment solutions (e.g., IPA compounds, leachates, or soil extracts) would have to be adjusted continuously or at given time intervals. Since changes in concentrations, pH, and solute potential of treatment solutions are essentially unpredictable, this is generally not on option. Fortunately for solution-culture bioassays there is another option. A fairly constant range for a given concentration of treatment solution, pH, and solute potential can be readily achieved by simply replacing the entire solution at regular intervals (e.g., every day or every other day) and by adding water (e.g., daily or twice daily) to the

system to compensate for the water lost by evapotranspiration (see Blum and Dalton 1985; Blum and Gerig 2005). For gravel, sand, vermiculite, etc. systems which are essentially solution bioassays complete solution changes can be achieved for open pot systems (i.e., with drainage holes) by flushing the systems at given time intervals (see Waters and Blum 1987; Shafer et al. 1998). There are, however, times when complete changes are not possible. For example when treatment solutions are recirculated in semi-closed systems to determine the effects of "root exudates plus" leachates (see Wilson and Rice 1968; Kochhar et al. 1980; Tang and Young 1982). For such systems continuous or frequent adjustments of inorganic compounds, pH values and solute potentials are required if cause and effect are to be determined.

When solutions of organic and inorganic compounds including IPA compounds are added to soil systems, a variety of processes (e.g., sorption, modification, utilization and synthesis by soil organisms, and uptake by seeds, roots and mycorrhizae) reduces and modifies the concentrations of organic and inorganic compounds of treatment solutions, and gravitational flow, capillary movement, and transpirational pull distribute and redistribute the compounds within the soil system in an unpredictable manner. The initial concentration gradients for the soil system are determined by the volume and concentration of solution added, nature and size of the soil system, gravitational flow, the affinity of individual compounds to soil particles, distribution of seeds, roots and mycorrhizae, evapotranspiration, etc. (Note: This is for container systems without drainage holes. For container systems with drainage holes an additional loss can also occur by gravitational flow.) The result is that local concentrations, as well as the overall available concentrations of compounds within a soil system, may decline with time at least initially, but can also increase over time if sufficient rates of evapotranspiration occur (see Sect. 3.2.2.2). Here also unpredictable changes in pH and solute potential will occur over time particularly within the immediate zones surrounding seeds, roots, and mycorrhizae. Unfortunately soil systems are much more difficult to deal with than solution bioassay systems. For consistent concentration ranges, pH values, and solute potentials of treatment solutions (i.e., the soil solution in this instance), the only option is to frequently (see Blum and Shafer 1988; Blum and Gerig 2006) or continuously supplement (e.g., drip irrigation; Blum et al. 1999a) soil systems. However, balancing inputs directly to compensate for losses within soil bioassay systems over time is extremely difficult, if not impossible, to achieve. If the time intervals between additions are such that the concentrations of the compounds of interest decline to some consistent and predictable state, then multiple additions to soil systems are an option particularly for IPA compounds. Multiple additions of complex mixtures such as leachates and soil extracts, however, are still problematic in that over time the accumulation of the more stable organic and inorganic compounds within a soil system will lead to problems with oxygen availability, salinity, pH, etc.

Addition of Tissues, Litter, and Residues Since tissues, litter, and residues frequently represent an input source that normally declines over time (at least in temperate regions of the world), the initial addition on or within the soil is all that is frequently required. The initial concentrations of organic and inorganic compounds

within tissues, litter and residues will depend on a variety of factors described in Sects. 2.1.3.1 and 2.1.3.2. Their rate of input (loss) to the soil system will depend on particle size (i.e., surface area) and location of tissues, litter, and residues on the surface and within the soil, soil characteristic such as pore size, activity of soil organisms and the associated release and production of water-soluble compounds, and the frequency and amount of water added to the soil system. (Note: Inputs of hydrophobic and volatile compounds may also occur (see Bradow 1991; Bradow and Connick 1988a, b).)

Increasing concentrations of treatment solutions can be adjusted initially so that they are similar except for the organic compounds of interest. This is not possible for tissues, litter, and residues added to the surface of a substrate or mixed into a substrate. Increasing amounts of tissues, litter, and residues will not only lead to changes in allelopathic compounds but also changes in other organic and inorganic compounds and the physical environment. The differences in the physical environment can be minimized by the addition of inactive materials similar in texture and structure of tissues, litter, and residues (see Putnam and DeFrank 1983; Putnam et al. 1983). Differences in pH, inorganic compounds, organic modifiers other than organic compounds of interest, etc. associated with increasing amounts of tissue, litter, and residues and changes due to leaching and decomposition over time, however, can presently only be accounted for indirectly (see Sect. 6.4). Another potential option, the addition of the same amount of tissue, litter, and residues to all treatments by using plant materials with different chemical compositions (e.g., see Hall et al. 1982, 1983).

3.2.4 Concentration and Dose

Total concentrations (i.e., available and unavailable) and total available concentrations of IPA compounds within a bioassay system are unlikely to be equivalent to dose. Dose is determined by the active IPA compounds that actually contact and directly act on bioassay species or in some cases indirectly by way of mycorrhizal hyphae. Thus, dose is not directly related and in some instances not related at all, to the concentrations of IPA compounds added to a bioassay system or to the total or available concentrations of IPA compounds within a bioassay system. There are really four concentration fractions in any bioassay system: (a) the total, (b) the available, (c) the active, and (d) the active that actually interacts with bioassay species. How these fractions are related to each other varies with the location of IPA compounds and the location of seed, root, and mycorrhizae of the bioassay species in the medium, the type of medium (e.g., solution, sand, or soil), and the physicochemical and the biotic environments of the medium. What is currently needed for all bioassays are estimates of the concentrations of active IPA compounds that actually interact with seeds, roots, and mycorrhizae of seedlings and older plants. Solution bioassays come closest to providing such estimates. Even educated guesses for soil systems are dubious. The reasons:

a. active fractions are readily and rapidly modified in bioassay systems over time (e.g., modification and utilization by microorganisms, sorption and uptake by seeds, roots and mycorrhizae, soil particle fixation, oxidation/reduction, environmental changes, etc.),
b. the relationships between available concentrations, potential active concentrations, and dose (active concentrations) in a medium are not necessarily linearly related and, thus, unpredictable,
c. active fractions that actually interact with seeds, roots and mycorrhizae, particularly in soil systems, are highly variable in time and location due the heterogeneity of their distribution, the reduced mobility of IPA compounds in soil, and the variability of the physicochemical and biotic environments of soil systems (Note: This is particularly a problem for compounds that are not systemic in action, e.g., phenolic acids.), and
d. observed effects are unlikely to be due to a single compound but rather to the actions and interactions of a complex set of promoters, modifiers, and inhibitors within leachates, "root exudates plus", tissues, litter, and residues (see Blum 2011).

This conundrum of concentration versus dose for plant-plant allelopathic interactions desperately needs resolution. It may be possible to obtain some approximation of this relationship with labeled compounds. For example Shann and Blum (1987) observed that depletion of ferulic acid was 2.5 times greater than the uptake of [U-ring-^{14}C] labelled ferulic acid by 14–18 day-old cucumber seedling treated with 0.5 mM ferulic acid (pH 5.5) in solution culture. However a word of caution, Lehman and Blum (1999) found that initial ferulic acid solution concentrations surrounding 9–15 day-old cucumber seedling roots (pH 4.5, 5.5, or 6.5) were more frequently and consistently related to the inhibition of net phosphorus uptake than to ferulic acid uptake by roots. Uptake of phosphorus and ferulic acid was determined by depletion from solution. (Note: Results from both experiments were based on 5-h exposures.)

3.2.5 Serial Dilutions of Complex Mixtures

Serial dilutions are a convenient way of obtaining a range of concentrations, particularly for individual or simple mixtures of IPA compounds, as long as pH, solute potentials, and nutrition are adjusted to be equivalent. For complex mixtures such as leachates and soil solutions the use of serial dilutions is a questionable practice. It could possibly be argued that a type of serial dilution takes place over time during extended rain events but even for extended rain events the amounts, types, and ratios of individual organic and inorganic compounds leached from living tissues, litter, and residues will vary over time in a compound specific manner. Highly soluble and mobile compounds are more likely to be leached than lower soluble and less mobile compounds and, thus, available pools of individual compounds will be depleted at different rates. Serial dilutions, therefore, create unnatural or atypical

combinations of ratios and concentrations for the individual organic and inorganic compounds of complex mixtures. Maintaining the correct concentrations and ratios of promoters, modifiers, and inhibitors in treatment solutions is essential since both concentrations and ratios determine the outcome. Thus, serial dilutions of complex mixtures can lead to an incorrect assessment of the dose response for complex mixtures of field systems. In addition, the pH, water potential, and nutrition for serial dilutions of complex mixtures will also be different then rain-generated dilutions in the field. For one way around this dilemma see Sect. 4.2.2.1.

Varying the amounts of plant tissues, litter, and residues on or in the soil, akin to serial dilution, comes with its own set of problems. The ratios and concentrations per unit of tissue will not change but decreasing or increasing the amount of plant tissue, litter, and residue on or within the soil changes a whole host of soil environmental factors (e.g., pH, aeration, water holding capacity, inorganic compounds, temperature, etc.) that need to be monitored and adjusted. A partial solution to this dilemma may be the addition of inert or inactive materials similar in texture and structure to actual tissues, litter, and residues (see Putnam and DeFrank 1983; Putnam et al. 1983), accounting for these changes indirectly (see Sect. 6.4), or by adding the same amount of tissue, litter, or residues but with different chemical compositions to each experimental unit (e.g., see Hall et al. 1982, 1983). Identifying inert or inactive materials, however, will require rigorous preliminary testing in the bioassay system to be used.

3.3 Field Inputs and Laboratory Treatments of Water-Soluble Compounds

The inputs of available (free and water-soluble) organic and inorganic compounds into soil by throughfall or fog drip (i.e., leaching of leaves, stems, etc.) vary considerably over time and location and consist mainly of pulse or acute input events in temperate regions of the world. Inputs occur each time there is a sufficient rain, condensation (e.g., fog drip), or above canopy irrigation event. Initial amounts of input vary with structure of the canopy, tissue type, chemistry of the leachate, time of year, etc. Inputs, however, decline over time for extended rain or irrigation events as pools of water-soluble compounds are depleted. Given sufficient time between rain, irrigation, and fog drip events pools recover.

Input by “root exudates plus” would be fairly continuous (low level, long term, chronic inputs) with the amounts of input varying with age of root tissue, growth and development of roots, presence or absence of mycorrhizae, soil type and environment, and the actions of microorganisms in the rhizosphere or mycorrhizosphere, etc. Inputs are greater for younger actively growing regions of roots and mycorrhizae than for older more mature roots. As roots mature, they lose their root hairs, may eventually lose their epidermis and cortex, and depending on species begin to form protective layers (e.g., periderm), and lose their ability to form mycorrhizae.

Inputs by aboveground litter and residues are largely seasonal in temperate regions of the world (normally a fairly short acute input event). Inputs by belowground materials are also seasonal but can also occur any time of the year particularly after extended periods of flooding or drought. Amounts and rates of inputs from litter and residues once on or within the soil vary with the nature of the materials (e.g., types of materials [e.g., leaves, stems, flowers, roots, mycorrhizae, etc.], chemical composition, state of weathering and fragmentation), rates of decomposition, and environmental conditions (e.g., rain and irrigation events [e.g., frequency, volume, duration, etc.], soil moisture, nutrition, etc.). Soil inputs by litter and residues would, thus, consist of various acute input events (i.e., pulses after rain events) followed by chronic inputs (low level longer term inputs). However, both pulses and chronic inputs decline with time as litter and residues fragment and/or decompose.

Given that all these inputs end up in the soil, it would appear the simplest and most logical thing to do would be to test field soil samples or their soil water-extracts for their potential allelopathic activity at various times of the year. Unfortunately, field soil samples and their water-extracts collected at a point in time only contain a fraction of the potential organic compounds that may be involved in allelopathic interactions since once organic compounds enter soil, they are sorbed to soil particles, polymerized, converted and utilized by soil organisms, taken up by roots and mycorrhizae, etc. What is present in or recovered from soil samples at a given point in time, thus, constitutes only a residual (net) concentration (see Chap. 1 and Sect. 4.4.4).

The complexity of field system inputs and our inability to integrate all these inputs into a single bioassay system has lead researchers to study the effects of leachates, "root exudates plus", litter, residues, soils (e.g., extracts) and IPA compounds from them separately by applying them to laboratory bioassay systems. (Note: The testing of plant tissue, litter, and residue extracts in bioassays is no longer recommended [see Sect. 2.1.3.3].) These types of treatment applications (i.e., inputs) to the laboratory bioassay systems, excluding possibly litter, residue, and some "root exudates plus" bioassays, represent only pulse or acute treatment (input) events. Treatments are frequently applied multiple times. Some preliminary attempts, however, have been made to create chronic treatments (e.g., continuous additions; see Blum et al. 1999a). Unfortunately we are a long way from creating treatments (inputs) for laboratory bioassay systems that are consistent with the nature, range, frequency, and location of inputs in field soils.

3.4 Mobility and Proximity of Compounds in Soil Media

Under field conditions, unless organic and inorganic compounds including allelopathic compounds are released by sources within the spermosphere of seeds (Giri et al. 2005), rhizosphere of roots, or the mycorrhizosphere (composed of rhizosphere and the hyphosphere [see Sect. 3.7.4.].) of mycorrhizae, some form of water transport, radicle, root, or mycorrhizal growth, or redistribution by the actions of

macro- and mega-fauna in the soil is required before seeds, roots, or mycorrhizae will contact these organic and inorganic compounds including allelopathic compounds. Transport of water solutions occurs by gravitational flow, capillary movement, flow generated by transpirational pull, or diffusion. Of these, gravitational flow and flow generated by transpirational pull are a form of mass flow that can occur over considerable distances. Capillary movement and diffusion by comparison tend to be relatively slow and, thus, are very likely to be important only over shorter distances. The growth of radicles, roots, or mycorrhizae towards or away from a source will also regulate contact or lack of contact with allelopathic compounds, respectively. Once radicles, roots, or mycorrhizae reach the zone of a source, their growth may be stimulated by the presence of promoters or slowed and in some cases entirely stopped by the presence of inhibitors.

Unfortunately in many laboratory bioassays, factors and processes regulating mobility (i.e., transport) and growth are radically altered because:

a. Soils are processed (e.g., sieved, dried, mixed) and adulterated (e.g., addition of sand, vermiculite, perlite, nutrients, etc.) changing the original soil structure (e.g., soil aggregates and, thus, pore sizes and organic matter distribution) and the physical, chemical, and biotic properties of soil.
b. The frequent addition of water or treatment solutions to laboratory soil systems overemphasizes the role of gravitational flow within soil bioassay systems.
c. The small volume of soil, the frequent additions of water and treatment solutions, and the compression of the root and mycorrhizal systems within a small volume of soil all tend to force frequent and direct contact with treatment solutions.
d. Once roots or mycorrhizae reach the bottom of a container with poor drainage or without drainage holes they can potentially have continuous contact with an artificial water table made up of modified treatment solutions. Modified in that compounds have been differentially removed by soil processes (e.g., differential sorption and microbial utilization) as treatment solutions migrate through the soil column.

The end product is that the rate of movement and the frequency of contact of allelopathic compounds by seeds, radicles, roots, and mycorrhizae are substantially greater in laboratory bioassay systems than in field systems. When we add that many laboratory soil systems do not even contain bulk soil but consist entirely of rhizosphere or mycorrhizosphere soil, the reason for the higher frequency of contact by radicles, roots, and mycorrhizae becomes even more apparent. As a result it is likely that microorganisms in the rhizosphere and in the mycorrhizosphere do not compete directly for resources such as organic and inorganic compounds including allelopathic compounds unless they are limiting. In other words they respond independently to the rapid influx of organic and inorganic compounds in laboratory bioassays (Blum et al. 1993; Staman et al. 2001; Blum 2011). It is unlikely that the rapid and frequent influxes of organic and inorganic compounds present in laboratory systems occur in field soils and, thus, such independence in response would probably not be a frequent occurrence in nature. The smaller the bioassay system and the more frequent the treatments (e.g., IPA compounds or leachates) to such

systems, the greater the chances of independent responses by bioassay species and soil microorganisms.

3.5 Seed and Seedling Densities

Weidenhamer et al. (1987) demonstrated that the amount of caffeic acid, ferulic acid, vanillic acid or juglone available per seed in Petri dishes with Whatman No. 1 filter paper determined the level of inhibition of radicle length of seeds. More specifically, they observed that lower concentrations produced equivalent or greater inhibition than higher concentrations when the available concentrations per seed were equivalent or greater, respectively (i.e., seed surface areas were competing for phenolic acids). Such effects could be achieved by manipulating either seed density in the Petri dishes or manipulating the concentration or volume of the solutions added to the Petri dishes. Clearly, the relationships between bioassay species density (actually contact surface area) and concentration and volume of treatment solutions added to Petri dishes cannot be ignored.

Similar relationships have been observed in soil systems for concentrations of gallic acid and hydroquinone (Weidenhamer et al. 1989) and several herbicides and seedling density (Burrill and Appleby 1978; Hoffman and Lavy 1978; Andersen 1981; Winkle et al. 1981; Thijs et al. 1994; Sinkkonen 2001). Seedling density is essentially a proxy for root and mycorrhizal surface area. But these relationships are even more complex for seedlings (and older plants) in that increasing or decreasing the density of seedlings will also increase or decrease, respectively, resource competition between seedlings including competition for allelopathic compounds (Weidenhamer et al. 1989; Thijs et al. 1994; Sinkkonen 2001, 2003, 2007; Weidenhamer 2008). The result is that the higher the seedling numbers, the lower the effects for a given inhibitor concentration but the greater the potential for resource competition and autotoxicity (e.g., see Sinkkonen 2007; Huang et al. 2013). The observation of hormesis suggests that similar concerns are also warranted for stimulation by promoters (Belz et al. 2005, 2007; Duke et al. 2006; Belz 2008). Clearly the relationships between bioassay species density (i.e., contact surface area), concentration and volume of treatment solutions, tissues, litter, and residues added to substrates, and volume of substrate (e.g., determined by pot size) also cannot be ignored.

As an aside, there is an additional density issue that is generally ignored in laboratory bioassays but is very important for field systems. That is the presence of sensitive and insensitive seeds, seedlings, or older plants from other species all present at their own individual densities. From a holistic perspective, all three types of species at their appropriate densities should, therefore, be included in bioassays (e.g., see Thijs et al. 1994). The seeds, roots, and mycorrhizae of all these individuals and their associated microorganisms not only release and produce organic compounds including allelopathic compounds but also compete directly or indirectly for limiting resources including allelopathic compounds. Unfortunately since the

inclusion of all these species at their individual densities will make it impossible to identify cause and effect, most cause and effect bioassays studying plant-plant allelopathic interactions have used single individuals or monocultures at a given density and growth stage (Note: Autotoxicity can be a concern for monocultures.). In other words, other species have been excluded and putative allelopathic species are represented only by IPA organic compounds, leachates, "root exudates plus", soil solutions (e.g., extracts), tissues, or residues added to bioassay systems. This type of approach is clearly in harmony with a reductionist perspective, an approach that can potentially identify cause and effect but such an approach provides little, if any, insight regarding what is actually occurring on a day to day basis in the field.

3.6 Symbiotic Relationships

Of particular interest are two common structures resulting from symbiotic relationships between vascular plants and microbes, legume nodules and mycorrhizae (see Fig. 2.1). Legume nodules are a product of the interactions between plant roots and bacteria (e.g., species of *Rhizobium* or *Bradyrhizobium*) and mycorrhizae are a product of the interactions between plant roots and fungi (e.g., aseptate endophytes in the Glomeromycota [arbuscular mycorrhizae] and septate fungi in the Ascomycetes and Basidiomycetes [ectomycorrhizae]). The metabolic/catabolic and transfer processes associated with these structures directly or indirectly regulate:

a. anatomy and morphology of roots,
b. the ability and efficiency of capturing resources,
c. plant growth, development, and reproduction (e.g., fitness),
d. the ability of plants to compete with each other, and
e. plant responses to environmental stressors, including allelopathic compounds.

Legume seedlings and older plants with functional nodules can fix atmospheric nitrogen, an essential element that is frequently a limiting resource in the field for growth and reproduction. Nodule induction, development, and function involves a complex stepwise set of processes which are regulated by a host of genetic, physicochemical, and biotic factors (Zaharn 1999; Tang et al. 2006; Garg and Geetanjali 2007; Goergen et al. 2009; Ferguson et al. 2010; Rinaudo et al. 2010; Denison and Kiers 2011; Rodríguez-Navarro et al. 2011; Sugawara and Sadowsky 2011; Mortier et al. 2012), including allelopathic compounds (Blum and Rice 1969; Rice 1984; Siqueira et al. 1991; Ramanujam et al. 1998; Mallik 1999, 2000).

Functional nodules require photosynthate from the plant but provide nitrogen products in return. For example, Herridge and Pate (1977) found for cowpea (*Vigna unguiculata* L.) that nodules consumed 9 % of the carbon from the plant's total net photosynthesis, 43 % which was respired, 6 % was made into dry matter, and 51 % was returned to the shoot as nitrogen products. In addition to nodules, legumes also have mycorrhizae (see Zahran 1999; Sprent and James 2007; Franzini et al. 2010). Depending on species and environment, infections of legume roots by mycorrhi-

zal fungi have been found to stimulate nodule development and nitrogen fixation (Ganry 1982; Redecker et al. 1997) or to inhibit nodule development and nitrogen fixation (Franzini et al. 2010).

The presence of mycorrhizae is fairly common among land plants (i.e., bryophytes, pteridophytes, gymnosperms and angiosperms). For example, 100% of gymnosperm families (12 surveyed) and 94% of angiosperm families (195 surveyed) have species with mycorrhizae, and 99% of the species (84 surveyed) and 72% of the species (2964 surveyed) surveyed in these families were obligate mycorrhizal species, respectively (see Wang and Qui 2006; Smith and Read 2008). There are, however, some plant families that characteristically do not have mycorrhizae, for examples: Chenopodiaceae, Brassicaceae, Caryophyllaceae, Polygonaceae, Juncaceae, Proteaceae and Solanaceae. Of the seven types of mycorrhizae identified for vascular plants, arbuscular mycorrhizae (AM) and ectomycorrhizae (ECM) are the most common. Of the two, the AM have the widest distribution among both herbaceous and woody species. Most plants with ECM are woody perennials. Mycorrhizal fungi have a range of functions that influences the growth and development of seedlings and plants. For example the:

a. hyphal networks utilize between 4 to 20% of net photosynthesis,
b. hyphal networks expand the volume of the soil surrounding roots that can be exploited for resources,
c. hyphae function like root hairs by taking up from the soil, as well as losing water, organic compounds, and inorganic compounds to the soil (e.g., nutrients such as phosphorus, and micronutrients),
d. hyphae and associated structures transfer organic compounds including allelopathic compounds and inorganic compounds to roots and among roots of identical or different plant species,
e. hyphal networks modify physical, chemical, and biological attributes of soil,
f. hyphal networks can help reduce plant disease,
g. mycorrhizal fungi can suppress aggressive agricultural weeds,
h. mycorrhizal fungi can stimulate the production of secondary plant products,
i. mycorrhizal fungi under certain conditions can also become root pathogens, and
j. the presence of mycorrhizae can effect nodulation and nitrogen fixation of legumes (Ganry 1982; Brownlee et al. 1983; Francis and Read 1984; Simard et al. 1997; Abbott and Gazey 1994; Read 1997; Redecker et al. 1997; Davies et al. 2001; Giri et al. 2005; Moyer-Henry et al. 2006; Tungate et al. 2007; Ultra et al. 2007; Nichols 2008; Siddiqui and Pichtel 2008; Smith and Read 2008; Franzini et al. 2010; Rinaudo et al. 2010; Gianinazzi et al. 2010; Barto et al. 2011, 2012; Furuno et al. 2012).

Finally, the formation and development of mycorrhizae, like nodulation and root growth, is regulated by a host of genetic and environmental factors (Danell 1994; Clark 1997; Gryndler et al. 2005; Gehring et al. 2006; Mummey and Rillig 2006; Tungate et al. 2007; Smith and Read 2008; Likar et al. 2009; Denison and Kiers 2011; Hasbullah et al. 2011; Sharma et al. 2011; Talaat and Shawky 2011), including potentially allelopathic compounds (Koide and Li 1991; Anaya 1999; Callaway et al. 2008).

Some additional comments are warranted at this point in regard to the transfer of organic compounds between plants (see d. above). Blum (2011) recently suggested that the transfer of organic compounds, including allelopathic compounds, between plants by way of root grafts and hyphae may be outside of the definition of allelopathy since root contact by allelopathic compounds must come by way of the environment (Molisch 2001). According to Molisch, allelopathic compounds must be released from one plant (i.e., released into the environment) and then exert an influence (i.e., stimulate or inhibit) on another associated plant. The transfers by root grafts clearly do not meet these criteria. In spite of that, the transfer of organic and inorganic compounds by way of root grafts still constitutes a chemical transfer between neighboring plants that will influence the competitive relationships of pairs of individuals or groups of plants (i.e., communities) and thus must be understood. What about transfers by mycorrhizal hypae? In fact the transfer of organic and inorganic compounds among plants including allelopathic compounds by mycorrhizal hyphae and associated structures is not direct. The interface structures of the fungi are located within the apoplast of root cortex, i.e., between cell walls for EMC and between cell walls and cell membranes for AM. The location, structure, and nature of these interfaces between fungal structures and cell membranes vary in considerable detail but the membranes of both producing and receiving root cells are separated from fungal structures by an apoplast. There is no cytoplasmic continuity between the fungal structures and root cells. When continuity does occur, the fungi function as pathogens. For more details regarding uptake and transfer by fungal hyphae associated with mycorrhizae see Smith and Read (2008). Consequently if the apoplast is considered as being part of the external environment of the root (i.e., the apoplast is contiguous with the external root environment), then the transfer of stimulatory or inhibitory compounds by mycorrhizae fits the definition of allelopathy. If not, such transfer must be excluded from plant-plant allelopathic interactions just as the direct transfer between plants by way of root grafts.

The action of allelopathic compounds on seedlings and older plants can be direct, for example, by modifying cell membranes or cell processes or be indirect by modifying symbiotic relationships of seedlings or older plants (see Macías et al. 2007; Bais et al. 2010; Blum 2011; D'Abrosca et al. 2013; Duke et al. 2013). In the absence of functional nodules or mycorrhizae only direct effects of allelopathic compounds, no matter their source, can be determined. The whole suite of effects (i.e., direct and indirect effects) requires the presence of functional nodules and mycorrhizae. Thus determining the effects of allelopathic compounds on seedlings and older plants without symbiotic relationships provides only one part of the story unless one is working with very young seedlings or non-mycorrhizal species. In most instances, unless it is contrary to experimental objectives, maturing seedlings and older plants should be inoculated with soil extracts or specific microbial species of the rhizosphere/rhizoplane, or mycorrhizosphere/rhizoplane, *Rhizobium* or *Bradyrhizobium* for nodulating species, and/or spores, infected root fragments, hyphae, or infected soil for mycorrhizae before testing IPA compounds, leachates (e.g., tissues, litter, residues, or "root exudates plus"), soil solutions, litter or residues in bioassays.

As a practical matter, the time it takes to develop functional nodules and mycorrhizae is something that needs to be considered when designing bioassays. Since it takes approximately a week or more for the initial development of functional nodules (Staley et al. 1992; Senaratne and Ratnasinghe 1993; Antunes et al. 2006) and mycorrhizae (see Staley et al. 1992; Antunes et al. 2006; Smith and Read 2008) and then an additional 4–9 weeks, depending on species, for the more extensive formation of root nodules and the colonization of roots by mycorrhizal fungi, it could be argued that the inclusion of an inoculum for the induction and development of symbiotic relationships in bioassays of seeds and very young seedlings are not necessary. However, the author suspects that the presence or absence of nodule bacteria and mycorrhizal fungi in the soil can make a difference in how IPA compounds, leachates, etc. will affect germination and young seedling growth and development. There is considerable evidence that microorganisms present in the soil can and do regulate the effects of IPA compounds on germination and plant growth (see Sect. 3.7).

3.7 Soil Microorganisms (Microflora and Fauna)

Species of microflora (e.g., actinomycetes, algae, bacteria, fungi, viruses), microfauna (e.g., protozoa, amoeba, nematodes, etc.), mesofauna (e.g., microarthropods, oligochaetes, etc.), and macrofauna (e.g., larger invertebrates, etc.) in soil are very diverse, have a broad range of metabolic (i.e., anabolic and catabolic) functions, have diverse patterns of distribution in the soil, and interact with each other in complex ways (Lavelle and Spain 2001). Their actions can for example:

a. modify the physicochemical properties of the soil,
b. determine rates of decomposition of litter, residues, and organic matter,
c. determine the release and availability of organic and inorganic compounds (i.e., carbon and nutrient cycles) in soils and soil solutions,
d. lead to the modification of organic compounds and the synthesis of new organic compounds including allelopathic compounds in soil,
e. lead to the formation of symbiotic relationships with roots (e.g., nodules and mycorrhizae),
f. modify seed, seedling, and older plant metabolic processes, modify uptake and losses of organic and inorganic compounds, and modify susceptibility to external stressors, and
g. determine rates of predation and root diseases (see Baker and Snyder 1965; Burges and Raw 1967; Alexander 1977; Jensen et al. 1986; Barazani and Friedman 1999a, b; Hoagland 2001; Lavelle and Spain 2001; Blum 2004, 2006; Liebeke et al. 2009; Badri et al. 2013; see Sect. 3.6).

From the standpoint of plant-plant allelopathic interactions, most studies have focused on the way soil microflora modify, utilize, and synthesize new putative allelopathic compounds and how their actions in turn may regulate plant-plant

allelopathic interactions. The effects of the direct or indirect actions (see above) of soil organisms on plant-plant allelopathic interactions have largely been ignored. The bottom line is that the community structure of soil organisms (e.g., population numbers, diversity, biomass, and distribution) surrounding seeds, roots, and mycorrhizae and the direct and indirect actions of organisms in soil are extremely important factors in the ultimate expression of plant-plant allelopathic interactions (see Heisey et al. 1985; Blum and Shafer 1988; Shafer and Blum 1991; Ekschmitt 1998; Barazani and Friedman 1999a, b; Blum et al. 1999a, b; Schmidt and Ley 1999; Hoagland 2001; André et al. 2002; Inderjit 2005; Zhang et al. 2009, Blum 2011, also see Sect. 3.6). Unfortunately due to their complex actions, distributions, and environmental requirements and our present inability to isolate, identify, and characterize a large segment of the organisms within soils particularly microflora, determining their direct and indirect roles in plant-plant allelopathic interactions is extremely difficult even under the best of circumstances. Some would, in fact, argue that it is presently impossible. However no matter the difficulties, the actions of organisms in the bulk soil, rhizosphere or mycorrhizosphere, rhizoplane, and endorhizosphere must be understood before any real progress can be made toward demonstrating the existence of plant-plant allelopathic interactions under field conditions.

To emphasize the importance of and to provide a coherent perspective for soil microorganisms and their roles in plant-plant allelopathic interactions, general comments regarding numbers, species diversity, biomass, distribution, function, measurements, and their relevance to laboratory bioassays are provided below. The following comments focus on microorganisms (microflora and fauna) in the soil since these are the organisms of primary interest in laboratory bioassays.

3.7.1 Numbers

Based on direct counts or culture techniques, bacteria are the most abundant microorganisms in the soil attaining populations in excess of 10^8/g soil, representing 10^4–10^6 species (Wollum 1998). Algae and cyanobacteria located primarily near the surface or just below the surface of the soil range from 10^3 to 10^8/g soil although 10^3 to 10^4 are more common. The actinomycetes and fungi range between 10^7 to 10^8 and 10^5 to 10^6/g soil, respectively. Viruses in fertile agricultural soils may reach 10^{11}/g soil (Angel and Gagliardi 1998; Wollum 1998). The estimates for fauna, excluding nematodes and protozoa, may total well over 1.5 million/m^2 for a typical mineral soil (Kevan 1965). Nematodes ranged from 1.8 to 120 million/m^2 in a European grassland soil (Kevan 1965) and from 1.7 to 6.3 million/m^2 in a variety of forest soils (Sohlenius 1980). Based on a variety of studies Macfadyen (1968) determined that soils contain just below 1 million protozoa per m^2. For number estimates of additional faunal species see Lavelle and Spain (2001).

Unfortunately many of these numbers significantly underestimate the actual total numbers in soil since many organisms in soil are too small to be seen and counted, cannot be isolated and grown in cultures, and the efficiencies of the methods used to

isolate and categorize organisms were frequently poor. For example, estimates suggest that less than 1 % of the bacterial species in soil can be isolated and grown in culture (Parekh and Bardgett 2002; Sylvia et al. 2004; Zhang and Xu 2008; Hirsch et al. 2010), although in areas such as the rhizosphere that percent may reach 10 % (Sørensen 1997) and for specific genera of bacteria that may reach 60 % or more (Alexander 1977; Bakken 1997). The bottom line is that obtaining realistic estimates of the total numbers of functional bacteria in soil based on direct counts or culture-dependent techniques are presently not possible. This is also clearly the case for obtaining total numbers of actinomycetes, cyanobacteria, fungi, and viruses. However for organisms that can be seen and identified or grown in culture, counts and culture-dependent techniques are still very useful tools for quantifying population numbers of these organisms in soil systems and determining potential functional relationships between numbers of these organisms and seed, seedling, and plant behavior (Phillipson 1971; Lavelle and Spain 2001; André et al. 2002). For example Blum et al. (2000) observed that a 500 % increase in the number (colony-forming units) of phenolic acid-utilizing bacteria in the rhizosphere of cucumber seedlings growing in Cecil A-horizon soil treated with an equal-molar mixture of 6 phenolic acids resulted in roughly a 5 % reduction in the inhibition of absolute growth rates of its leaves.

3.7.2 Species Diversity

Species diversity describes the qualitative variation among organisms. Diversity is composed of two components: the number of species present (species richness) and the evenness of the distribution of individuals among the species (equitability). A diverse range of techniques has been used to obtain estimates of the diversity of organisms in soil including counts, culture-dependent techniques, and culture-independent techniques such as fatty acid methyl ester analysis, phospholipid analysis, and nucleic acid-based techniques for microflora (Øvreås 2000; Parekh and Bardgett 2002; Robinson and Bruce 2002; Xu 2006; Zhang and Xu 2008; Hirsch et al. 2010; Lombard et al. 2011). Estimates for faunal diversity have been primarily based on actual counts (Phillipson 1971; Ekschmitt 1998; André et al. 2002). All of these techniques have their own limitations and efficiencies (see reference cited for details). Thus as with numbers of organisms (see Sect. 3.7.1), obtaining values for total diversity of soil organisms are presently not possible.

It should be stated, however, that communities of soil organisms are enormously diverse. For example, using reassociation kinetics of denatured DNA Torsvik et al. (1990) estimated that beech forest soil in Norway contained 4000 bacterial genome equivalence per gram of soil. Whitman et al. (1998) estimate that there are 2.6×10^{26} prokaryotes (i.e., bacteria and cyanobacteria) distributed in the soils of the world and according to Robinson and Bruce (2002) using the logic and calculations of Dykhuizen (1998) there are more than 10^{29} bacterial species. Examples of some estimates for the diversity of fauna in temperate communities are as follows:

a. Protists (formerly protozoa) may comprise several tens of species for each of the three phyla within the Kingdom of Protoctista (see Lavelle and Spain 2001). In New Zealand soils Stout and Heal (1967) reported species richness values (number of species per site) of 14–19 in grassland soils and 33–45 in forest litter for rhizopods (e.g., amoeba), and 29–43 in grassland soils and 21–59 in forest litter for ciliates.
b. Nematode species richness values of 70–100 species have been reported in pastures (71), beech-woods (75), prairies (80), deciduous forests (92), and wheat-soybean fields (100). See Petersen and Luxton (1982) and Bernard (1992) for sources.
c. Collembola species richness values of 16–32 for grasslands and 37–80 for deciduous forests have been reported by Petersen and Luxton (1982).
d. Lower numbers of fauna, however, are not uncommon. For additional diversity values for these fauna and other soil fauna the reader may wish to consult Petersen and Luxton (1982) and Lavelle and Spain (2001).

Furthermore it can be stated that the distribution of individuals among species within a given community is highly variable in time and space since individual species of microflora and fauna respond differently to specific biotic and physicochemical environments of soil (see Burges and Raw 1967; Alexander 1977; Paul and Clark 2007; Lavelle and Spain 2001; Van Elsas et al. 2007). For example, the addition of carbon and energy sources from IPA organic compounds, leachates, tissue, litter, and residues will change the diversity of microorganisms in soil (see Blum and Shafer 1988; Shafer and Blum 1991; Blum et al. 2000; Wardle et al. 2006). What follows are examples of what happens to diversity when soils are treated with IPA compounds such as phenolic acids.

a. Using molecular techniques and cluster analysis Qu and Wang (2008) observed that phenolic acid amendments caused a significant shift in fungal species diversity, i.e., changes in the dominant fungal types and fungal structural diversity.
b. Treating soil or soil-microbe-cucumber seedling systems (Portsmouth or Cecil soils) with phenolic acids resulted in the induction and selection of phenolic acid-utilizing microorganisms (e.g., bacteria, actinomycetes, and fungi) within soil and the rhizosphere (Blum and Shafer 1988; Shafer and Blum 1991; Blum et al. 2000).
c. Treating Cecil A-horizon soil with different phenolic acids (e.g., *p*-coumaric acid or vanillic acids) resulted in a preferential induction and selection of phenolic acid-utilizing bacteria. Isolates selected from vanillic acid-treated soil were more successful in utilizing either phenolic acid than isolates selected from *p*-coumaric acid treated soil. The difference was due to a larger preferential selection of *p*-coumaric acid-only utilizers for isolates selected from *p*-coumaric acid treated soil (Blum et al. 2000).
d. For soil columns containing nutrient poor Cecil A-horizon soils, the addition of increasing concentrations of nutrient solution (0–50% Hoagland's solution; at 2–3.5 ml/h) had no apparent effect on the number (colony-forming units) of *p*-coumaric acid-utilizing microorganisms but stimulated oligotrophic

microorganisms. However, when columns were treated with *p*-coumaric acid (53.5 ± 0.342 μg/ml), increasing nutrient concentrations stimulated the numbers of both oligotrophic and *p*-coumaric acid-utilizing microorganisms (Blum et al. 1999a).

e. Induction, selection, and stimulation or inhibition of microorganisms (e.g., bacteria, actinomycetes, or fungi) in Portsmouth A- and B-horizon soils treated with different phenolic acids (ferulic or *p*-coumaric acid) varied with soil horizon and with phenolic acid. In addition the communities of bacteria, actinomycetes, and fungi responded differently to the treatment of phenolic acids (Blum and Shafer 1988).

Clearly species diversity, both richness and the evenness, can provide considerable insight regarding the potential organismal processes and functions of soil systems and how these may relate to or modify plant-plant allelopathic interactions. In addition, changes in diversity of soil systems at specific locations, particularly the bulk soil, rhizosphere, and mycorrhizosphere, can provide insight to the following:

a. the overall health of the bulk soil, rhizosphere and mycorrhizosphere,
b. the stability or volatility of soil systems based on shifts in abundance of microfloral and faunal species in the bulk soil, rhizosphere and mycorrhizosphere,
c. how the bulk soil, rhizosphere, and mycorrhizosphere community responds to environmental changes and treatments including allelopathic compounds, and
d. the presence of potential indicator or keystone species (Baker and Snyder 1965; Perry et al. 1989; Bej et al. 1991; Bernard 1992; Bongers and Bongers 1998; Yeates and Bongers 1999; Knoepp et al. 2000; Zhang and Xu 2008; Øvreås 2000; Lavelle and Spain 2001; Yeates 2003).

3.7.3 Biomass

A balanced perspective requires more than just numbers. Population numbers tend to overestimate the importance of the smaller organisms while biomass tends to overestimate the importance of larger organisms. Biomass values have frequently been based on biovolume which is determined from diameter and length of microflora and fauna and conversion values determined from laboratory cultures (Phillipson 1971; Alef 1995a; Paul and Clark 2007). For microflora, various indirect methods have also been used such as fumigation incubation, ninhydrin nitrogen reaction, respiration, oxygen consumption, and ATP content (Alef 1995b; Joergensen 1995a, b, c; Sparling 1995; Van der Werf et al. 1995). For fauna of sufficient size, individuals have been extracted from soil or litter, average individual weight (AIW) has been determined, and the number/unit area has been multiplied by AIW. For details regarding these procedures, the reader may wish to consult Phillipson (1971) and Petersen and Luxton (1982).

Values for microbial biomass in temperate and tropical soils have been found to range from 300 to 2240 kg dry weight/ha (see Lavelle and Spain 2001). This rep-

resents anywhere from 1 to 5% of the total soil carbon (Theng et al. 1989; Insam 1990). Fungi and bacteria are the main components of microbial biomass and their contribution ranges from 10 to 90% depending on carbon content of the soil. The higher the carbon content of the soil, the higher their contribution to the biomass (Anderson and Domsch 1980). The fresh weight of bacteria, actinomycetes, fungi, protozoa, and nematodes in kg wet mass/ha of soil has been estimated to be 300–3,000, 300–3,000, 500–5,000, 5–200, and 1–100, respectively (see Wollum 1998). For additional biomass values see Petersen and Luxton (1982) and Lavelle and Spain (2001).

3.7.4 Distribution

For roots without mycorrhizae, four zones have been recognized:

a. bulk soil,
b. rhizosphere,
c. rhizoplane, and
d. endorhizosphere. (Note: Seeds also have zones that are somewhat analogous to roots: b. the spermosphere (Giri et al. 2005) would be analogous to the rhizosphere, c. the outer surface of the seed coat would be analogous to the rhizoplane, and d. the embryo and associated tissues would be somewhat analogous to the endorhizosphere of roots (see Mundt and Hinkle 1976).)

The bulk soil or root-free soil has been defined as soil that is not influenced by the activities (e.g., exudation, secretion, lysis of cells, uptake, etc.) and modifications (e.g., pH, moisture, compaction, aeration, and organic matter) of roots (Sylvia et al. 2004). The rhizosphere has been defined as the soil matrix immediately surrounding roots (Rovira and Davey 1974; Rovira et al. 1979; Rovira 1991; Schmidt 1991; Kennedy 1998; Lavelle and Spain 2001; Sylvia et al. 2004; Blum 2006). The thickness of the rhizosphere is on the order of a few mm. It is frequently characterized by gradients in soil moisture, pH, organic matter, nutrients, microflora, fauna, and soil gases. There are, thus, distinct differences between the bulk soil and the rhizosphere. The rhizoplane has been defined as the surface of the root and the very closely adhering/imbedded particles of soil (Rovira et al. 1979; Giri et al. 2005). In general bacterial and fungal populations, i.e., microflora, are the greatest on the rhizoplane and decline rapidly with distance from the root (Rouatt et al. 1960; Papavizas and Davey 1961; Rouatt and Katznelson 1961; Rovira et al. 1974; Curl and Harper 1990). Microorganisms can also be found within the root apoplast of the cortex. This zone has been referred to as the endorhizosphere (Whipps and Lynch 1986; Giri et al. 2005). Populations of microflora in this zone are generally small. However, the actual numbers and patterns of distribution of individual species within these various root zones (i.e., rhizosphere, rhizoplane, and endorhizosphere) are quite variable (Papavizas and Davey 1961; Rovira and Davey 1974; Miller et al. 1990; Hawkes et al. 2007). Fauna such as protozoa and nematodes also tend to be

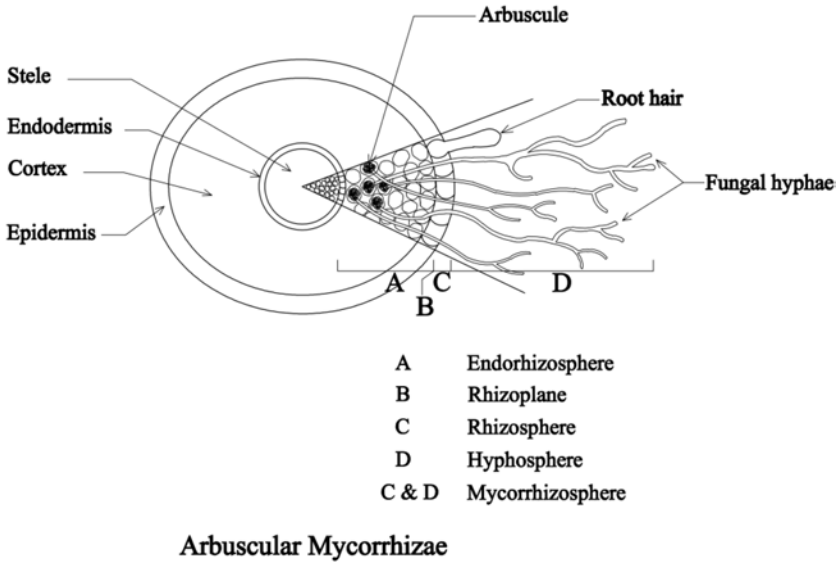

Fig. 3.1 Zones for arbuscular mycorrhizae. (Illustration by Amy Blum Grady, used with permission)

highest in the rhizosphere but here as well their numbers and patterns of distribution within these zones are quite variable (Sheu et al. 2005; Griffiths et al. 2007).

For roots with mycorrhizae, five zones have been recognized (Fig. 3.1). These are:

a. bulk soil: soil that is located beyond the hyphosphere and is not influenced by roots and the mycorrhizal fungi associated with roots,
b. hyphosphere: soil zone that extends beyond the rhizosphere and is under the influence of mycorrhizal hyphae,
c. rhizosphere: soil zone that is under the joint influence of the root and the mycorrhizal hyphae immediately adjacent to the root,
d. rhizoplane, surface of the root and closely adhering soil particles, and
e. endorhizosphere: zone composed of mycorrhizal hyphae and associated structure as well as other microbes located within the cortex of the root (see Giri et al. 2005; Smith and Read 2008).

The hyphosphere and the rhizosphere together comprise the mycorrhizosphere (Giri et al. 2005). Some others include the roots as well in their definition. For example, Timonen and Marschner (2006) make a further distinction by recognizing two zones, the inner (roots [endorhizosphere and rhizoplane] and rhizosphere) and outer (hyphosphere) mycorrhizosphere. The author will use the definition of Giri et al. throughout this volume. The mycorrhizosphere can range from a few mm to a considerable distance particularly if rhizomorphs (multi-hyphal linear aggregates) are included (Smith and Read 2008).

Given these differences, it should not be surprising that the zones described for roots and mycorrhizae have recognizable differences in the presence, number, diversity, biomass, and distribution of microflora and fauna. For example, Rouatt et al. (1960) found wheat rhizosphere to bulk-soil ratios of 23 for bacteria, 7 for actinomycetes, 12 for fungi, 2 for protozoa, 125 for bacterial ammonifiers, 1260 for bacterial denitrifiers, and 2 for bacterial anaerobes. Population estimates of bacteria, actinomycetes, fungi, protozoa, bacterial ammonifiers, bacterial denitrifiers, and bacterial anaerobes in the rhizosphere for this study were 10^9, 10^7,10^6, 10^3, 10^8, 10^8, and 10^7/g soil, respectively. Ratios of bacteria and fungi for soybeans were 12 and 4, respectively. Rhizosphere populations in this case were estimated at 10^8 and 10^5/g soil, respectively. These ratios were based on field samples and, thus, substituting mycorrhizosphere for rhizosphere of mycorrhizal species is probably appropriate. In the past the term rhizosphere has been used for both roots with and without mycorrhizal fungi. However because there are considerable differences between roots and mycorrhizae in anatomy and morphology, habitats, associated soil organisms, and functions, the term mycorrhizosphere has now been substituted for the term rhizosphere of mycorrhizal plants (Meyer and Linderman 1986; Linderman 1988; Andrade et al. 1997, 1998; Budi et al. 1999; Johansson et al. 2004; Giri et al. 2005; Duponnois et al. 2008; Koele et al. 2009). Microflora and fauna are also different for mycorrhizal roots when compared to non-mycorrhizal roots. The reasons for the difference are that the presence of mycorrhizal fungi alters water and nutrient uptake, production of "root exudates plus", the physicochemical properties of soils within the mycorrhizosphere, and the selection pressure (both positive and negative selection depending on species) on other soil organisms (Katznelson et al. 1962; Oswald and Ferchau 1968; Ames et al. 1984; Meyer and Linderman 1986; Linderman 1988; Secilia and Bagyaraj 1987; Andrade et al. 1997, 1998; Budi et al. 1999; Johansson et al. 2004; Giri et al. 2005; Duponnois et al. 2008; Koele et al. 2009, also see Sect. 3.6).

So why are differences in microflora and fauna between bulk soil, hyphosphere, rhizosphere, rhizoplane, and endorhizosphere important to plant-plant allelopathic interactions? Because the biotic and physicochemical environments, the microflora and fauna, and the metabolic and catabolic processes for these regions or zones are unique and, thus, their influence on plant-plant allelopathic interactions is also unique.

3.7.5 Function

In bulk soil a substantial number of the soil organisms (i.e., bacteria, actinomycetes, fungi, viruses, microfauna, and mesofauna) are very likely to be inactive (quiescent) or dormant and become active only when environmental conditions exceed minimum conditions required for their metabolism, growth, and reproduction. According to Lavelle and Spain (2001) the average bulk-soil bacteria may be active 3 years out of 30 years. Inactive microorganisms would also be expected in the rhizosphere

and the mycorrhizosphere but because of the continuing inputs of organic compounds by roots and mycorrhizae and associated organisms, the numbers of inactive individuals there are very likely to be much smaller. The dilemma for researchers studying the functions of soil organisms is identifying and then quantifying which, when, and where soil organisms are active. For those organisms that are active, the functions, aside from their own growth, reproduction, and survival, are enormously variable and frequently highly interrelated in soils. Interrelated functions include decomposition (i.e., fragmentation and turnover of litter, residues, and organic particles and compounds), formation of soil aggregates, carbon and mineral cycles, transformation of elements, production of new and sometimes unique organic compounds, formation of symbiotic relationships with roots, diseases, and predation just to name a few (Baker and Snyder 1965; Carson 1974; Alexander 1977; Insam and Rangger 1997; Lavelle and Spain 2001; Paul and Clark 2007; Smith and Read 2008). There are, thus, a large number of ways by which the functions of organisms in soil can be characterized and estimated. For example, one could monitor changes in enzyme activity, organic and inorganic substrates, respiration, and population sizes of various functional groups or species of organisms, the production of unique compounds or breakdown products, the incorporation of probes, the development of symbiotic relationships, disease, or the levels of predation, etc. (see Baker and Snyder 1965; Phillipson 1971; Jensen et al. 1986; Marshall 1986; Alef and Nannipieri 1995; Insam and Rangger 1997; Lavelle and Spain 2001; Blum 2004, 2006; Paul and Clark 2007;Tracey et al. 2007; Wallenstein and Weintraub 2008; Carlsen et al. 2012). However, relating these functions to seed, seedling, and plant behavior and determining whether such relationships constitute cause and effect are another matter since observed activities of soil organisms and seeds, seedlings, and older plants may not even be related, i.e., the observed effects are independent and collinear in a positive or negative manner.

The primary point to be made here is that the presence, numbers, biomass, diversity, distribution, community structure, function, and location of microorganisms in the soil of field or laboratory systems are never trivial but enormously important to, not only germination, growth, reproduction, stress tolerance, competitive ability, and survival of seeds, seedlings, and older plants, but also the overall health of soil systems. Thus, the characterization of the functions of soil microorganisms are essential if we are to understand how organic compounds released by putative allelopathic species are supplemented (added to), modified, and/or eliminated by microorganisms and how sensitive seeds, seedlings and older plants are affected by the resulting complex mixtures of promoters, modifiers, and inhibitors within the soil. Recent developments in omics particularly metabolomics (profiling and fingerprinting of primary and secondary metabolites present in cells) may provide the tools necessary for doing just that (Narasimhan et al. 2003; Wallenstein and Weintraub 2008; Fan et al. 2009; Leão et al. 2009; Liebeke et al. 2009; Wallenstein et al. 2010; Reaves and Rabinowitz 2011; Nguyen et al. 2012; Badri et al. 2013; see Sect. 6.6).

3.7.6 Relevance to Laboratory Bioassays

In most laboratory bioassays particularly when soil volumes are small and seedling root and mycorrhizal root systems are well developed bulk-soil organisms are not an important component in soil- seedling/older plant bioassay systems since the entire soil system essentially functions as a rhizosphere or mycorrhizosphere. Thus, for most small laboratory bioassay systems, the regulatory influence of bulk soil is absent. (Note: For seed-soil bioassays, bulk soil will likely be present unless the density is too high and spermospheres overlap or the soil is too shallow [see Sect. 3.7.4].) In addition the artificial and over-processed soils used in laboratory bioassay systems tend to be deficient of fauna and contain only highly resilient microflora (e.g., actinomycetes, bacteria, and fungi).

Unfortunately the action of rhizosphere and mycorrhizosphere microbial populations turn out to be a particular pernicious problem in laboratory bioassay systems because the enrichment or treatment of environmentally rich bioassay systems (e.g., adequate nutrients, moisture, and temperature) with carbon and energy sources, such as IPA compounds, leachates (e.g., tissues, litter, residues, or "root exudates plus"), soil solutions (e.g., extracts), tissues, litter, and residues, dramatically alters the qualitative and quantitative nature of the microorganisms within the soil over relatively short time intervals, i.e., hours or days. Changes on the order of hours or days can also occur at times in the field but the speed, magnitude, and frequency of the changes in laboratory bioassays and the portion of microorganisms within the rhizosphere and mycorrhizosphere impacted by treatments in the laboratory are very likely to be at least an order of magnitude greater. In addition the speed, rate, and frequency of organic treatments in the laboratory can readily overwhelm the microorganisms in the rhizosphere and mycorrhizosphere of seedlings or older plants (i.e., input of organic compounds is greater than can be utilized by microorganisms). That could also be the case for the microorganisms on the rhizoplane and in the endorhizosphere. When those conditions occur, the responses of microorganisms and the responses of seedlings or older plants within bioassay systems will become uncoupled from each other, i.e., essentially independent of each other (Blum et al. 2000; Staman et al. 2001; Blum 2011). The author suspects that such independence of action is not uncommon for laboratory bioassays.

3.8 Herbivory and Disease

Under laboratory conditions, herbivory and disease are generally not a problem as long as disease-free seeds, seedlings, older plants, and media are utilized and the growth environment is kept clean and free of herbivores and pathogens. Both of these, however, can become a problem for bioassays carried out in greenhouses or out-of-doors. If they occur during a bioassay and were not part of the original experimental design, the bioassay should be terminated since the actions of both herbivores and pathogens will not be uniformly distributed throughout the experi-

mental population. The use of chemicals (e.g., pesticides and fungicides) to control herbivores such as insects, nematodes, and various types of pathogens should be avoided unless they are to be part of the original experimental design since these chemicals can potentially modify system processes (e.g., soil sorption, microbial utilization, susceptibility of bioassay species to allelopathic compounds, etc.) even when no obvious effects on growth are observed for the bioassay species.

3.9 Physicochemical Environments

The lack of proper emphasis and clear description of physicochemical environments provided for most bioassays in the literature belies the importance of the physicochemical environment on the outcome of a particular bioassay. In fact for species of plants, microbes, or fauna which are sensitive to allelopathic compounds, the presence or absence, the magnitude, and the direction (i.e., stimulation or inhibition) of allelopathic effects are regulated by their physicochemical (and biotic) environments. Thus, for all intents and purposes, physicochemical environments must be properly characterized for bioassays in order to maximize their value to present and future researchers. Furthermore if we are to understand plant-plant allelopathic interactions under field conditions, bioassays with a range of physicochemical environments relevant to field systems are required. The use of bioassays with a single physicochemical environment or a narrow range of physicochemical environments (e.g., 1 day/night temperature and/or a narrow range of soil moisture and nutrition) provides only a very small piece of the environmental response surface or response cloud (multidimensional space) for a field system under study and, thus, provides very limited insight regarding the possible range and magnitude of the effects of allelopathic compounds within that system.

In general when researchers select the environment for a bioassay, they tend to select an environment that is suitable for their specific species. The selected environment, thus, tends to maximize seed germination or seedling and older plant growth and development and tends to ignore the actual field environment. Furthermore, this selection process also tends to ignore how that environment may influence the functions of other organisms (e.g., microorganisms) within that bioassay system. The necessity to adjust the environment of bioassay systems to be consistent with the field environment should, therefore, be obvious. Adjusting the environment to regulate the functions of microflora and fauna in bioassay systems turns out to be equally important if not more important (see Sect. 3.7). Most researchers recognize the need for such adjustments when symbiotic relationships are involved (e.g., reduced nitrogen and phosphorus to stimulate nodulation and nitrogen fixation and enhance the formation of functional mycorrhizae, respectively; see Sect. 3.6) but tend to ignore them in the absence of symbiotic relationships. From a practical stand point there are limits to what can actually be done in the laboratory. In addition most researchers are limited by resources, facilities, and time. For some things that can be done to make bioassays more holistic and, thus, more relevant to the field environment see Sect. 1.5.2 and Chap. 4, 5, and 7.

3.10 References or Controls

Since a primary tool for the study of allelopathic effects has been and is the dose-response bioassay, it will be used as an example to describe the characteristics of a controlled experiment. For simplicity let us use a seedling nutrient culture dose-response bioassay as a model. Dose-response bioassays for an identified putative allelopathic (IPA) compound require the inclusion of experimental units that function as negative controls (seedlings are not affected by the IPA compound; i.e., the absence of the IPA compound) and as positive controls (concentration of IPA compound at which an effect is expected). For maximum benefit experimental units with a series of increasing concentrations between the negative and positive controls should also be included. Imbedded in this concentration gradient should be concentrations that are consistent with those expected in field systems. In addition in order to isolate the effects of increasing concentrations of an IPA compound on seedlings, all other factors (e.g., inorganic ions, pH, solute potential, microbial populations, and aeration) of the experimental units must stay constant as the concentration of the IPA compound is increased. If that is not the case (i.e., more than one factor is simultaneously changing) then it will be impossible to conclude with any confidence that the effects observed on seedlings are entirely due to the increasing concentration of the IPA compound. In fact the observed effects on seedlings could turn out to be primarily due to the simultaneous or collinear changes in other factors (e.g., pH and solute potential) as the concentration of the IPA compound is increased. It is also possible that no effects of increasing concentrations of the IPA compound are observed on seedlings due to the selection/induction and actions of increasing microbial populations with increasing treatment concentrations of the IPA compound. When we add that concentrations of IPA compounds function only as promoters or inhibitors when the physiochemical and biotic conditions are appropriate (see Sect. 2.1.3.1 for details) the use of proper controls or adjustment for bioassay systems become even more important.

Isolating the effects of one factor by adjusting all other factors so that they are constant (i.e., controlled experiments) is not always an option, particularly when testing more complex mixtures of organic and inorganic compounds in soil-bioassay systems. Researchers have, thus, taken a variety of approaches depending on their experimental objectives. For example they have designed experiments where potentially all known active factors are represented by a range of values (i.e., factorial treatment grids) or they have quantified a range of potentially active factors and effects of bioassay systems (i.e., no adjustments for changing collinear factors) at various times after treatment and used regression analysis to identify which factors and factor interactions might be responsible for the observed effects (see Sect. 6.4). However, with the possible exception of solution-culture dose-response bioassays, most dose-response bioassays consist of a complex set of undefined gradients in factors in addition to the factor of interest. For these types of bioassays most researchers simply include a reference treatment or negative control as a baseline for determining the effects of a given series of treatments (e.g., increasing concentra-

tions of leachates or residues). Reference or baseline treatment is actually a more appropriate term than control for these types of bioassays since such bioassays are not really controlled experiments and at times do not even have a zero treatment. Since some type of control or reference is required for all bioassays, it is essential to identify, create, and include proper negative and positive controls for controlled experiments or proper references (baselines) for experiments with multiple or collinear changing factors. For controlled experiments causes and effects can be determined directly and for other types of experiments only hypothetical causes and effects can be determined.

3.11 Measurements

3.11.1 Seeds/Seedlings/Older Plants

Responses of seeds, seedlings, or plants to allelopathic compounds can be measured at a variety of levels ranging from the molecular level to the whole plant level (see Sect. 2.1.4). Effects determined at the molecular (e.g., gene products), cellular (e.g., membrane depolarization, enzyme activity, and metabolic products), and physiological (e.g., photosynthesis, transpiration, or water and ion uptake) levels immediately after treatments can provide insight about potential primary sites and modes of action of allelopathic compounds. However, many of these products and processes are fundamentally very unstable or dynamic. Any delay in taking measurements after treatment and the products and processes measured will very likely represent secondary, tertiary, etc. effects, not primary effects. Thus, when measurements are taken is extremely important. The unstable or dynamic nature of these processes and products also makes it very difficult to directly relate changes in these processes and products to the effects observed at the seed, seedling, or plant levels.

In the last 10 years or so new tools, techniques, and methods have been introduced to identify and characterize primary sites and modes of action of allelopathic compounds. Among them are omics methods (genomics, proteomics, metabolomics, and physionomics; see Gidman et al. 2003; Dunn and Ellis 2005; Macías et al. 2007; Duke et al. 2008, 2013; Chen et al. 2009; Leão et al. 2009; Metz 2011), the use of silenced transgenic plants (Interjit et al. 2009), and mutant lines (Xu et al. 2012). It is hoped that once such tools, techniques, and methods, as well as others are fully implemented that considerable progress will be made in identifying and characterizing the primary sites and modes of action of allelopathic compounds and the active organic compounds found in and released from plants (e.g., Schmelz et al. 2003; Chen et al. 2009; D'Abrosca et al. 2013) and microbes (Narasimhan et al. 2003; Wallenstein and Weintraub 2008; Fan et al. 2009; Leão et al. 2009; Liebeke et al. 2009; Wallenstein et al. 2010; Reaves and Rabinowitz 2011; Nguyen et al. 2012; Badri et al. 2013) and found in soils (e.g., Wallenstein and Weintraub 2008; Fan et al. 2009; Liebeke et al. 2009; Wallenstein et al. 2010).

Effects measured at the seed (e.g., germination, radicle growth), seedling (e.g., seedling emergence, growth, biomass), or older plant (e.g., growth, biomass, nodule number, leaf symptoms) levels are far removed from primary effects and are a result of a complex cascade of effects that started at the molecular level. Furthermore, since plants have considerable buffering capacity (e.g., alternative pathways, the ability to adjust i.e., acclimate), effects initially observed at the molecular and cellular levels may not be evident at the seed, seedling, or plant levels. So what should be measured? That really depends on the experimental objective. However no matter what is measured, a proper context of a given measurement is required before any conclusions can be drawn regarding potential cause and effect particularly at the seed, seedling or older plant levels

On occasion measurements are presented as percent change. Such values are of little value unless a reference or baseline value (i.e., an actual value) is also provided since percent change can represent a broad range of observed changes that may or may not be biologically significant. In fact, all relative values require a reference or baseline value for proper interpretation.

Both destructive and non-destructive measurements have been utilized to quantify the influence of individual or complex mixtures of allelopathic compounds on seeds, seedlings, older plants, symbiotic relationships, etc. Destructive measurements (e.g., shoot and root biomass, etc.) require that different experimental units be harvested at each point in time. Non-destructive measurements (e.g., leaf area, transpiration, photosynthesis, etc.) may be repeated over time on the same experimental units (e.g., leaf). Repeated, non-destructive measurements will, however, require different analytical techniques than destructive measurements because repeated measurements do not meet the underlying criteria for standard statistical techniques (see Steel and Torrie 1997). Each, however, provides useful and possibly unique information about allelopathic effects.

3.11.2 Microorganisms

Common measurements found in the literature for soil microorganisms are:

a. rates of soil respiration (frequently a combination of roots and microorganisms),
b. colony-forming units (CFU) of bacteria, actinomycetes, and fungi per gram of root, mycorrhizae, rhizosphere soil, mycorrhizosphere soil, or bulk soil,
c. CFU of specific carbon utilizing bacteria, actinomycetes, and fungi,
d. rates of specific enzyme activity or changes in metabolites associated with microorganisms (by way of conventional biochemical methods or metabolomics; see Sect. 6.6), nodulation, infection of roots by mycorrhizal fungi, and
e. plant disease, among others. (see Baker and Snyder 1965; Phillipson 1971; Jensen et al. 1986; Marshall 1986; Alef and Nannipieri 1995; Insam and Rangger 1997; Lavelle and Spain 2001; Blum 2004, 2006; Paul and Clark 2007; Tracey et al. 2007; Smith and Read 2008).

These measurements for soil microorganisms (primarily actinomycetes, bacteria, and fungi) in laboratory bioassay systems are vital to our interpretation and subsequent understanding of how, when, and where IPA compounds, leachates, soil solutions (e.g., extracts), tissues, litter and residues impact seed germination, seedling emergence, growth, etc. (see Sects. 3.6 and 3.7). In addition such measurements provide insight into whether the actions of soil organisms within a specific bioassay system are normal (i.e., rates that one might reasonably expect in the field) or atypical (i.e., rates that are too high or too low).

Unfortunately separating the actions of soil microorganisms on the rhizoplane, in the rhizosphere, or in the mycorrhizosphere from the actions of roots and/or mycorrhizal fungi are difficult even under the best of circumstances and extremely time consuming. In spite of that, every effort should be made to characterize the actions of microorganisms in laboratory bioassays. Their actions are so central to plant-plant allelopathic interactions that without adequate characterization of microorganisms in bioassay systems the identification of cause and effect would be highly suspect.

The author is not aware of any studies on how the actions of soil faunas may influence plant-plant allelopathic interactions. However, since faunas are important facilitators in the turnover of organic compounds, organic matter, residues, and litter on and within the soil, measurements of their actions and potential influences on the expression of plant-plant allelopathic interactions also deserves attention.

3.11.3 Media

A whole range of measurements are possible for media. The ones for liquid-culture bioassays are pH, solute potential, content of organic and inorganic compounds, aeration, temperature, inputs and losses of test compounds from solutions, etc. These can be readily measured by taking small subsamples for analyses at appropriate time intervals. For solid media such as soil, possible measurements would include: soil texture and structure, mineral constituents, surface area for sorption, pH, moisture, temperature, organic and inorganic compounds, inputs and losses of test compounds, etc. Obtaining such measurements, however, are much more destructive for soil cultures than solution cultures since larger samples are required for analyses of soils than for solutions. Frequently complete destructive sampling of soil systems is required to obtain desired measurements. No matter what type of media are used, such information is vital to our understanding of the ways that media characteristics modify and regulate the response of seeds, seedlings, older plants, and microorganisms to treatments of IPA compounds, leachates (e.g., tissues, litter, residues, or “root exudates plus”), soil solutions (e.g., extracts), tissues, litter, and residues.

3.11.4 General Environment

Characterizing the general environment before, during, and after treatments is essential (see Sect. 3.9). Measurements that come to mind are photoperiod, light intensity, relative humidity, and day/night temperatures. In addition variations and gradients for the general environment under light banks, within growth chambers, or greenhouses should also be determined. Once identified, the effects of such variation and gradients on bioassay systems can at times be accounted for by positioning or blocking of treatments.

3.12 Final Comments

Up to this point, effects of leachates (e.g., tissues, litter, residues, or "root exudates plus"), tissues, litter, and residues have been treated as if their actions were independent of each other. That is clearly not the case in the field. In fact, data from bioassays might suggest that leaf leachates are neutral, "root exudates plus" leachates are stimulatory, and plant litter and residues are inhibitory. Other scenarios are, of course, possible. Clearly such differences in behavior of leaf leachates, "root exudates plus" leachates, and litter and residues must be taken into consideration when one concludes that a particular species is a putative allelopathic species. To conclude that a particular species is potentially allelopathic based on a single type of bioassay is thus debatable. Most organic and inorganic compounds released from living plants and their litter and residues end up in the soil where they have the potential to interact with roots of associated species that are stimulated, unaffected, or inhibited by the actions of the resulting total promoters, modifiers, and inhibitors within the soil (Blum 2011). The total promoter/modifier/inhibitor complex within the soil would include all active organic and inorganic compounds initially in the soil, those added by leachates, tissues, litter, and residues, and those generated by microbial and faunal activity.

So what constitutes an appropriate experimental design when studying plant-plant allelopathic interactions? Unfortunately there are no simple answers. However when designing a field-relevant bioassay system, the following knowledge regarding the field system of interest is very beneficial:

a. the range of variations of physicochemical (e.g., temperature, light intensity, soil type, nutrition, moisture, solute potentials of soil solutions or treatment solutions, aeration, pH, and organic matter) and biotic (e.g., species, densities, and microbial populations) environments,
b. a baseline (i.e., net concentrations) for free organic and inorganic compounds present within the soil,
c. potential sources of organic and inorganic inputs (e.g., location, types, timing, frequency, and concentrations), and
d. the turnover rates of free organic and inorganic compounds in the soil.

Some insights regarding irreversible sorption, microbial utilization, and seed and root uptake (i.e., potential source-sink relationships) within field soils would also very useful. The idea is not an attempt to duplicate field environments in the laboratory since that is not possible but to run bioassays with the essential features and conditions that are relevant to field systems. Once determined in the field, selecting and implementing many of these features (e.g., the right bioassay species and density, light intensity, photoperiod, thermal periods [i.e., day/night temperature], and soil type, nutrition, moisture level, and pH) in bioassays are fairly straight forward. Determining the types, rates, frequencies, compositions, and concentrations of inputs (i.e., treatments) to bioassay systems, losses from bioassay systems, and the role of other species including microorganisms present are another matter (see Sects. 2.1.1.5, 2.1.3, and 3.2).

When designing bioassays, the underlying question should be: Will the concentrations of available and active allelopathic compounds (promoters and inhibitors) and modifiers in leachates, soil solutions (e.g., soil extracts), tissues, litter, and residues added to or present in the bioassay system (designed to be relevant to field conditions) affect (i.e., stimulate or inhibit) the species of interest under the environmental conditions of the bioassay? Furthermore, how will the effects be modified as the environment changes? These are theoretical question that will lead to a better understanding of potential plant-plant-allelopathic interactions under a range of field environments. Answering such theoretical questions is one primary function of laboratory bioassays. Other functions for laboratory bioassays are to identify and characterize:

a. potential putative allelopathic species and associated susceptible species,
b. sources of and pathways for potential active allelopathic compounds,
c. the influence of the physicochemical and biotic environments on the expression of allelopathic interactions,
d. the nature and function of promoter/modifier/inhibitor complexes in soil solutions (e.g., soil extracts), leachates (e.g., tissues, litter, residues, or "root exudates plus"), tissue, residues, litter, and soils,
e. the modes of action for and interactions between promoters, modifiers, and inhibitors among others, and
f. specific unique symptoms of sensitive species associated with the presence and actions of promoters, inhibitors, or promoter/modifier/inhibitor complexes.

What laboratory bioassays cannot do, given the requirements of scientific methods, the accepted rules of science, and the nature of laboratory bioassays, is to provide unequivocal evidence for the day to day absence or presence of plant-plant allelopathic interactions in the field. Harper (1977) suggested that the more successful attempts to understand interference (in our case allelopathic interactions) have involved the study of specific symptoms, the more specific and unique the symptom the better. If such symptoms can be observed in the field and can be experimentally produced in the laboratory and the field, a researcher may hope to come closer to a procedure equivalent to Koch's postulates in establishing a potential causal relationship between specific allelopathic compounds and plant symptoms.

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Chapter 4
Hypothetical Standard Screening Bioassays

Abstract This chapter describes in some detail a number of standard (common or classical) laboratory screening bioassays for identifying putative allelopathic plants and sensitive species. These include bioassays for the following: identified putative allelopathic compounds, leachates, "root exudates plus", litter and residues, and soils. The procedures of each bioassay have been adjusted, where possible, to be consistent with the tenets of Chap. 3. Comments regarding potential benefits and limitations of these bioassays are also provided throughout the chapter.

4.1 Introduction

There are several major types of standard bioassays that have been utilized by researchers to demonstrate the presence of potential plant-plant allelopathic interactions involving water-soluble compounds:

a. bioassays of leachates from living plants (e.g., leaves, stems, bark, twigs, and reproductive structures),
b. bioassays of "root exudates plus" from living plants,
c. bioassays of dead plant materials (e.g., litter, residues, and decomposing organic matter),
d. bioassays of extracts from plant tissues, dead plant materials, and soils,
e. bioassays of field soils, and
f. bioassays of identified putative allelopathic (IPA) compounds (i.e., promoters and inhibitors) isolated and identified from extracts or leachates of living tissues, "root exudates plus", litter, residues, and soils.

Of these, bioassays of living and dead plant tissue extracts are no longer recommended (see Sects. 2.1.3.2 and 2.1.3.3). What follows are a few chosen examples of hypothetical laboratory bioassays which are consistent whenever and wherever possible with the tenets of Chap. 3. The author hopes that these will provide some additional guidance for designing relevant screening bioassays for plant-plant allelopathic interactions. These examples are not intended to be viewed or used as cook-book or cookie-cutter bioassays but simply as a starting point for designing better and more innovative bioassays. For simplicity and convenience

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let us assume that all the options for the hypothetical experiments have been completed, the data have been collected, and that the data are now ready to be analyzed. However since these are hypothetical experimental designs, the reader will find at times only general design details and a number of appended comments describing cautions, adjustments, modifications, or improvements to the experimental designs.

Helianthus annuus (sunflower; Sect. 4.2), a dicot, and *Triticum aestivum* (wheat; Sect. 4.3), a monocot, were chosen as the putative allelopathic species for these hypothetical bioassays. Both species have long and extensive histories in the plant-plant allelopathic interaction literature and are important crop species with weed suppressing properties (Overland 1966; Wilson and Rice 1968; Schon and Einhellig 1982; Leather 1983a, b; Rice 1984; Worsham 1984, 1989, 1990; Shilling et al. 1985; Blum 2011). Sunflower is also an important member of the early stages of old-field succession in the prairie states of North America (Wilson and Rice 1968). Living sunflower plants were used to generate the leaf leachates and the "root exudates plus" leachates tested in bioassays (see Sect. 2.1.3.2). Wheat straw, stubble, and root residues were used as the plant litter and residues tested in bioassays. Crabgrass (*Digitaria sanguinalis* L.), ivyleaf morningglory (*Ipomoea hederacea* L.), redroot pigweed (*Amaranthus retroflexus* L.), and prickly sida (*Sida spinosa* L.) were chosen as bioassay species. All four weed species are problem weeds in agricultural systems of the temperate regions of North America as well as other regions of the world (Weed Science Society of America 1971; Holm et al. 1977; Lorenzi and Jeffery 1987; Zimdahl 1989) and are members in the early stages of old-field succession in North America (Oosting 1942; Wilson and Rice 1968). Although all the chosen species are herbaceous annuals, this does not mean that the bioassays described here are limited to those types of species. In fact most of these bioassays with minor modifications can be used for a variety of other types of species including herbaceous and woody perennials. Finally, four of the six species, the exceptions being ivyleaf morningglory and redroot pigweed, have been documented as arbuscular mycorrhizal plants (Harley and Harley 1987; Davies et al. 2001; Moyer-Henry et al. 2006; Wang and Qiu 2006; Tungate et al. 2007; Ultra et al. 2007; Hasbullah et al. 2011; Sharma et al. 2011; Talaat and Shawky 2011). A number of other species of *Ipomoea* have been documented as arbuscular mycorrhizal species but ivyleaf morningglory was not listed among them (Anaya 1999; Wang and Qui 2006). Wang and Qiu (2006) categorized redroot pigweed weakly to non-mycorrhizal while Schreiner and Koide (1993a, b) and Sanders and Koide (1994) categorized redroot pigweed as non-mycorrhizal. Sanders and Koide (1994) observed that the presence of mycorrhizal fungi actually inhibited the growth of redroot pigweed.

4.2 Living Plants

There are several ways by which water-soluble organic and inorganic compounds can be released from living plants and enter the soil (see Sect. 1.4). Among them are leaf, stem, flower, and fruit leachates produced during rain, dew, fog, or mist

events (Long et al. 1956; Tukey et al. 1957; Tukey 1966; Wilson and Rice 1968; del Moral and Muller 1969; Rice 1971, 1984) and active and passive losses from roots (Rice 1968, 1984; Wilson and Rice 1968; Rovira 1969; Leather 1983a, b). Once in the soil, these compounds serve a variety of functions including stimulation or inhibition of weed germination, seedling emergence, plant growth, nodulation, formation of mycorrhizae, and as a source of carbon and energy for soil flora and fauna (Tukey and Mecklenburg 1964; Overland 1966; Wilson and Rice 1968; del Moral and Muller 1969; Blum and Rice 1969; Rice 1968, 1971, 1984; Leather 1983a, b). The focus here is on one function, the potential stimulation or inhibition of weeds by leaf leachates and "root exudates plus" of sunflower plants (*Helianthus annuus* L), i.e., plant-plant allelopathic interactions. Wilson and Rice (1968) found that leaf leachates of old-field sunflower plants inhibited the growth of horseweed (*Erigeron canadensis* L), black-eyed susan (*Rudbeckia hirta* L*)*, crabgrass, pigweed, and *Haplopappus ciliates* Nutt and that leachates of "root exudates plus" of sunflower plants were inhibitory to the growth of sunflower (self-inhibiting, autotoxic), horseweed, black-eyed susan, crabgrass, and pigweed. Leather (1983a) found that leaf and stem leachates of several cultivars of sunflower inhibited growth of a broad range of weeds. Among them were velvetleaf (*Abutilon theophrasti* Medic), jimson weed (*Datura stramonium* L), tall morningglory (*Ipomoea purpurea* L), and green foxtail (*Setaria viridis* L). He also found that "root exudates plus" leachates inhibited the growth of velvetleaf and jimsonweed but stimulated tall morningglory and green foxtail.

4.2.1 Solutions Used to Collect Leachates and "Root Exudates Plus"

As previously pointed out in Sect. 2.1.3.2 leaf leachates have been collected by:

a. immersing leaves in distilled water (Tukey and Mecklenburg 1964),
b. misting or spraying leaves with distilled water (Long et al. 1956; Tukey et al. 1957;Tucky 1966; Chou and Muller 1972), cistern water (Wilson and Rice 1968, Al-Naib and Rice 1971), or other types of solutions (Scherbatskoy and Klein 1983),
c. collecting fog drip in the field (del Moral and Muller 1969),
d. collecting throughfall in the field (Gallet and Pellisier 1997; Nilsen et al. 1999), and
e. applying simulated-rain droplets to leaves (Cogbill and Likens 1974; Percy 1986; DuBay and Heagle 1987; Shafer et al. 1985; Shafer 1988, 1992).

Of these, collecting throughfall and fog drip in the field provide the most realistic leachates and dipping, misting, or spraying leaves with distilled water in the field or laboratory provide the least realistic leachates largely due to the difference in the physical and chemical characteristics of rain/fog drip and distilled water. In the laboratory, using rain water collected in the field would be an excellent choice. However, rain events are frequently unpredictable particularly in the temperate

regions of the world and the physical and chemical characteristics of rain water are unpredictable from one rain event to another. For consistency, availability, and the potential of fine-tuning its chemistry, simulated-rain water is an excellent substitute for rain water. Information on physical and chemical composition of natural rain water is available in the literature (Cogbill and Likens 1974). Formulations of and adjustments for simulated-rain water (e.g., ion content and pH) have been described by DuBay and Heagle (1987), Shafer et al. (1985), Percy (1986), and Shafer (1988, 1992).

Solutions used to collect "root exudates plus" from living plants range from distilled water (Wu et al. 2000a, b; Noguchi et al. 2002; see Sect. 2.1.3.2) to various types of nutrient solutions (Martin and Rademacher 1960; Wilson and Rice 1968; Bell and Koeppe 1972; Kochhar et al. 1980; Tang and Young 1982; Tang 1986; Tang et al. 1989; Pérez and Ormeño-Nuñez 1991) depending on experimental objective and substrate (e.g., liquid, agar, vermiculite, or sand) in which roots were growing (for additional details see Sect. 2.1.3.2). Hoagland's nutrient solution has been the most commonly used nutrient solution (Hoagland and Arnon 1950; for basic formula also see Blum 2011). Hoagland's nutrient solution was formulated to provide adequate nutrition for plant growth in solution and soil bioassay and its nutrient content, pH, and water potential can be readily adjusted to meet experimental objectives. What constitutes the most realistic solution to collect "root exudate plus" is difficult to say (see Sect. 2.1.3.2). However, the use of distilled water to obtain "root exudate plus" is not recommended since distilled water can be very disruptive to cells of roots and microorganisms.

The physical and chemical nature of solutions (e.g., temperature, inorganic compounds present, pH, and solute potential) used to collect leachates and "root exudates plus" is extremely important (see Sect. 2.1.3.2). Adjustments to temperature, inorganic compounds including nutrients and pH can easily be made in most instances. Adjustments to water potentials are unfortunately much more complicated. There are, however, times when adjustments of solute potential are essential. Water potentials of solutions can be adjusted, if necessary, by addition of polyethylene glycol or inorganic compounds (e.g., nutrient strength; Steuter et al. 1981; Blum et al. 1992). Data from a model by Steuter et al. (1981) for polyethylene glycol and data from Lang (1967) for sodium chloride in conjunction with freezing-point depression data can be used to adjust and estimate the solute potentials of the polyethylene glycol and nutrient solutions, respectively. However, Blum et al. (1992) found that phenolic acid solutions adjusted with polyethylene glycol impacted seedling radicle length differently than phenolic acid solutions adjusted by changing nutrient solution strength. For additional insight regarding the use of polyethylene glycol as an osmoticum see Lawlor (1970), McClendon (1981), and Plaut and Federman (1985). Mannitol has also been used to adjust solute potential but it is best used under sterile conditions (see Sect. 5.2.3.1). Freezing-point depression of solutions can be determined with an Osmette Precision Osmometer (Precision Systems Inc. Natick, Massachusetts). A more direct option for determining water potential is to use of a thermocouple psychrometer (Brown 1970).

No matter the approach taken, the solutions used to collect leachates or "root exudates plus" should have realistic, or at least consistent, inorganic content, water

Fig. 4.1 Field of sunflowers in Minnesota in August

potentials, and pH values. If solutions are not consistent for these factors, their differences must be accounted for by the inclusion of an appropriate range of treatments (i.e., series of control or reference solutions) or by adjusting solutions so that these factors are similar for all treatment solutions.

4.2.2 Leaf Leachates

4.2.2.1 Sources and Collections of Leaf Leachates

Objectives To simulate a normal range of rain events by applying simulated-rain water with defined physical and chemical characteristics (e.g., droplet size, pressure, solute potential, nutrient content and pH) to sunflower leaf canopies.

To collect representative samples of leaf leachates, determine physical and chemical characteristics of the resulting leachates, and utilize those leachates in bioassays.

Materials and Methods Sunflower (*Helianthus annuus* L) plants with a density of 5 plants/m of row and a row spacing of 20 cm (Fig. 4.1) were cultivated in field plots using normal agricultural practices (e.g., tilling, disking, fertilizer application, etc.; Unger 1980; Fernandes et al. 2011; Moroke et al. 2011; Zheljazkov et al. 2008, 2011). Field plots without sunflower were included and cultivated in a similar manner to that of the sunflower plots. Plots (5×5 m) with (treatment plots) and without (reference plots) sunflowers were located randomly within blocks of a field located on a University farm five miles south of the campus. Blocking was based on landscape position and soil texture. Leachates were collected once flowers were fully developed. Simulated rain (see Sects. 4.2.1 and 4.3.3) of 6.25, 12.5, 18.75, or

25 mm was applied 1 m above the canopy for the sunflower plots and at the same height for the reference plots at 25 mm/h (Note: Rates and times (volumes) may need to be adjusted depending on the canopy of the sunflower plants. Collection times for leachates were 15 min for 6.25 mm, 30 min for 12.5 mm, 45 min for 18.75 mm, and 1 h for 25 mm). See Cogbill and Likens 1974; DuBay and Heagle (1987), Shafer et al. (1985), and Shafer (1988, 1992) for details regarding the nature and composition of the simulated-rain water and the type of spray nozzle and pressure used to apply the simulated-rain water (also see Sects. 4.2.1 and 4.3.3). Actual composition, pH and solute potential of the simulated-rain water were based on rain water collected in the field and analyzed prior to the study. Simulated-rain water was collected just above the sunflower canopy and leachates were collected at the soil level and at identical positions for the reference plots with 25 cm diameter glass funnels inserted into 4-l glass bottles. Funnels and bottles were acid washed, rinsed thoroughly with distilled water, and sterilized before each use. Since sunflower leaves from adjacent plants were in close proximity to each other at the time of collection, ten funnel units were placed at random between plants in each treatment plot. Ten funnel units were also placed at random in the reference plots. Plants and areas on the perimeter of the plots were excluded. The volume for each funnel unit was determined and the content of the funnel units for each plot were combined. Collected simulated-rain water and leachates from each plot were then transferred to the laboratory, filtered with Buchner funnels containing Whatman #1 filter paper to remove particles, filter sterilized (0.2 µm membrane filters), and stored in a refrigerator at 4 °C. The basic experimental design was as follows: randomized-block design of 5 treatments (4 rain levels and reference) × 6 replicates (blocks) = 30 experimental units. The following data were collected:

a. mm of ambient daily precipitation up to a week prior to simulated-rain events,
b. average solar radiation during daylight hours above canopy and day length,
c. mm of simulated-rain water just above the sunflower canopy and leachates at the soil level below the canopy were collected to determine retention by the sunflower canopy,
d. mm of simulated-rain water at canopy height and at soil level in reference plots,
e. ml of leachate collected in sunflower plots and simulated-rain water collected in reference plots for each funnel unit,
f. leachate and simulated-rain water (reference) pH values, solute potential, total N, NO_3^-, NH_4^+ and available P and K content (Note: N, NO_3^-, NH_4^+ and available P and K will simply be referred to NPK from now on), an estimate of carbon or organic matter content, and Folin–Ciocalteau (F–C) reactive compounds,
g. specific potential putative allelopathic compounds (i.e., promoters, inhibitors) within or profiles of primary and secondary metabolites (metabolic profiling) of leachates could also be determined,
h. the presence or absence of sunflower mycorrhizae,
i. pH, total nitrogen (N), nitrate-N (NO_3), ammonium-N (NH_4), phosphorous (P), potassium (K), and organic matter of the upper 15 cm of soil for each treatment plot,

j. minimum, maximum, and average daily air temperatures just prior and during simulated-rain events, and
k. average light intensity at noon above and below the canopy as an indicator of canopy cover.

See Kormanik and McGraw (1982) for quantifying mycorrhizae. See Rodier (1975), Allen (1989), Buurman et al. (1996), and Nollet (2000) for solution analyses. See Pearcy et al. (1989) for environmental measurements. For soil analysis 5 random soil core samples were taken per treatment plot with a 2 cm diameter × 15 cm soil corer. Core samples for each treatment plot were batched, mixed, and sieved (0.4 mm) prior to analysis. See Bear (1964), Black (1965a, b), Page (1982); Klute (1986); Allen (1989), Carter (1993), Sparks (1996); Tan (1996), and Margesin and Schinner (2005) for soil analyses.

The F–C reactive compounds are surrogates for the promoter/modifier/inhibitor complex in leachates. The Folin–Ciocalteau reagent is reduced by substances with phenolic hydroxyl groups (e.g., cinnamic acids, hydroxybenzoic acids, gallic acid, tannic acids, and tyrosine), some substances without phenolic hydroxyl groups (e.g., pyridines, purines, alanine, cysteine, tryptophan, ascorbic acid, fructose, and proteins), some inorganic substances [e.g., iron sulphate, manganese sulphate, and potassium nitrate], and some other compounds (Anderson and Todd 1968; McAllister 1969; Box 1983; Ikawa et al. 1988; Blum et al. 1991, 1992). F–C equivalent values could be expressed in terms of any of these compounds although substances with phenolic hydroxyl groups have been the primary reference compound used in the literature.

Conventional biochemical techniques (Waller et al. 1999; Yu and Dahlgren 2000; Sampietro et al. 2009) or metabolic profiling could be used to identify and quantify a range of primary and/or secondary compounds within the leachate (Summer et al. 2003; Dunn and Ellis 2005; Xi et al. 2008; Prince and Pohnert 2010; Sardans et al. 2011).

Leachates could also be collected at given time intervals over the growing season. However, there should be at least a week or longer between simulated-rain events or ambient precipitation events and the next simulated-rain event to allow for the plants to recover. Collecting and testing leachates after ambient or simulated-rain events or at different stages of development are additional options. Finally, different rates of simulated-rain water could also be applied to the canopies.

Hypotheses If the pools of leachable organic and inorganic compounds within the leaf canopy at a given point in time are finite or these pools require an extended period of time for recovery, then the concentration of promoters, modifiers, and inhibitors within the leachate collected should decline with increasing mm of simulated rain.

If the pools are not finite or the pools recover fairly rapidly, then concentrations of promoters, modifiers, and inhibitors within the leachates will remain fairly constant or decline more slowly than the leachates for the first hypothesis, respectively, with increasing mm of simulated rain.

If multiple simulated-rain events are used, then the organic and inorganic compounds within leachates (and solute potential, pH, etc.) will depend on the stage of development and the physiology ("health") of the sunflower plants, the time intervals and weather (e.g., ambient rain events, amount of sunlight, and temperatures) before and between rain events, and the rates of recovery between rain events.

Changes in solute potential, pH, etc. are also likely to occur in conjunction with the decline in promoters, modifiers, and inhibitors (i.e., organic and inorganic compounds) of leachates. The organic and inorganic gradients within the leachates collected by different levels (i.e., mm) of simulated-rain water during a given rain event do not correspond to laboratory dilutions series (see Sect. 3.2.5). They are independent of each other since they were collected from different sunflower plots. However, multiple rain events from the same plants within the same plot are not independent of each other.

4.2.2.2 Leaf Leachate Bioassays

The potential effects of organic and inorganic compounds (i.e., promoter/modifier/inhibitor complex) and the physical nature (e.g., solute potential and pH) of leachates from leaf canopies obtained by simulated-rain events (or by fog drip or natural-rain events [throughfall]) may be determined by testing leachates in Petri dish, solution, and soil bioassay systems. Proper collection, processing, storage, and application of leachates, however, are essential (see Sects. 2.1.3.2, 2.1.3.3, and 2.1.3.4).

Objectives To determine if leaf leachates collected at one point in time with simulated-rain water under field conditions could potentially influence weed seed germination and radicle length in Petri dishes without soil (single application) and weed seedling emergence and biomass in the presence of soil (multiple applications) under laboratory conditions.

To determine if there are relationships between the physicochemical properties of the leachates collected and germination, radicle length, seedling emergence, and biomass of weed species.

Materials and Methods *Option 1:* Sunflower leaf leachates obtained with simulated-rain water (treatments) or simulated-rain water (see Sects. 4.2.1 and 4.3.3; reference) were added to sterile glass Petri dishes (Fig. 4.2; 100 mm × 25 mm) containing sterile Whatman #1 filter paper and weed seeds (crabgrass [*Digitaria sanguinalis* L.], morningglory [*Ipomoea hederacea* L.], or pigweed [*Amaranthus retroflexus* L.]). Weed seeds were not surface sterilized. The volume of leachates or simulated-rain water added to Petri dishes was based on seed size and number but was identical for all experimental units for a given weed species. Solutions added to Petri dishes were at room temperature. Number of weed seeds per Petri dish was constant for each species and based on seed size (see Sect. 3.5). Germination (number of seeds with radicles) and radicle length were monitored just after germination ceased for the reference treatment of a given weed species. The total number of germinated weed seeds and the radicle lengths for germinated seeds

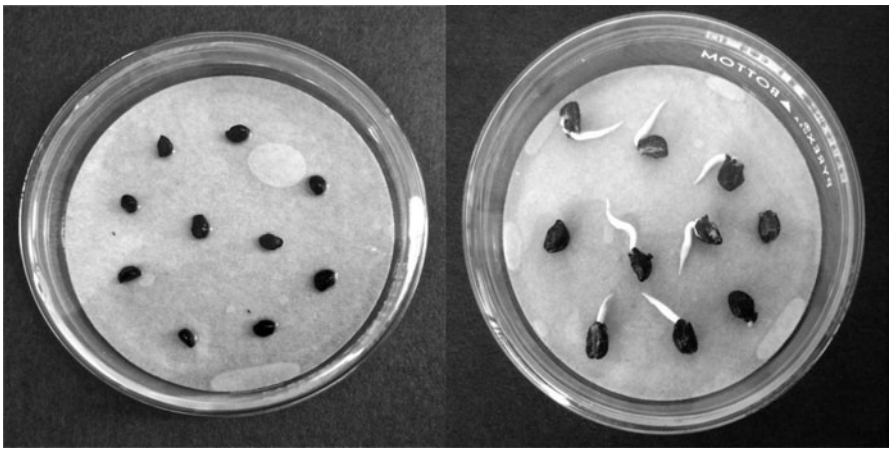

Fig. 4.2 Petri dish system with morningglory seeds, water, and filter paper on day one and day four

in each Petri dish were determined (Note: Daily emergence or cumulative daily numbers could also be used for time series studies). Bioassays were carried out in a growth chamber in the dark with a relative humidity of 70 % and day/night temperatures mimicking the times when leachates were collected. The basic experimental design was as follows: randomized-block design of 5 treatments (4 rain levels and reference) × 6 replicates (blocks) = 30 experimental units per weed species per rain event.

Seeds can be surface sterilized. However, obtaining seed lots where all the seed surfaces are sterile is extremely cumbersome and time consuming. In addition, species sterilization procedures can impact germination and radicle growth (e.g., see Blum et al. 1984) and surface sterilization will not eliminate microorganisms within seeds (Leben 1961; Mundt and Hinkle 1976). After seed imbibition a small amount of free solution should still be evident in the Petri dishes. Multiple additions of leachates and simulated-rain water should be avoided, if at all possible, because the organic and inorganic content, water potential, etc. of solutions will change with each addition. Relative humidity should be kept as high as possible to minimize evaporation from Petri dishes. Petri dishes may also be sealed to minimize evaporation but the potential creation of anaerobic conditions may be a concern. The use of a single reference solution could lead to false interpretation of the data particularly when the physical and chemical composition (e.g., pH values, content of inorganic compounds, and solute potentials) of the leachates for each rain level are different. In that case properly adjusted control or reference solutions are required for the different leaf leachates. Another option would be to adjust the pH, inorganic compound content and solute potential of each treatment solution (leachates and reference) so that they are similar.

A delay in seed germination due to leachate treatments will impact radicle length (i.e., estimated growth rates for radicles). Thus, if radicle growth rates are to be

determined independently of germination time, then pre-germinated seeds with roughly equal radicle lengths will need to be utilized in the leachate bioassays.

Option 2: Soil for this bioassay was taken from the sunflower reference plots. Air-dried and sieved (5 mm mesh) soil was placed into 15 cm pots with a 1,767 cm^3 volume (no drainage holes in the bottoms of the pots), and surface seeded with weed seeds (crabgrass [*Digitaria sanguinalis* L.], morningglory [*Ipomoea hederacea* L.], or pigweed [*Amaranthus retroflexus* L.]). Soil and seed surfaces were not sterilized. Seeds were placed roughly equidistant from each other in each pot to minimize competition between seeds. Number of weed seeds per pot was constant for each species and based on seed size (see Sect. 3.5). After surface seeding, seeds were covered with 1 cm of soil. The final g/pot of soil was identical for all pots.

Pots were treated with identical amounts of sunflower leachates or simulated-rain water (reference) to bring the soil moisture level to a predetermined level based on pot weight. It was assumed that a change of 1 gm for a pot system was equal to 1 ml change in water content of the pot system. The predetermined level was chosen so that soil moisture was adequate for germination but below field capacity. After the initial treatment, solutions were added only when soil moisture of all pots reached a level that would accommodate the total volume of the treatment solution to be added. After addition of treatment solution all pots weights were then brought back to the predetermined level by addition of simulated-rain water. Solutions added to pots were at room temperature.

Bioassays were carried out in growth chambers with a light intensity of 140–180 µmol/cm^2/sec and a relative humidity of 70 % and a photoperiod and day/night temperatures mimicking the times when leachates were collected. Light intensity at pot level, air and soil temperature, and relative humidity were monitored at regular intervals during the experimental period. The number of weed seedlings per pot was recorded daily. Weed seedlings were removed after each count to minimize competition. When removing seedlings, soil disturbance was minimized as much as possible. The daily averages, total numbers at end of the experimental period, and slopes based on cumulative numbers of weed seedlings per day were determined for seedling emergence. The number of days for the experimental periods were determined by the cessation of seedling emergence for the reference treatment of a given weed species. The basic experimental design was as follows: randomized-block design of 5 treatments (4 rain levels representing a concentration range and reference) × 6 replicates (blocks) = 30 experimental units per weed species per rain event.

Option 3: Transplanted weed seedlings of equal size were used instead of weed seeds (see *Option 2*) to determine the effects of sunflower leaf leachates on weed seedling biomass. For this bioassay seedlings of equal size germinated, grown in quartz sand (or vermiculite), and watered with nutrient solution were transplanted into 150 ml cups containing 150 g soil supplemented with NPK. One seedling was transplanted per cup (Fig. 4.3). Shoot, root, and total weed seedling biomass per cup was determined at the end of the experimental period. At least an experimental period of a week was required to observe an obvious visual effect by leachates on seedling size.

Due to the small volume of soil, the soil was supplied with nutrients, i.e. NPK. Nutrient additions were based on soil analysis. For soil analyses see Sect. 4.2.2.1.

Fig. 4.3 Morningglory seedlings growing in soil cup system

Weed seedlings could also be inoculated with mycorrhizal fungi before transplanting (See Menge and Timmer 1982).

Depending on the nature of the soil (Option 2 and 3), the addition of sand may be required for adequate percolation and aeration. Based on experimental objective, leachates and reference solutions added to pots or cups may or may not be adjusted for pH, nutrient content, and solute potential. Although cup system weights for Option 3 will increase over time due to seedling growth and the predetermined soil moisture level will decline, these changes turn out to be relatively small over short time intervals (see Blum and Gerig 2006). By picking the right predetermined moisture level, multiple daily additions of treatments can be precluded. A greenhouse with the appropriate photoperiod and day/night temperatures or the out-of-doors could also be used. See Pearcy et al. (1989) for environmental measurements.

Hypotheses If the pools of leachable organic and inorganic compounds within a leaf canopy given a simulated-rain event are actually or functionally finite (i.e., recovery of organic and inorganic compounds including promoters, modifiers, and

inhibitors takes longer than the rain event), then the stimulation or inhibition of germination, radicle length, emergence, and biomass of sensitive weed species will change (i.e., increase or decline) as increasing mm of simulated-rain water are applied to a leaf canopy (i.e., resulting organic and inorganic compounds within leachates will be diluted).

If the pools are not actually or functionally finite (there is complete or partial recovery of the pools during a rain event), then stimulation or inhibition of germination, radicle length, emergence, and biomass of sensitive weed species will not change or will change at a slower rate than for Hypothesis 1 leachates as increasing mm of simulated-rain water are applied to the leaf canopy (i.e., resulting leachates are not diluted or are diluted at a slower rate than for Hypothesis 1 leachates).

If sunflower leaf leachates or simulated-rain water (reference solution) have pH values, inorganic contents, and solute potentials that are approximately similar for all the treatment solutions or, if necessary, adjusted so that they are similar, then any observed effects (i.e., stimulatory or inhibitory) of sunflower leaf leachates on germination, radicle length, emergence, and biomass of sensitive weed species will be a direct or indirect product of the organic promoters, modifiers, and inhibitors present in the sunflower leachates.

If germination, radicle length, emergence, and biomass of sensitive weed species are stimulated, then organic promoters dominate the promoter/modifier/inhibitor complex of the leachates and if inhibited, then inhibitors dominate the complex of the leachates. (Reminder: Theoretically any given compound may be a promoter, modifier, or inhibitor depending on its concentrations and physicochemical state and the species process being monitored [Sect. 2.1.3.1]). This also assumes that any differences in inorganic compounds, solute potential, pH, etc. other than promoters, modifiers, and inhibitors of leachates have been accounted for or mitigated.

If sunflower leaf leachates and reference solutions have different pH values, organic and inorganic contents, solute potentials, and organic promoters, modifiers, and inhibitors (subset of organic compounds), then the observed effects on weed seeds and seedlings will be a product of all the direct or indirect actions and interactions of all the factors of the sunflower leaf leachates, not just the organic promoters, modifiers, and inhibitors of the leachates. The use of the word indirect here is an attempt to include the action of any active altered or produced organic compounds by microorganisms.

Analyses *Step 1:* Do leachates have an effect on weed germination, radicle length, emergence, and biomass? If the leachates produced by a single rain event of 6.25, 12.5, 18.75, and 25 mm constitute a series of gradients (e.g., organic and inorganic concentrations, solute potentials, pH values, etc.), then linear and polynomial regression analyses can be used to determine the potential relationships between mm of simulated-rain water applied to or recovered under the sunflower canopy (X, independent variable) and the observed increase or decrease (Y, dependent variable) in germination and radicle length (Option 1), seedling emergence (Option 2; average and total seedling numbers and slopes), and seedling biomass (Option 3). For more complex statistical analyses, such as time series (Option 2), modeling, etc. (all

Options), the reader is advised to consult a statistician for the proper statistical and analytical tools and techniques.)

Step 2: What are the potential causative agents? Multiple regressions could be used to determine potential roles for and interactions of organic compounds (individually, if identified, or combined [e.g., carbon, organic matter content, F–C reactive compounds, compounds identified by conventional biochemical techniques, or metabolic profiling]), inorganic compounds (individually, if identified, [e.g., NPK] or combined [e.g., salinity]), solute potentials, pH values, etc. of leachates and control or reference solution(s) in determining the potential factor, factors, and interactions of factors that may be responsible for the observed increase or decrease of germination and radicle length (Option 1), seedling emergence (Option 2), and seedling biomass (Option 3). If organic compounds individually or in combinations appear to account for some or all of the observed response, then further studies are warranted (see Chap. 5). If not, the presence of potential allelopathic effects by canopy leachates under the conditions collected, processed, and tested must be rejected.

See Sect. 4.2.1 for adjusting and monitoring solute potentials. See Sect. 4.2.2.1 for solution analyses, determining F–C reactive compounds, conventional biochemical techniques, and metabolic profiling.

Comments Option 1 provides information on the effects (i.e., stimulation or inhibition) of a single application of sunflower leachates on germination and radicle length in the absence of soil. Given the Petri dish environment (e.g., direct immersion of seeds in leachates), the results represent maximum potential stimulation or inhibition that is possible for the conditions of that bioassay (e.g., volume and concentration of treatment solutions, density of seeds, seed size, temperature, microbial populations, time period of the bioassay, etc.). Options 2 and 3 provide information on the effects (i.e., stimulation or inhibition) of multiple applications of sunflower leachates from a single rain event on seedling emergence and biomass when seeds, radicles, and seedling roots are in contact with soil. For all intents and purposes, the soil functions as a modifier (barrier) regulating types and concentrations of compounds reaching the seeds, radicles, and roots. Thus, the behavior observed in the pot and cup systems represents a soil-modified response to the leachates. As with the Petri dish bioassay, the observed increase or decrease, in this case seedling emergence or biomass, is valid for only the conditions of that bioassay (e.g., soil type, soil layer above seeds, volume and concentration of leachates applied, seed density, temperature, light intensity, microbial populations, time period of the bioassay, etc.). The bottom line is that these options provide a means of screening for potential effects of leachates and the physical and chemical makeup of leachates in the absence and presence of soil with the caveat that the microbial actions within these bioassay systems are an unknown factor. However, the overall artificial nature of these bioassay systems will provide little, if any, direct insight regarding how leaf leachates are actually impacting seed germination, radicle length, seedling emergence, and biomass under field conditions. Furthermore, the use of a single reference solution may lead to false interpretation of the data particularly when the physical and chemical composition (e.g., pH, water potentials, and inorganic

compound content) of the leachates for each simulated level and event are different. In that case an appropriate range of treatment solutions (i.e., series of control or reference solutions) will be required for each rain level and event. Another option would be to adjust the pH, inorganic compound content and solute potential of each treatment solution (leachates, reference solution) so that they are similar.

The use of multiple leachate treatments for soil systems is, of course, somewhat atypical since in nature rain events, at least in temperature regions of the world, do not occur on a regular daily basis. Multiple additions are, however, required to maintain soil moisture for germination and growth and to refresh leachates in the soil since organic and inorganic compounds added by leachates are bound, leached, utilized by microbes, etc. (see Sect. 1.4). Refreshing treatments solutions appears to be important for meaningful dose response bioassays because the effects of most IPA compounds studied are not systemic in action and are lost as concentrations decline.

4.2.3 "Root Exudates Plus"

Two classical systems commonly used to collect and test for potential stimulatory or inhibitory activity of "root exudates plus" have been the step system of Martin and Rademacher (1960) and the XAD-4 resin system of Tang and Young (1982), Tang 1986, and Tang et al. (1989). Modified versions of the step system (Wilson and Rice 1968; Bell and Koeppe 1972; Kochhar et al. 1980) and the XAD-4 resin system are described below (also see Sect. 6.3).

4.2.3.1 Stair-Step System

Objective To determine if sunflower "root exudates plus" leachates could potentially influence seedling emergence and biomass of several weed species in the absence of soil.

Materials and Methods *Option 1:* Sunflower plants were grown from seed in flats containing quartz sand in a shade-free area of a greenhouse. Flats were watered with Hoagland's nutrient solution daily or as needed (Hoagland and Arnon 1950). Light intensity at plant level, air and soil temperature, and relative humidity were monitored at regular intervals during the growth period. Once sunflower seedlings were established, seedlings of the same size were gently removed from the sand and transplanted (one seedling per pot) into 15 cm pots (3,535 cm^3 volume) containing quartz sand. Sunflower seedling roots were inoculated with fungal spores during the transplanting process to allow for the development of mycorrhizae. For procedural details regarding inoculation see Menge and Timmer (1982). When the sunflower plants were 25–30 cm tall, the pots were placed on steps of a 3 stair-step system (Fig. 4.4) in the greenhouse. Pots (15 cm; 3,535 cm^3 volume) containing quartz sand and weed seeds (crabgrass [*Digitaria sanguinalis* L.], morningglory

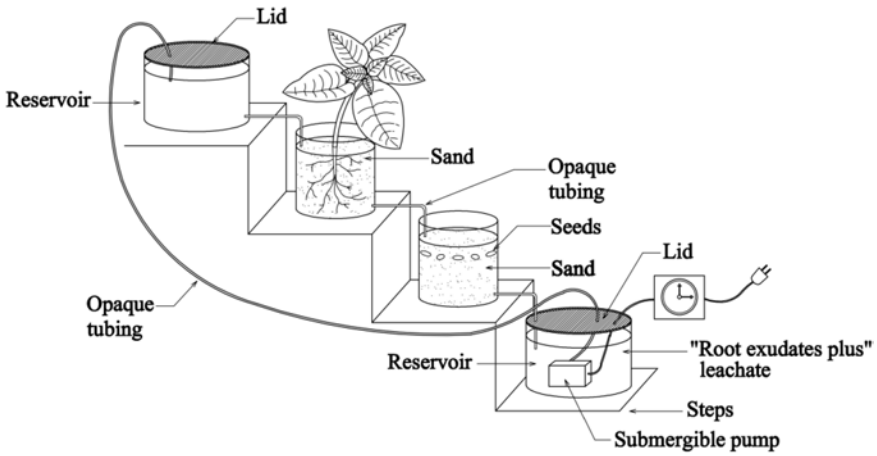

Fig. 4.4 Stair-step system for studying recirculating "root exudates plus" leachates. (Illustration by Amy Blum Grady, used with permission)

[*Ipomoea hederacea* L.], or pigweed [*Amaranthus retroflexus* L.]) placed 1 cm below the surface of the sand were also placed on steps of the stair-step system. Numbers of weed seeds per pot were constant and based on seed size. Seeds were placed roughly equidistant from each other in each pot to minimize competition (see Sect. 3.5). System components were not sterilized. The arrangements on the stair-step system were as follows:

a. treatment 1—on step 2 a pot with sunflower and on step 1 a pot with quartz sand (sunflower reference),
b. treatment 2—on step 2 a pot with quartz sand and on step 1 a pot with weed seeds (weed reference), and
c. treatment 3—on step 2 a pot with sunflower and on step 1 a pot with weed seeds (treatment).

The locations of sunflower pots and weed pots on the stair-step systems can actually occur in any order since this is a semi-closed recirculating system. Pots were connected by tubing so that Hoagland's solution and "root exudates plus" (actually "root exudates plus" leachates) could flow by gravity from the bottom of a pot (step 2) to the sand surface of the pot below (step 1). Hoagland's solution was supplied to the pots in the following manner.

Independent reservoirs at the bottom of each stair-step system (step 0) were filled with 2 l of Hoagland's solution (pH 5.5). (Note: Reservoirs, tubing, and pots were impermeable to light. Volume of reservoirs may be adjusted as needed.) Hoagland's solution from these reservoirs was pumped by submersible pump and tubing to independent reservoirs located at the top of the stair-step system (step 3) for 5–10 min (sufficient time for the 2 l to be pumped to the top reservoirs) at 4 h intervals using a timer. The reservoirs on step 3 were connected to the pots on step 2 by tubing. The flow rates from the upper reservoirs were regulated by a clamp to

minimize sand surface disturbance and to extend the time period during which pots were supplied with Hoagland's solution containing "root exudates plus" leachates. Since each stair-step system (treatment or reference) consisted of interconnecting reservoirs and pots, Hoagland's solution and "root exudates plus" leachates were circulated from the reservoir on step 3 to the sand surface of the pot on step 2 to the sand surface of the pot on step 1 to the reservoir on step 0 and back again to the reservoir on step 3 for each individual stair-step system. The stair-step system was designed to minimize competition between plants for water, nutrients, light, and space. In order to achieve that, the following was required:

a. the pH of the solutions in each reservoir (step 0) was monitored and adjusted daily,
b. the water lost by evaporation and transpiration was replaced each morning, afternoon, and evening by additions to the reservoirs at the bottom of the stair-step system (step 0),
c. the nutrients (e.g., nitrogen, phosphorus, and potassium) in each reservoir were closely monitored and adjusted so that nutrients were not limiting,
d. the solute potentials of the solutions in each reservoir (step 0) were monitored and adjusted as needed.

In addition, average light intensity at the top of sunflower plants and the sand surface of weed pots, daily minimum, maximum, and average day/night air temperature, and daily seedling emergence were recorded. Weed seedlings were removed after each count to minimize competition. When removing weed seedlings, disturbance of the remaining seeds was minimized as much as possible. The daily average, total number at the end of the experimental period, and slopes based on cumulative number of weed seedlings per day were determined for seedling emergence (Note: Daily emergence or cumulative daily numbers could also be used for time series studies.). The number of days for the experimental periods were determined by cessation of seedling emergence for the reference treatment of a given weed species. In addition root, mycorrhizae and shoot biomass of sunflower plants in each pot were determined. For plants with mycorrhizae, estimates of percent infection were also determined (see Kormanik and McGraw 1982). The basic experimental design was as follows: randomized-block design for 3 treatments × 5 replicates (blocks) = 15 experimental units per weed species.

Multiple sunflower seedlings were not used in this system because sunflowers have been shown to be autotoxic (i.e., self-inhibiting; Wilson and Rice 1968) and to eliminate potential competition between sunflower plants. Biomass and surface area of roots and mycorrhizae of sunflower plants are important determinants in this system. The bigger the systems of roots and mycorrhizae, the more likely there will be an effect. Due to potential rapid drying of the upper portion of the sand in the pots, seeds may have to be covered with more than 1 cm of sand or the sand surface can be covered by wet filter paper to reduce evaporation from the sand surface. A layer of gravel could also be placed on the sand surface. Gravel has an additional benefit in that it will reduce algal growth on the surface of the sand. Replacing solutions at various time intervals is not recommended since accumulated "root

exudates plus" leachates would be lost each time solutions are replaced. All indications are that some accumulated level (i.e., threshold) of original and modified (e.g., modified by microbial activity and oxidation) "root exudates plus" leachate is required before effects are observed. See Sects. 4.2.1 and 4.2.2.1 for adjusting and measuring solute potential, for environmental measurements, and for solution analyses. Sunflower plants could also be grown out-of-doors to increase the phenolic acid content of the plants (Hall et al. 1982, 1983). Phenolic acids have frequently been labeled as IPA compounds (see Rice 1984).

Option 2: Transplanted weed seedlings of equal size were used instead of weed seeds (see Option 1) to determine the effects of sunflower "root exudates plus" leachates on weed seedling biomass. For this bioassay, seedlings of equal size grown in quartz sand and watered with nutrient solution were transplanted into the pots at a density of four seedlings per pot (see Sect. 3.5). Weed seedlings were inoculated with fungal spores prior to transplanting to allow for the formation of mycorrhizae (see Menge and Timmer 1982 for details). Final shoot, root, and total seedling biomass was determined for the weed seedlings. An experimental period of at least a week was required to observe an obvious visual effect of the "root exudates plus" on seedling size.

The experimental designs of both Options 1 and 2 could be considerably improved by using sunflower plants of different ages or sunflower plants grown under different environmental conditions (Hall et al. 1982, 1983; also see Sect. 2.1.3.1) and by monitoring the sunflower plants at harvest and/or "root exudates plus" leachates for F–C reactive compounds or isolating, identifying, and quantifying organic compounds by conventional biochemical techniques or metabolic profiling. The different types (e.g., age) of sunflower plants, the F–C reactive compounds, and/or the organic compounds identified could then be used as independent quantitative variables to describe response surfaces for weeds. The basic experimental design would then be as follows: randomized-block design for 3 treatments × 5 sunflower types × 5 replicates (blocks) = 75 experimental units per weed species. This assumes that pH, solute potential, and inorganic compounds (e.g., NPK) have been properly maintained. See Sect. 4.2.2.1 for determining FC-reactive compounds, conventional biochemical techniques and metabolic profiling.

Hypotheses If sunflower "root exudates plus" leachates with similar pH, solute potentials, and adequate or similar nutrient content are recirculated, then any observed effects (i.e., stimulation or inhibition) of sunflower "root exudates plus" leachates on weed seedling emergence and biomass in the absence of soil will be a direct or indirect product of the organic promoters, modifiers, and inhibitors of the sunflower "root exudates plus" leachates.

If weed seedling emergence and biomass are stimulated, then promoters dominate the promoter/modifier/inhibitor complex of the leachates and if inhibited, then inhibitors dominate the complex. (Reminder: Theoretically any given compound may be a promoter, modifier, or inhibitor depending on its concentrations and physicochemical state and the species process being monitored [Sect. 2.1.3.1]).

If sunflower "root exudates plus" leachates with different pH values, nutrient contents, solute potentials (i.e., were not adjusted) and organic promoters, modifiers, and inhibitors are recirculated, then the observed effects will be a direct or indirect product of all the actions and interactions of all the factors present in the sunflower "root exudates plus" leachates, not just the organic promoters, modifiers, and inhibitors present in the sunflower "root exudates plus" leachates. The use of the word indirect here is an attempt to include the action of any active altered or produced organic compounds by microorganisms.

Weed seedlings (or seeds) are unlikely to impact sunflower plants in this instance, but in the unlikely event that this happens, all Hypotheses would apply after substituting weed "root exudates plus" leachates for sunflower "root exudates plus" leachates and sunflower plant biomass for weed seedling emergence or biomass at the appropriate places.

Analyses *Step 1:* Do sunflower "root exudates plus" leachates have an effect on weed seedling emergence or biomass? Do weed "root exudates plus" leachates have an effect on sunflower biomass? Given this design we are limited to a means comparison of the following:

a. Treatment 2 weed data (reference) to Treatment 3 weed data (sunflower "root exudates plus" leachate treatment), and
b. Treatment 1 sunflower data (reference) to Treatment 3 sunflower data (weed seed leachate treatment).

Step 2: What are the potential causative agents for a.? If the recirculating solutions have been properly monitored and adjusted, then a significant effect for a. would imply that the organic compounds released by sunflower roots and mycorrhizae directly or indirectly are responsible for the observed effects on weeds (seedling emergence [Option 1] and biomass [Option 2]). On the other hand if nutrients were limiting and there were differences in pH, solute potential, etc. in the recirculating solutions, then no conclusions could be drawn regarding causation. Similar observations regarding sunflowers (b.) could also be made for that system.

Comments for Weeds The effects observed in the stair-step system are the product of the actions and interactions of both organic and inorganic compounds released (actually net release since both release and uptake occurs) from sunflower roots and mycorrhizae and to some extent weed roots and mycorrhizae depending on the system. Microbial actions within the systems are an unknown factor. If the inorganic compounds, pH, and solute potentials of the nutrient solutions in the reservoirs have been properly maintained (not limiting and similar for all treatments), then we may hypothesize that the effects observed (i.e., stimulation or inhibition) were directly or indirectly due to the organic compounds released from sunflower roots (i.e., "root exudates plus" leachates). Additional circumstantial evidence to support or to reject this hypothesis could be provided by analyzing the "root exudates plus" leachates for specific organic compounds and determining if there were any correlations between weed seedling emergence or weed seedling biomass and the organic compounds identified. However, some caution regarding the significance of these

observations is essential since stair-step systems provide information on the effects (i.e., stimulation or inhibition) of sunflower "root exudates plus" under somewhat different conditions than found in soil systems. For example, in the stair-step system with quartz sand as substrate there is:

a. an absence of irreversible and reversible sorption of both organic and inorganic compounds to recalcitrant organic matter, clays, etc.
b. an absence of numerous anaerobic sites,
c. a much greater likelihood of oxidation of both organic and inorganic compounds due to the porosity of the quartz sand and the transfer between pots,
d. a much lower resistance to belowground growth and, thus, a different anatomy and morphology of roots and mycorrhizae, and a lower loss and lysis of cells,
e. a very rapid transfer and distribution of "root exudates plus" leachates (i.e., organic and inorganic compounds) throughout the substrate,
f. an atypical nutrient content, pH, and solute potential of Hoagland's solution compared to soil solutions,
g. considerable dilution of the water-soluble "root exudates plus" due to the volume of nutrient solutions within the system, and
h. microbial populations that differ from those found in soils.

Furthermore, the stair-step system tests for the effects of water-soluble compounds and with minor exceptions for the effects of hydrophobic and volatile compounds. As a result, the actual production, distribution, oxidation/reduction, and microbial utilization of "root exudates plus" leachates in the stair-step systems are substantially different from "root exudates plus" found in soil systems. The bottom line is that the stair-step system provides a means of screening for potential effects of "root exudates plus" leachates in the absence of soil. However, the overall artificial nature of this bioassay system will provide little, if any, direct insight regarding actual effects of sunflower "root exudates plus" on weed seeds or seedlings under field conditions. Finally, since the actual expression of stimulation or inhibition will also be determined by the physicochemical and biotic environments of the bioassay system, the results will also be limited to the environmental conditions of the bioassay and the species process being monitored.

4.2.3.2 XAD-4 Resin System

Objectives To determine if sunflower "root exudates plus" leachates could potentially stimulate or inhibit weed germination and radicle length of several weed species in Petri dishes without soil.

To determine if XAD-4 resin removes stimulatory or inhibitory substances from "root exudates plus" leachates.

To recover, isolate, and identify potential stimulatory or inhibitory substances sorbed by XAD-4 resin.

The first and second are primary objectives and the third is a secondary objective. XAD-4 resin is known to sorb a variety of organic compounds including

phenolic acids that have been labeled as identified putative allelopathic (IPA) compounds. For additional details see Sects. 2.1.3.2, 6.3, and 7.1.

Materials and Methods The bottoms of 4-liter glass bottles were removed and bottles were inverted. The neck of each bottle was sealed with a 2 mm plastic screen. Glass wool was placed above the screen and clean quartz sand was added to the inverted-glass bottles until the sand reached approximately 5 cm from the top. All inverted-glass bottles contained the same amount of sand. Sunflower plants were grown and inoculated as described in Sect. 4.2.3.1 and transplanted into these inverted 4-liter glass bottles (see Tang and Young 1982; Tang 1986; Tang et al. 1989). A set of inverted-glass bottles without sunflower plants were included as reference or baseline. All inverted-glass bottles (plus or minus transplanted seedlings) were supplied with full strength Hoagland's nutrient solution daily until sunflower plants were at the desired stage of maturity (see Sect. 4.2.3.1). Prior to converting the inverted-glass bottle systems to recirculating systems, the systems were flushed with 0.1 strength Hoagland's nutrient solution to remove any excess nutrients that may have accumulated. Each inverted-glass bottle system (plus or minus sunflower plants) was then connected directly to a glass column containing either glass beads or XAD-4 resin (Fig. 4.5). The columns in turn were connected by tubing to a reservoir which was connected to the top of the inverted-glass bottles by tubing which was placed just below the surface of the sand. All inverted-glass bottles, tubing, and columns were covered with aluminum foil. The reservoirs were filled with 2.5 l of 0.1 strength Hoagland's nutrient solution (pH 5.5) and a peristaltic pump was attached to the tubing between the reservoir and the tops of the inverted-glass bottles. Flow rates were adjusted for continuous drip irrigation of approximately 600 ml/h. Water was added to each reservoir as frequently as necessary to compensate for evapotranspiration. Frequency of water additions depended on the rates of evapotranspiration but volume within the reservoirs was not allowed to go below 90 % of the original volume. Glass beads were cleaned and XAD-4 resin was replaced or reactivated at regular intervals during the week (see Sect. 6.3). After a week solutions from the reservoirs were brought to their original volumes, filter sterilized, and monitored for nutrient content (e.g., NPK), solute potential, and pH before solutions were tested in Petri dishes containing filter paper and weed seeds (crabgrass [*Digitaria sanguinalis* L.], morningglory [*Ipomoea hederacea* L.], or pigweed [*Amaranthus retroflexus* L.]). For additional details regarding the Petri dish bioassay, see Option 1 Sect. 4.2.2.2. The basic experimental design was as follows: randomized-block design of 4 treatments (plus or minus XAD-4 resin with and without sunflower plants) $\times 3$ replicates (blocks) $= 12$ experimental units per weed species.

XAD-4 resin and glass beads were cleaned prior to use. For details regarding cleaning and reactivation of XAD-4 resins see Tang and Young (1982) and Sect. 6.3. The volume and mass of resin to be used in the columns will vary with the size of the root and mycorrhizal systems. A preliminary bioassay should be used to determine the correct volume and mass of resin required. Tang and Young (1982) used a 12-g slurry to fill their column. Glass beads should theoretically have a similar

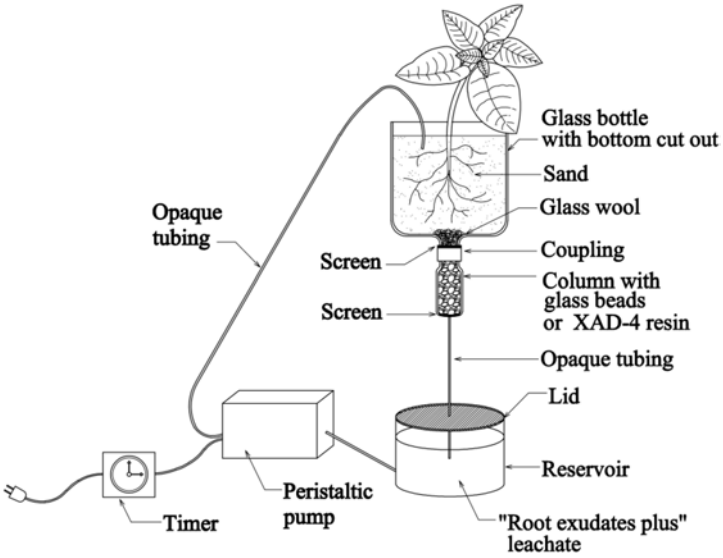

Fig. 4.5 XAD-4 resin and glass bead systems for collecting "root exudates plus" leachates. A modified version of figure by Tang and Young (1982) used with permission of American Society of Plant Biologists. (Illustration by Amy Blum Grady, used with permission)

surface area as the resin. Volume in a reservoir may need to be adjusted in order to maintain the prescribed flow rate. The time interval before samples are taken from reservoirs for use in Petri dish bioassays may need to be adjusted depending on the sizes of the sunflower root and mycorrhizal systems; the larger the root and mycorrhizal systems, the shorter the time period required before samples can be taken for testing. If NPK, solute potential, and pH of the solutions from the reservoirs vary substantially, then solutions should be adjusted so that they are roughly equivalent before being used in the Petri dish bioassay. See Sects. 4.2.1 and 4.2.2.1 for solution analyses and adjusting and monitoring solute potentials.

Hypotheses If solutions (i.e., sunflower "root exudates plus" leachates) from sunflower systems without XAD-4 resin (system with glass beads) are stimulatory or inhibitory to weed germination and radicle length when compared to solutions from systems without sunflower and without XAD-4 resin (system with glass beads), then the observed effects are due to the organic compounds released from sunflower roots and any subsequent modifications and synthesis by microorganisms.

If the stimulatory or inhibitory effects of test solutions are reduced or lost in the presence of XAD-4 resin, then the resin is sorbing allelopathic compounds (see Sect. 2.1.3.1) from the "root exudates plus" leachates.

If solutions with different pH values, nutrient contents, solute potentials and organic promoters, modifiers, and inhibitors are tested (i.e., solutions were not properly maintained or adjusted), then the observed effects will be a direct or indirect product of all the actions and interactions of all the factors present. The use of the

word indirect here is an attempt to include the action of any active altered or produced organic compounds by microorganisms.

The first and second hypotheses assume that solution levels have been properly maintained and NPK content, solute potentials and pH values of the solutions have been properly adjusted as needed. The second and third hypotheses assume that XAD-4 resin does not itself have an effect on weed germination and radical length.

Analyses Three mean comparisons for weed germination and radicle length can be made for leachates from the following systems:

a. XAD-4 resin systems without sunflower (XAD-S) vs glass bead systems without sunflowers (G-S) to determine potential effect of XAD-4 on weed germination and radicle length,
b. Glass bead systems with sunflower (G + S) vs G-S to determine the effects of sunflower "root exudates plus" leachate on weed germination and radicle length, and
c. XAD-4 resin systems with sunflower (XAD + S) vs G + S to determine if XAD-4 resin reduces or eliminates sunflower "root exudates plus" leachate effects on weed germination and radicle length.

Comments Both the stair-step system and the XAD-4 resin system test leachates of "root exudates plus" produced in the absence of soil and, thus, have essentially similar concerns and problems (see Comments for Weeds under Sect. 4.2.3.1) but the resin system has an advantage over the stair-step system. If the resin sorbs potential allelopathic compounds, these compounds can be released from the resin and analyzed for organic compounds or organic compounds or XAD-4 treated and untreated leachate solutions can be analyzed for organic compounds to determine their differences. For the released compounds and the treated and untreated leachates conventional biochemical techniques or metabolic profiling can be used to provide insight regarding the organic compounds that may be responsible for the observed effects. See Waller et al. (1999), Yu and Dahlgren (2000), and Sampietro et al. (2009) for conventional biochemical techniques. See Summer et al. (2003), Dunn and Ellis (2005), Xi et al. (2008), Prince and Pohnert (2010), and Sardans et al. (2011) for metabolic profiling.

4.2.4 Significance

What we know at this point from these bioassays is that canopy (or leaf) leachates and leachates of "root exudates plus" in the absence of soil have or do not have the potential to stimulate or inhibit germination, radical length, seedling emergence, and biomass of weed species under the conditions of the bioassays. If that is the only objective, then there is no need to go further. However, if the objective is to establish the range of biotic (e.g., different stages of sunflower maturity plus or minus mycorrhizae, etc.) and environmental conditions under which these potential effects may be expressed, then additional experiments using a range of biotic and

environmental conditions will be required. Furthermore, if one wishes to establish that the observed stimulation or inhibition of canopy (or leaf) leachates and "root exudates plus" leachates are due to allelopathic compounds released by the canopy (or leaves) and roots and mycorrhizae, respectively, then specific roles for active organic compounds (i.e., promoters and inhibitors) within these leachates and subsequently created active organic compounds resulting from oxidation, reduction, or the action of microorganisms would also need to be demonstrated individually and within the context of the leachates and exudates being tested (Willis 1985, 1994, 2007; Blum et al. 1999; Molisch 2001; Blum 2011; also see Sect. 1.1 and Chap. 6). This may be readily achievable if the observed effects are due to a small number of highly active compounds (i.e., the herbicide paradigm) but may not be so readily achievable if the observed effects are due to a large number of compounds with low activity and low concentrations acting in concert (i.e., the more holistic paradigm). In the latter case metabolic profiling (metabolomics) may provide some answers (Summer et al. 2003; Dunn and Ellis 2005; Prince and Pohnert 2010; D'Ambrosca et al. 2013; Duke et al. 2013; see Sect. 6.6).

4.3 Plant Litter and Residues

A number of researchers have observed that aboveground and belowground small grain cover crop litter and residues, such as rye or wheat, are at times effective in reducing early emergence and lowering the number of broadleaf weed seedlings, such as morningglory, pigweed, and prickly sida in no-till cropping systems (Barnes and Putnam 1983; Barnes et al. 1986; Lieble and Worsham 1983; Putnam et al. 1983; Shilling et al. 1985; Worsham 1989, 1990; Yongoing 2005; Blum 2011). For convenience the terms plant litter and residues will be combined under the term residues in the remaining chapters. The term residues will now include all recognizable dead plant materials aboveground and belowground unless specified otherwise. Field experiments suggest that this inhibition of early weed seedling emergence is likely due to some combination of the following factors:

a. the physical barrier and shading associated with residues (Fester and Peterson 1979; Worsham 1989),
b. the modification of soil temperature, pH, and moisture by residues (Fester and Peterson 1979; Lehman and Blum 1997; Xu et al. 2006),
c. the reduction in soil disturbance and changes in soil compaction of no-till systems (Worsham 1989),
d. the immobilization of nutrients such as nitrogen (Kimber 1973; Hadas et al. 2004),
e. increased seed predation (Brust and House 1988; Reader 1991; Brust 1994), and
f. the release of inhibitory compounds such as phenolic acids (Guenzi and McCalla 1966; Liebl and Worsham 1983; Shilling et al. 1985; Blum et al. 1991; Wu et al. 2000a, b, 2001a, b; Tapin et al. 2006), organic acids (Patrick 1971; Lynch

1977; Tang and Waiss 1978; Shilling et al. 1985), hydroxamic acids (Willard and Penner 1976; Niemeyer et al. 1989; Wu et al. 2000c; Macías et al. 2005; Mathiassen et al. 2006), volatile compounds (Buttery et al. 1985; Bradow 1991) and other compounds (Nakano et al. 2006).

Since the actual importance of the individual factors just listed in reducing weed seedling emergence by cover crop residues is not clearly understood, we might ask the following question: What role, if any, does each of these factors have in reducing early weed seedling emergence in no-till cropping systems? This question, however, is too broad and too complex to be addressed here. A more manageable and more appropriate question consistent with the subject of this volume might be: What role, if any, do allelopathic compounds have in the reduction of early broadleaf weed seedling emergence by rye or wheat cover crop residues in no-till systems? However since the presence of wheat residues in no-till systems can also at times stimulate weed seedling emergence (Blum et al. 1997, 2002; Blum 2011), the question becomes: What role, if any, do allelopathic compounds have in the stimulation or inhibition of early broadleaf weed seedling emergence by rye or wheat cover crop residues in no-till systems? Finally to simplify the following presentation, "rye or wheat" was replaced by "wheat" in the last question.

This is a good place to pause and caution the reader regarding the hypothetical bioassays described so far and for those that follow. Any observed potential allelopathic effects for sunflower leaf leachates and "root exudates plus" leachates as well as for residue leachates and soil extracts (representing soil solutions) that follow, are primarily due to hydrophilic (water-soluble) compounds since these leachates and extracts are water based. The potential role of most hydrophobic compounds and volatile compounds are excluded. On the other hand, the potential allelopathic effects of wheat residues on and in soil and soils with wheat residues removed cannot be solely described by the actions of hydrophilic compounds since the potential actions of any hydrophobic and volatile compounds present cannot be excluded.

4.3.1 Field Study

Objectives To determine how increasing biomass of wheat residues (i.e., straw, stubble, root and mycorrhizae) generated by three different wheat seeding rates affects weed seedling emergence over time. This assumes that the wheat seeding rates chosen resulted in a range of increasing biomass for wheat residues. Formation of tillers by wheat, however, can at times compensate for lower seeding rates.

To determine potential relationships between weed seedling emergence and the physicochemical states generated by the presence of different amounts of wheat residues.

To determine how weed seedling emergence changes with aging aboveground and belowground wheat residues. Aging here refers to the state of weathering, leaching, fragmentation, and decomposition of residues. This assumes that adequate

Fig. 4.6 Wheat and reference plots just before harvest in central North Carolina in June

rain events and soil moisture occur during the experimental period. If rain events are too infrequent, some aboveground surface irrigation may be required.

To generate test materials for residue bioassays.

Materials and Methods (Blum et al. (1997); Henry A Wallace Institute of Alternative Agriculture Inc, summarized with permission of Cambridge University Press) Wheat (*Triticum aestivum* L. Southern States 555) was planted conventionally in the fall at three different seeding rates and harvested in June (Fig. 4.6). What remained after harvest within the wheat plots were wheat residues consisting of straw (i.e., stems and leaves), stubble, and root and/or mycorrhizal fragments. Reference plots without wheat were included in the field trials. Both the wheat and reference plots were managed using normal agricultural practices (e.g., tilling, disking, fertilizer application, etc.). There was one 5×5 m (25 m^2) plot for each treatment per block (i.e., 3 wheat seeding rates and one reference per block = 4) and there were 5 blocks. The plots within a block were arranged in a complete randomized design. Blocking was based on landscape position and soil texture. The total number of plots was 20. In each plot three broadleaf weed species (morningglory [*Ipomoea hederacea* L.], pigweed [*Amaranthus retroflexus* L.], or prickly sida [*Sida spinosa* L.]) were surface seeded (500 seeds/m^2) into randomly located subplots (1×5 m) shortly after the wheat was harvested. Subplots near the borders of the plots were excluded. Locations of weed subplots were randomized within each plot.

Weed seedling numbers per subplot were counted biweekly for 4 months (Fig. 4.7). The following were also determined at biweekly intervals using five randomly located soil core samples taken from each treatment plot: soil water-soluble Folin–Ciocalteau (F–C) reactive compounds (Note: Specific organic compounds or profiles of primary and secondary compounds [metabolic profiling] could also be determined.), soil moisture, soil pH, soil NPK, and soil carbon/nitrogen ratio. In addition for the first set of soil cores (i.e., right after wheat harvest), soil particle size (sand, silt and clay), soil organic matter, and soil cation exchange capacity were also

Fig. 4.7 Morningglory (*top*), pigweed (*middle*), and prickly sida (*bottom*) seedlings in wheat residue field subplots in July. (*middle and bottom* figures taken from Blum (2011), figures used with permission of Springer Science and Business Media)

determined. The biomass of aboveground and belowground wheat residues (down to 15 cm) was determined biweekly with 2 random 0.25 m^2 quadrates per treatment plot. Surface residues were cut at the soil surface and removed and roots and mycorrhizae were recovered from the excavated soil. Air-dry weights of aboveground and belowground biomass were determined. Water-soluble Folin–Ciocalteau (F–C) reactive compounds were determined on subsamples of the residues and soils collected. Soil temperatures at the soil surface and at 15 cm and percent light intensity

reaching the soil surface were determined at 5 random locations within each treatment plot. Samples and environmental data were collected on the same day for each sampling time. All samples and environmental data were taken from the subplots without weeds. Data on rain events (date, time, and amount), rain water samples for analysis, and daily max-min air temperatures were also collected during the experimental period.

This field bioassay was designed to demonstrate that the presence of wheat residues for a particular cultivar stimulates or inhibits weed seedling emergence (i.e., increasing or decreasing numbers over time due to emergence and mortality) and to provide a source of test materials for subsequent bioassays. Thus, no crops, such as soybeans for example, were planted in the wheat or reference plots since that would have made both the chemical and physical environments for weed seed germination and emergence even more complex. Finally depending on materials and soil cores required for subsequent laboratory bioassays, the size of the treatment plots may need to be larger than the 5×5 m plots utilized. Also depending on quiescence, dormancy, mortality, and predation, the weed seed number per m^2 may need to be modified (see Blum et al. 1997; Blum 2011). Changes in water-soluble F–C reactive compounds in aboveground and belowground residues and soil were used as indicators of aging (i.e., weathering, leaching, fragmentation, and decomposition) for residues, for an estimate of the potential transfer of water-soluble substances to the soil, and for the residual concentrations within the soil. See Sect. 4.2.2.1 for soil sampling, soil analyses, environmental measurements, and determining F–C reactive compounds. Metabolic profiling on a representative number of residue and soil extracts could provide additional insight regarding causation (see Summer et al. 2003; Dunn and Ellis 2005; Wallenstein and Weintraub 2008; Xi et al. 2008; Liebeke et al. 2009; Prince and Pohnert 2010; Wallenstein et al. 2010; Sardans et al. 2011; Carlsen et al. 2012).

Hypotheses If increasing biomass of wheat residues creates a series of increasing or decreasing physicochemical states in the immediate area surrounding weed seeds, seed radicles, and seedling roots or mycorrhizae, then weed seedling numbers (i.e., emergence minus mortality) depending on the sensitivity of a weed species to such physicochemical states will either be higher, the same, or lower in the wheat plots than in the reference plots.

If the changes in the physicochemical states measured for the soil from weed-free subplots and the soil environment immediately surrounding weed seeds, seed radicles, and seedling roots or mycorrhizae within the weed subplots are related and if the first hypothesis is true, then a significant pattern of relationships between seedling numbers of sensitive weed species in the weed subplots and the physicochemical states of the soil from the weed-free subplots should be evident.

If the physicochemical states change sufficiently over time as wheat residues are weathered, leached, fragmented, and decomposed, then weed seedling numbers, depending on species sensitivity to such physicochemical changes, will respond accordingly, i.e., numbers will either increase, stay the same, or decrease with time.

If weed seedling emergence of sensitive weed species are initially suppressed by wheat residues and the suppression is subsequently released over time as inhibit-

ing factors decline, then weed seedling numbers should initially be small (or zero). Then as suppression of wheat residues declines, seedling numbers should increase with time until active weed seeds in the soil become limiting. This assumes that no major changes in quiescence and dormancy of seeds and mortality of seedlings occurred during the experimental period.

If weed seedling emergence of sensitive weed species are initially enhanced by wheat residues, then weed seedling numbers should initially increase rapidly (e.g., no delay) and eventually, given enough time, level off as promoting factors decline and active weed seeds in the soil become limiting. This also assumes that no major changes in quiescence and dormancy of seeds and mortality of seedlings occurred during the experimental period.

Changes in the physicochemical environment brought about by the different amounts and states of wheat residues will also modify the populations of active soil organism (flora and fauna). The changes in the actions of soil organisms will influence the rates and types of organic and inorganic compounds leached from the residues, the concentrations of organic and inorganic compounds in the soil that are available to interact with seeds, roots, and mycorrhizae, and the synthesis of new, sometimes unique, organic compounds. This assumes that exceptional or unusual environmental changes do not occur during the experimental period. (Note: Finally, for the last three hypotheses comparisons of trends between wheat treatment plots and reference plots should be made to determine if the changes observed were significantly different from the reference plots).

Analyses *Step 1:* How does biomass of wheat residues on and in the soil affect weed seedling emergence as measured by seedling numbers over time? Weed seedling numbers could be analyzed by univariate analysis of variance for each biweekly interval and by repeated measures analysis (multivariate profile analysis) to test for parallelism of response over time, mean response over time, and trends over time (orthogonal polynomial contrasts).

Step 2: What are potential causative agents or factors? Covariate, correlation, and principle components analyses (with reference plots included and excluded) could be used to determine significant relationships between weed seedling numbers and water-soluble F–C reactive compounds in residues and soil physicochemical factors quantified (e.g., light penetration, temperature, soil moisture, pH, NPK, water-soluble F–C reactive compounds, and C/N ratio.).

Comments Even if increasing or decreasing weed seedling numbers (i.e., emergence minus mortality) are found to be related to the biomass of wheat residues of Southern States 555 and the aging of wheat residues, the actual cause or causes is/are still unknown. Significant relationships between soil physicochemical states associated with increasing biomass and aging of residues may suggest potential factors or processes that could be responsible and, thus, deserve further study (see Sect. 6.6). However, a too heavy reliance on only water-soluble compounds (e.g., F–C reactive compounds) as the causative agents can be misleading since the potential role of hydrophobic and volatile compounds present would not be included.

4.3.2 Test Materials

Wheat straw and stubble residues were collected at monthly intervals for 4 months with 0.25 m^2 quadrates from the weed-free subplots and freeze-dried. Once aboveground residues were removed, residues of roots and mycorrhizae down to 15 cm were collected. Soil was carefully removed from the residues of roots and mycorrhizae to minimize fragmentation and the recovered residues were freeze-dried. Any remaining soil particles on the residues of roots and mycorrhizae were removed after freeze-drying. Weights of aboveground (i.e., straw and stubble) and belowground (i.e., roots and mycorrhizae) residues for each quadrate were determined before residues were stored in desiccators in the dark at room temperature. Sufficient samples were collected at each monthly interval in order to simulate field wheat residue levels in laboratory bioassays. Representative samples of residues were analyzed for water-soluble F–C reactive compounds and available NPK content. Profiles for primary and secondary compounds (metabolic profiling) could also be determined for representative residue samples.

Soil to a depth of 15 cm was collected from areas without wheat (reference plots), air-dried, sieved (1 cm^2), mixed, and stored in the laboratory for analysis and bioassays. To characterize this soil, 5 subsamples of soil were analyzed for particle size (sand, silt and clay), organic matter, mineral constituents (e.g., NPK), cation exchange capacity, and pH.

In addition, 3 soil cores per plot (approximately10 cm deep and 15 cm in diameter) for a total of 60 soil cores were also collected from all plots at monthly intervals for 4 months and used immediately in bioassays. Core samples were collected in the weed-free subplots.

Samples were taken immediately after wheat harvest, and 1, 2, 3, and 4 months after wheat harvest. Sequential monthly residues and core samples represent different stages of weathering, leaching, fragmentation, and decomposition of residues. See Sect. 4.2.2.1 for soil analyses and environmental measurements. See Robinson (1980), Harborne (1984), Allen (1989), Pearcy et al. (1989), Walinga et al. (1995), Kalra (1998) for residue analyses. See Summer et al. (2003), Dunn and Ellis (2005), Wallenstein and Weintraub (2008), Xi et al. (2008), Liebeke et al. (2009), Prince and Pohnert (2010), Wallenstein et al. (2010), Sardans et al. (2011), and Carlsen et al. (2012) for metabolic profiling of residue samples.

4.3.3 Simulated-Rain Water

In most laboratory studies there is a considerable reliance on distilled water as the medium for solution bioassays and as a source of water in soil bioassay. Due to its water potential (approximately zero) distilled water can damage or disrupt cells and can, thus, be a confounding factor, particularly as a reference for solution-culture bioassay. Rain water collected in the field would be a much better choice than distilled water. However, rain events are frequently unpredictable particularly in

the temperate regions of the world and the chemical composition of rain water is somewhat unpredictable from collection time to collection time. For consistency, availability, and the potential of fine-tuning its chemistry, simulated-rain water is an excellent substitute for natural rain water. Information on physical and chemical composition of natural rain water is available in the literature (Cogbill and Likens 1974). Formulations (e.g., ion content and pH) and applications (e.g., droplet size and pressure) for simulated-rain water have been described by DuBay and Heagle (1987), Shafer et al. (1985), Percy (1986), and Shafer (1988, 1992). For additional information regarding adjustments for simulated-rain water see Sect. 4.2.1.

4.3.4 Residue Bioassays

Objective To determine how different amounts of wheat residues (i.e., straw, stubble, and root and mycorrhizae) on and in soil or on and in soil cores at different stages of weathering, leaching, fragmentation, and decomposition effects weed seedling emergence (i.e., numbers and slopes) when treated with multiple 6.25, 12.5, 18.75, or 25 mm simulated-rain events.

Materials and Methods *Option 1:* Residues of wheat roots and mycorrhizae from each monthly sampling period simulating the range of field levels were mixed into the soil and placed into 15 cm pots with a 1,767 cm^3 volume and drainage holes. Three biomass levels were chosen within that range. Weed seeds (morningglory [*Ipomoea hederacea* L.], pigweed [*Amaranthus retroflexus* L.], or prickly sida [*Sida spinosa* L.]) were surface seeded to each pot and lightly pressed into the soil to increase soil contact. Number of weed seeds per pot was constant for each species and based on seed size (see Sect. 3.5). Seeds were located equidistant from each other in the pots. Residues of wheat straw and stubble from each monthly sampling period were then placed on top of the soil surface of these pots. The ratio of aboveground and belowground biomass found in the field was maintained. Pots with only soil (no residues) were used as a reference (baseline) for comparison. For soil used see Sect. 4.3.2. The final g/pot of soil was identical for all pots but the pot system weights varied with the amount of residue added. Fragmentation of residues was minimized as much as possible. The attempt was to simulate the field conditions as closely as possible but to minimize competition between the seeds. Simulated-rain water (see Sect. 4.3.3) equivalent to 6.25, 12.5, 18.75, or 25 mm was applied on alternate days to the stubble and straw residues on the surface of the pots (i.e., above surface residues when present) with a pressurized spray nozzle to mimic rain drops (see Cogbill and Likens 1974; Shafer et al. 1985; DuBay and Heagle 1987). Pots were brought to a constant predetermined weight for each residue treatment each time simulated rain was applied, i.e., different predetermined pot weights were maintained for 0 g residue plus 6.25 mm, x g residues plus 6.25 mm, 2x g residues plus 6.25 mm, 3x g residues plus 6.25 mm, 0 g residues plus 12.5 mm, etc. Bioassays were carried out in a greenhouse. Light intensity at pot level, temperature, and relative humidity were monitored during the experimental period. The number of weed seedlings per pot was recorded daily. Weed seedlings were removed after each

count to minimize competition. When removing weed seedlings, soil and aboveground residue disturbance was minimized as much as possible. The daily averages, total numbers at the end of the experimental period, and slopes based on cumulative numbers of weed seedlings per day were determined for seedling emergence (Note: Daily emergence or cumulative daily numbers may also be used for time series studies.). The number of days for the experimental periods were determined by the cessation of seedling emergence for the reference treatment of a given weed species. At the beginning and end of the experimental period representative samples of aboveground and belowground residues were analyzed for water-soluble F–C reactive compounds and their metabolic profiles. The basic experimental design was as follows for each monthly sampling period: randomized-blocked design of 4 biomass levels (3 levels of biomass based on wheat seeding levels and reference) × 4 rain events × 5 replicates (blocks) = 80 experimental units per weed species. Bioassays (5 sample periods [0 to 4 months] × 80 = a total of 400 units) for each weed species were run separately to make the bioassays more manageable.

The sequential monthly harvests represent different stages of weathering, leaching, fragmentation, and decomposition of residues. Size of residue particles was not modified because using modified (e.g., ground) residues is generally not recommended due to the fact that surface areas and release rates of organic and inorganic compounds from such residues are much greater than surface areas and release rates of actual residues in the field (see Blum 1999). The actual number of weed species tested was based on their response during the field study (see Sect. 4.3.1). Straw, stubble, and residues of roots and mycorrhizae can also be tested individually but that cannot provide data on the combined effects.

Depending on the soil, the addition of sand may be required for adequate percolation and aeration. Also depending on evapotranspiration, the frequency of the simulated-rain events may need to be altered. In addition depending on soil type, the mm of simulated-rain water applied to the pots may need to be adjusted. Soil moisture content should not exceed field capacity. The use of multiple applications of simulated-rain water is, of course, atypical since in nature rain events, at least in temperature regions of the world, do not occur on a regular every other day basis. Multiple applications are, however, required to maintain soil moisture for germination and growth and to continue/refresh inputs of aboveground and belowground leachates into and in the soil. Differences in the predetermined weight were due to the different amounts of residue added. See Sects. 4.2.2.1 and 4.3.2 for soil analyses, environmental measurements, and residue analyses.

The experimental design could be considerably improved by:

a. the addition of chemically inactive materials mimicking aboveground and belowground residues to reference soils. Amounts added should be equal to the amount of wheat residues added (see Comments section below). This would add 3 inactive residue reference treatments to the design of the bioassay. Another option would be to add chemically inactive materials to all systems so that the total amount of both the wheat residues and the inactive material are identical for all treatments.

b. By monitoring soil characteristics such as pH, temperature, moisture, residue, NPK, water soluble F–C reactive compounds, and/or metabolic profiles at given intervals during the experimental period. This would require additional experimental units which could be harvested at intervals.

Option 2: Three soil cores per plot with and without wheat residues were taken randomly from plots at monthly intervals (5 sampling periods in this case), placed into pots, and surface seeded with weed seeds (morningglory [*Ipomoea hederacea* L.], pigweed [*Amaranthus retroflexus* L.], or prickly sida [*Sida spinosa* L.]) that were lightly pressed into the soil. Number of weed seeds per core was constant for each species and based on seed size (see Sect. 3.5). Seeds were located equidistant from each other in the soil. Bioassays were carried out in the greenhouse. Since the wheat residue levels of the soil cores were unknown, approximations of the wheat residue levels on and in the soil cores were determined after the bioassay was completed. Simulated-rain water (see Sects. 4.2.1 and 4.3.3) equivalent to 6.25, 12.5, 18.75, or 25 mm was applied on alternate days to the soil cores with a pressurized spray nozzle to mimic rain drops (Cogbill and Likens 1974; Shafer et al. 1985; DuBay and Heagle 1987). Light intensity at pot level, temperature, and relative humidity were monitored during the experimental period. The number of weed seedlings per pot was recorded daily. Weed seedlings were removed after each count to minimize competition. When removing seedlings, soil and aboveground residue disturbance was minimized as much as possible. The daily averages, total numbers at the end of the experimental period, and slopes based on cumulative numbers of weed seedlings per day were determined for seedling emergence. The number of days for the experimental periods were determined by the cessation of seedling emergence for the reference treatment of a given weed species. At the end of the bioassay residues of weed straw, stubble, root and mycorrhizae were removed from the soil cores, cleaned, freeze-dried and weighed. See Sect. 4.3.2. for analyses and environmental measurements. The basic experimental design was as follows for each sampling period: randomized-blocked design of 4 biomass levels (3 potential wheat biomass levels and reference) $\times 3$ soil cores per plot $\times 4$ rain events $\times 5$ replicates (blocks) $= 240$ experimental units for each weed species per sampling date. Bioassays for each weed species were run separately each month to make the bioassays more manageable. The actual number of weed species tested was based on their response during the field study (see Sect. 4.3.1). The experiment could be improved by determining FC- reactive compounds or for metabolic profiles of the residues recovered from soil cores.

Hypotheses For simplicity and clarity hypotheses are written for Option 1. However, with slight modification they will also apply to Option 2.

General If increasing aboveground and belowground biomass of wheat residues collected at different points in time (i.e., at different states of weathering, leaching, fragmentation, and decomposition) and treated with increasing amounts of simulated-rain water create a range of physicochemical and biotic environments that influence weed seedling emergence and survival, then weed seedling numbers and slopes of sensitive species should generate a recognizable pattern related to treatments.

Potential Roles of Promoters, Modifiers, and Inhibitors For both soil residue and soil core systems the input by aboveground residues consists primarily of water-soluble (hydrophilic) compounds but some volatile compounds may also be released and enter the soil. Input of belowground residues will include not only water-soluble compounds but also hydrophobic and volatile compounds. Effects observed for residue bioassay will, therefore, very likely be due to a combination of active hydrophilic, hydrophobic and volatile promoters, modifiers, and inhibitors. (Reminder: Theoretically any given compound may be a promoter, modifier, or inhibitor depending on its concentration and physicochemical state and the species process being monitored [see Sect. 2.1.3.1]). The following hypotheses assume that there are no major physical and biotic differences for the systems or that such differences have been equalized or accounted for in the experimental design.

If the promoters, modifiers, and inhibitors released by wheat residues for a given simulated-rain event change as the amounts of aboveground and belowground wheat residues are increased, then stimulation or inhibition of weed seedling emergence for sensitive species should respond accordingly. The expectation for increasing residues would be that water-soluble promoters, modifiers, and inhibitors released to the soil would increase unless retention of simulated-rain water by increasing amounts of residues reduces the volume of water reaching the soil.

If the promoters, modifiers, and inhibitors released by a given amount of aboveground and belowground wheat residues change as the amount of simulated-rain water applied to the systems are increased, then stimulation or inhibition of weed seedling emergence for sensitive species should respond accordingly. The expectation for a given amount of residues would be that the water-soluble promoters, modifiers, and inhibitors in the soil would be diluted by increasing amounts of simulated-rain water, i.e., the total amount may be the same or even increase slightly depending on the solubility of compounds but the concentrations in the soil solution will likely be lower since soil moisture content will be higher.

If the promoters, modifiers, and inhibitors released by aboveground and belowground wheat residues change as residues collected from the field become progressively more weathered, leached, fragmented, and decomposed, then stimulation or inhibition of weed seedling emergence for sensitive species should respond accordingly. The expectation would be that water-soluble promoters, modifiers, and inhibitors leached from residues would decline as residues become progressively more weathered, fragmented, and decomposed.

If promoters dominate the promoter/modifier/inhibitor complex, weed seedling emergence for sensitive species should be greater when compared to the reference. If inhibitors dominate the promoter/modifier/inhibitor complex, seedling emergence for sensitive weed species should be lower when compared to the reference.

Potential Role of Physical Factors The hypothesis below assumes that there are no differences in promoters, modifiers, and inhibitors and biotic factors for the systems or that such differences have been equalized or accounted for in the experimental design.

If the physical nature (e.g., pH, temperature, and soil moisture) of soil changes as aboveground and belowground wheat residues collected from the field are

progressively more weathered, leached, fragmented, and decomposed, as the amounts of residues are increased for the systems, and/or as the simulated-rain water applied to these systems are increased, then stimulation or inhibition of seedling emergence for sensitive species should respond accordingly. The expectation would be that the physical nature of the soil will change depending on the treatment or treatments applied to the soil. For example, Lehman and Blum (1997) found that pH of the Cecil A horizon soil was directly related to the increasing amounts of wheat residues mixed into this soil. Given a sufficient increase in soil pH, the expectation would be that the effects of phenolic acids, for example, would decline. They also observed that pigweed seedling emergence was inhibited directly by soil moisture above 0.133 g water/g soil and that there were significant interactions between soil moisture and wheat residue treatments for pigweed seedling emergence.

Potential Role of Biotic Factors The hypothesis below assumes that there are no differences in promoters, modifiers, and inhibitors and physical factors for the systems or that such differences have been equalized or accounted for in the experimental design.

If microbial populations of soil change as aboveground and belowground wheat residues collected from the field are progressively more weathered, leached, fragmented, and decomposed, as the amounts of residues are increased for the systems, and/or as the simulated-rain water applied to these systems are increased, then stimulation or inhibition of seedling emergence for sensitive species should respond accordingly. The expectation would be that microbial species and their population numbers in the soil will change (e.g., increase or decrease) depending on the nature (e.g., types and amounts) of the carbon sources leached from the residues, the nutrient status of the soil, and the changes of the physical environment. Increases or decreases in microbial populations which utilize promoters, modifiers, and/or inhibitors or synthesize new active compounds will influence the nature (e.g., types, combinations, and concentrations of active compounds) of the promoter/modifier/ inhibitor complex in the soil which interacts with seeds, roots, and mycorrhizae.

Combined Roles If the physicochemical and biotic factors of the soil are changed as progressively more aboveground and belowground residues at different stages of weathering, leaching, fragmentation, and decomposition are added and as the simulated-rain water applied to the systems is increased, then stimulation or inhibition of seedling emergence for sensitive species will be the product of all these factors acting and interacting with each other in modifying seedling numbers and slopes. Present evidence suggests that some changes in most, if not all, of these factors will occur for such residue and simulated-rain treatments unless specific efforts are made to adjust these factors to be roughly equivalent.

Analyses *Step 1*: How does increasing aboveground and belowground biomass of wheat residue at different stages of weathering, leaching, fragmentation, and decomposition treated with increasing amounts of simulated-rain water affect weed seedling emergence? Multiple regression analyses may be used to determine the potential relationships between the stages of residue weathering, leaching, fragmentation, and decomposition (e.g., time collected after harvest, average particle

size, F–C reactive compounds, or metabolic profiles) and the mm of simulated-rain water applied to the systems (X, independent variables) and the observed increase or decrease (Y, dependent variable) of weed seedling numbers and slopes. For more complex statistical analyses such as time series, modeling, etc., the reader is advised to consult a statistician for the proper statistical and analytical tools and techniques.

Step 2: If chemical (e.g., individual or mixtures of compounds identified, F–C reactive compounds, or metabolic profiles), physical (e.g., pH, soil moisture, etc.), and biotic (e.g., colony forming units of specific organic compound utilizers) states of soils or soil cores are monitored, multiple regression analysis may be used to identify potential causative agents or factors.

Comments Both Options 1 and 2 provide a simulation of the way increasing amounts of different weathered, leached, fragmented, and decomposing wheat residues on and in the soil treated with multiple applications of increasing amounts of simulated-rain water may modify weed seedling emergence but do not clearly identify which factors are responsible since chemical, physical, and biotic environments are modified differently by the various amounts and states of wheat residues and the amounts of simulated-rain water. Putnam has suggested that the addition of chemically inactive materials mimicking texture and structure of treatment residues can help in isolating potential chemical effects from physical effects by reducing or minimizing physical effects (see Putnam and DeFrank 1983; Putnam et al. 1983; Putnam 1994). Chemically inactive materials mimicking aboveground residues may be readily added to both options. The addition of chemically inactive materials mimicking belowground residues could be readily done for Option 1 but is not possible for Option 2 without excessive disturbance of the soil core. For Option 1 such additions would bring all the pots to the same weight and would simplify maintaining predetermined pot weights for each simulated rain event.

The potential for meeting the objective for Options 1 and 2 are somewhat different. This is because Option 1 has identical soil and soil structure with a series of increasing field aged wheat residues (collected at different times but tested at the same time), while Option 2 has a range of soils, soil structures, and wheat residue levels that are initially unknown. In addition the core samples of Option 2 were collected and tested at different times. In other words, Option 1 utilizes a processed artificial soil system and Option 2 utilizes a series of natural soil system cores. Both systems have identical simulated-rain treatment events although differences between the two systems in soil water penetration and evapotranspiration may be expected. Also if by chance the levels of wheat residues for the soil cores taken are too similar for a given monthly collection, then only the effects of plus or minus wheat residues can be determined for that sampling period. To improve the potential for meeting the objective of Option 2, soil cores could be specifically selected so that an appropriate range of wheat residues are included at each monthly collection. Such selection of soil cores, however, would tend to further uncouple the effects observed for the soil cores from the normal range of effects observed in field plots (other uncoupling factors, greenhouse, rain events, etc.). Finally, since soil cores were collected and tested at different times for Option 2, the general effects of weathering, leaching, fragmentation, and decomposition of wheat residues are more difficult to characterize than for Option 1 since the greenhouse environment varied

over time. Some of the environmental variation over time could be eliminated by using growth chambers.

In many ways these experiments are similar to the field study (see Sect. 4.3.1) in that they provide the same type of information regarding weed seedling emergence but under different environmental conditions (i.e., greenhouse plus atypical rain events versus the field). So why repeat the field study in the greenhouse or growth chamber? Nature is very complex and highly variable over time. Experiments in the greenhouse or growth chamber can provide the following benefits:

a. Option 1: choosing the nature of the substrate (i.e., soil type, texture, structure, compaction),
b. Options 1 and 2 to some extent: control over the amounts (i.e., concentrations) and types of wheat residues on and in the soil,
c. Options 1 and 2: greater control over various environmental factors such as physical and chemical composition of simulated rain, frequency and duration of rain events, soil moisture, soil nutrition, soil temperature, etc.,
d. Options 1 and 2 to some extent: control of weed species, seed numbers (e.g., residual seeds in seed bank are essentially absent in Option 1 but not in Option 2), seed location, and reduced or complete reduction of seed predation, and
e. Options 1 and 2: confounding factors such as seedling mortality can be monitored and competition, predation, and other stresses can be regulated and/or standardized.
f. To sum it up, greenhouse experiments (or growth chamber experiments) provide for simpler, less noisy, and more regulated environments that increase the chances of identifying potential effects of wheat residues on weed seedling emergence. These benefits, however, come at a cost. The observed effects in a greenhouse (or growth chambers) are in many ways uncoupled from those observed in the field.

4.3.5 Residue Leachates

4.3.5.1 Sources and Collections of Residue Leachates

Monthly collected and stored freeze-dried aboveground wheat straw and stubble residues (see Sect. 4.3.2) simulating the range found within the field system were placed into plastic pans containing screened drainage holes. Three residue biomass levels were chosen for testing. Simulated-rain water (see Sect. 4.3.3) was applied to the wheat residues with a pressurized spray nozzle to mimic rain drops (Cogbill and Likens 1974; Shafer et al. 1985; DuBay and Heagle 1987). Simulated-rain events representing 6.25, 12.5, 18.75, or 25 mm of rain were applied at 25 mm/h to a set of different pans containing the 3 residue biomass levels. Leachates were, therefore, collected from 12 pans (3 residue bioassay levels $\times 4$ levels of simulated rain) for each monthly sampling period. Wheat residue leachates were filtered to remove soil and organic particles, filter sterilized, and stored in a refrigerator. The pH values, water potentials, NPK, F–C reactive compounds, metabolic profiles, and potential

allelopathic compounds (i.e., promoters and/or inhibitors) of the resulting leachates were determined.

What about the residues for roots and mycorrhizae? A similar approach to that of aboveground residues was taken. However, such an approach ignores a fundamental difference between the two types of leachates. After all, organic and inorganic compounds leached from aboveground residues enter the soil as distinct entities before interacting with soil components while organic and inorganic compounds released from belowground residues (and residual organic matter) are a product of a complex series of soil processes, leaching being only one of them. Such an approach would, however, allow for the isolation, identification, and comparison of active compounds (i.e., promoters, modifiers, and inhibitors) that are leached from aboveground and belowground residues and provide a means for direct comparisons of the potential allelopathic activity of aboveground and belowground residues. Another option would be to collect leachates from a mixture of aboveground and belowground residues. That would integrate their individual effects.

There were 5 sampling dates (i.e., right after wheat harvest and 1, 2, 3, and 4 months after wheat harvest). Wheat straw and stubble residues may also be tested individually but that would not provide data on their combined effects. Collection times for leachates were 15 min for 6.25 mm, 30 min for 12.5 mm, 45 min for 18.75 mm, and 1 hr for 25 mm. To obtain adequate amounts of leachates for the bioassays, replicate samples may be required. See Sect. 4.2.2.1 for references for soil and solution analyses.)

4.3.5.2 Residue Leachate Bioassay

Objective To determine if the promoters, modifiers, and inhibitors (organic and inorganic) leached from different quantities of different weathered, leached, fragmented, and decomposed aboveground and belowground wheat residues could potentially influence weed seed germination and radicle lengths in Petri dishes without soil (single application) and weed seedling emergence (i.e., number and slopes) and biomass in the presence of soil (multiple applications) under laboratory conditions. Aboveground and belowground leachates were tested separately.

Materials and Methods *Options:* Aboveground and belowground wheat residue leachates were tested in Petri dishes and in soil as described for sunflower leaf leachates (Sect. 4.2.2.2; Options 1, 2, and 3) using morningglory (*Ipomoea hederacea* L.), pigweed (*Amaranthus retroflexus* L.), or prickly sida (*Sida spinosa* L.) as bioassay species. The effects of leachates from increasing amounts of residues for different weathered, leached, fragmented, and decomposed wheat residues obtained with 6.25, 12.5, 18.75, and 25 mm of simulated-rain water were determined. The amount of solution added to each experimental unit for each species was identical. The amount added for each species was based on seed size. For the Petri dish bioassay solutions were added once and for the soil bioassay, added approximately daily. The basic experimental design was as follows for each sampling period: randomized-block design of 3 residue levels × 5 treatments (4 simulated-rain leachates and

simulated-rain water [reference]) × 2 residue types (aboveground and belowground) × 3 replicates = 90 experimental units per monthly sample and weed species. Bioassays (5 monthly [0–4] samples × 90 = a total of 450 units) for each weed species were run separately to make the bioassays more manageable. See Sect. 4.2.2.2 for additional details.

Leachates from appropriate mixtures of aboveground and belowground residues could also be tested. The actual weed species tested were based on the responses observed during the field study (see Sect. 4.3.1). The use of a single reference solution could lead to false interpretation of the data particularly when the physical and chemical compositions (e.g., pH values, content of inorganic compounds, and solute potentials) of the leachates for each rain level are different. In that case properly adjusted reference solutions would be required for the different quantities of wheat residue and simulated-rain level. Another option would be to adjust the pH, inorganic compound content and solute potential of each treatment solution (leachates and reference) so that they are similar or at least not limiting.

Hypotheses The pool of organic and inorganic compounds that can be leached from a given sample of aboveground and belowground wheat residues by a simulated-rain event is finite or fixed and that finite or fixed value will likely get smaller as wheat residues are weathered, leached, fragmented, and decomposed. In addition as increasing mm of simulated-rain water are applied to a residue sample, the promoters, modifiers, and inhibitors within the leachates collected will be diluted. This dilution series, however, is not a serial dilution since each simulated rain event is applied to a different residue sample.

If aboveground and belowground wheat residue leachates and simulated-rain water have pH values, inorganic compound contents, and solute potentials that are approximately similar for all the treatment solutions or, if necessary, adjusted so that they are similar, then any observed effects (i.e., stimulatory or inhibitory) of wheat residue leachates on weed seeds or seedlings will be a direct or indirect product of the actions and interactions of the organic promoters, modifiers, and inhibitors present in the residue leachates.

If weed germination, radicle length, emergence, and biomass are stimulated, then promoters dominate the promoter/modifier/inhibitor complex and if inhibited, then inhibitors dominate the complex. (Reminder: Theoretically any given compound may be a promoter, modifier, or inhibitor depending on its concentration and physicochemical state and the species process being monitored [see Sect. 2.1.3.1]).

If aboveground and belowground wheat residue leachates and reference solutions have different pH values, content of organic and inorganic compounds, solute potentials, and organic promoters, modifiers, and inhibitors (subset of all organic compounds), then the observed effects will be a product of all direct or indirect actions and interactions of all the factors present in the residue leachates, not just the organic promoters, modifiers, and inhibitors in the leachates.

Since the actual expression of stimulation or inhibition will be adjusted or modified by the physicochemical and biotic environments of the bioassay system, the results are limited to those environmental conditions. The use of the word indirect

here is an attempt to include the actions of any microbial altered or produced compounds.

Analyses Initial assumption: The residue leachates obtained with 6.25, 12.5, 18.75, and 25 mm of simulated-rain water for the residue types, various biomass levels, and states (e.g., levels of weathering, fragmentation, decomposition, and leaching) of residues will generate concentration gradients of organic and inorganic compounds.

*Step 1:*Do leachates from increasing biomass of aboveground and belowground wheat residues and states of wheat residues treated with a range of simulated-rain water affect germination and radicle length of weeds in the absence of soil or seedling emergence and biomass of weeds in the presence of soil? Multiple regression could be used to determine the potential relationships between leachate types (i.e., mm of simulated-rain water applied to wheat residues, biomass of residues, and states of residues [X, independent variables]) and the observed increase or decrease (Y, dependent variable) of germination and radicle length (Option 1), seedling emergence (Option 2; numbers and slopes), and seedling biomass (Option 3). For more complex statistical analyses, such as time series (Option 2), modeling, etc. (all Options), the reader is advised to consult a statistician for the proper statistical and analytical tools and techniques.

An index such as the number of days residues were collected after the wheat harvest, average particle size of residues, F–C reactive compounds or metabolic profiles for residue leachates could also be used to represent the states of weathering, fragmentation, decomposition, and leaching of the residues.

Step 2: What are potential causative agents or factors? Multiple regressions could also be used to determine potential roles for and interactions of organic compounds (individual or mixtures of compounds identified, F–C reactive compounds, or metabolic profiles), inorganic compounds (NPK), solute potential, pH, etc. in determining the stimulation or inhibition of germination, radicle length, and seedling emergence and biomass.

Comments Option 1 provides information on the effects (i.e., stimulation or inhibition) of a single application of leachates from aboveground or belowground wheat residues on germination and radicle length in Petri dishes in the absence of soil. Given the Petri dish environment (e.g., direct immersion of seeds in leachates), the results represent maximum potential stimulation or inhibition that is possible for the conditions of that bioassay (e.g., volume and concentration of treatment solutions, density of seeds, seed size, temperature, microbial populations, etc.). Options 2 and 3 provide information on the effects of multiple applications of aboveground or belowground wheat residue leachates on seedling emergence and biomass when seeds are surrounded by soil. For all intents and purposes the soil surrounding seeds and roots functions as a differential permeable barrier (modifier) for organic and inorganic compounds regulating types and concentrations of compounds reaching the seeds and roots. The soil also regulates the amount of light reaching the seeds and roots. Thus, the behavior observed in the pot systems represents a soil modified response to residue leachates. As with the Petri dish bioassay, the observed stimulation or inhibition of seedling emergence (and biomass) are valid for only the

conditions of that bioassay (e.g., soil type, soil layer above seeds, volume and concentration of leachates applied, seed density, temperature, light intensity, microbial populations, etc.). The bottom line is that Options 1, 2 and 3 provide a means of screening for potential effects of aboveground and belowground residue leachates in the absence and presence of soil with the caveat that the microbial actions within these systems are an unknown factor. The overall artificial nature of these bioassay systems, however, will provide little, if any, direct insight regarding how residue leachates actually impact seed germination, seedling emergence, and biomass under field conditions.

4.3.6 Significance

What we know at this point from these bioassays in the field and the laboratory is that aboveground wheat residues (straw and stubble) and belowground wheat residues (roots and mycorrhizae) and their leachates in the presence or absence of soil have or do not have the potential to stimulate or inhibit weed seedling germination, radicle length, seedling emergence, and seedling biomass under the environmental conditions of these bioassays in the field and in the laboratory. If that is the only objective, then one need go no further. However, if in the laboratory one wishes to establish the range of environmental conditions under which these potential effects may be expressed, then additional experiments using a range of environmental conditions will be required. Furthermore if one wishes to establish that the observed stimulation or inhibition of residues and their leachates are directly or indirectly due to organic compounds, then specific roles for active organic compounds released from residues and subsequently created compounds resulting from oxidation, reduction or the action of microorganisms would also have to be demonstrated individually and within the context of the system being tested (Willis 1985, 1994, 2007; Blum et al. 1999; Molisch 2001; Blum 2011; also see Sect. 1.1 and Chap. 6). This may be readily achievable if the observed effects are due to a small number of highly active compounds (i.e., the herbicide paradigm) but may not be so readily achievable if the observed effects are due to a large number of compounds with low activity and low concentrations acting in concert (i.e., the more holistic paradigm). In the latter case metabolic profiling (metabolomics) could provide some answers (Summer et al. 2003; Dunn and Ellis 2005; Prince and Pohnert 2010; D'Ambrosca et al. 2013; Duke et al. 2013; see Sect. 6.6). Finally as indicated previously, some caution is required when interpreting results of these field and laboratory bioassays. For residue bioassays the input by aboveground residues consists primarily water-soluble (hydrophilic) compounds although some volatile compounds may also be released and enter the soil. Input of belowground residues will not only include water-soluble compounds but also hydrophobic and volatile compounds.

4.4 Field Soils

Once water-soluble organic and inorganic compounds are released from living plants and dead plant materials (litter and residues) into the soil, they are rapidly acted upon by soil processes (e.g., reversible or irreversible sorption, utilization and modification by soil organisms, taken up by seeds, roots and mycorrhizae, transferred within the soil by gravitational flow, capillary movement, transpirational pull, etc.). The same things happen to water-soluble organic compounds synthesized and released by soil organisms. Thus, the concentrations of free (in solution) or available (free and reversibly sorbed) organic and inorganic compounds in soil samples used in bioassays consist of what is left after soil processes have taken place. In other words, the free or available water-soluble organic and inorganic compounds in soil samples constitute only a fraction (i.e., residual or net concentration) of the total free or available water-soluble organic and inorganic compounds that are potentially available in field soils. In addition to water-soluble compounds field soil samples also contain residual concentrations of hydrophobic and volatile compounds. The bottom line is that the total potential impact of the active organic and inorganic compounds on seeds, seedlings, or older plants cannot be adequately determined by soil sample bioassays alone. (Note: Reasons for testing soil and soil extracts in spite of this limitation are provided in the comment sections and Sect. 4.4.4, in particular). Three common bioassays have been used in the past to test for the presence of potential promoters and inhibitors within soil: direct soil bioassays (Wilson and Rice 1968), bioassays of soil with and without activated carbon (Mahall and Callaway 1992; Nilsson 1994**;** Ridenour and Callaway 2001; Bertin et al. 2009), and bioassays of soil extracts (for example see Blum et al. 1992; Muscolo et al. 2013). Soil water extracts will, of course, contain primarily only residual hydrophilic (water-soluble) compounds.

4.4.1 Soil

Objective To determine the potential stimulation or inhibition of field soil from which a range of recognizable dead aboveground and belowground wheat residues have been removed.

Materials and Methods *Option:* Four soil cores (10 cm deep × 15 cm in diameter) were collected at random from each field treatment and reference plot (see Sect. 4.2.1 and 4.2.2 for details) 2 weeks after the wheat harvest. Aboveground wheat residues were carefully removed from the soil cores and oven-dried (100 °C). Soil cores from each plot were then combined, gently sieved (1 cm × 1 cm) to remove rocks and recognizable belowground wheat residue fragments, and mixed. Belowground wheat residues were oven-dried. Weights of the aboveground and belowground residue fragments recovered from the combined soil cores from each field plot were determined. A subsample from each combined soil core sample was analyzed for particle size (sand, silt and clay), organic matter, water-soluble F–C

reactive compounds, metabolic profiles, mineral constituents (e.g., NPK), cation exchange capacity, and pH. The freshly processed soil was placed into 15 cm plastic pots (1,767 cm^3) with drainage holes and transplanted with morningglory (*Ipomoea hederacea* L.), pigweed (*Amaranthus retroflexus* L.), or prickly sida (*Sida spinosa* L.) seedlings, five seedling per pot (see Sect. 3.5).

Seedlings for transplanting were produced prior to the collection of soil cores. Weed seeds were seeded into trays with drainage holes containing sand. Sand in the trays was watered daily with Hoagland's solution (pH 5.5) or more frequently if needed. Since seedling emergence varies with species, seeding times were adjusted to accommodate for this variance in emergence. Emerging seedlings were grown for 1 week.

Four seedlings of roughly equal size for each weed species were chosen, carefully removed, and transplanted equidistant from each other into pots containing identical amounts of soil. In order to obtain identical amounts of soil in each pot, the moisture content of the soil was determined (100 °C) and the fresh soil in the pots was adjusted accordingly. Pots containing soil and seedlings were initially treated with simulated-rain water (see Sect. 4.3.3) so that the soil was 25, 50, and 75% below field capacity. Pots were weighed and weights were recorded. Subsequently, sufficient simulated-rain water was added daily to each pot to maintain its initial weight. Production of seedlings and soil bioassays were carried out in a greenhouse. Average light intensity at seedling level, temperature, and relative humidity were monitored during the experimental period. Seedlings were allowed to grow for 2 weeks at which time seedlings were harvested and dry weight (100 °C) of aboveground and belowground biomass was determined. The basic experimental design for each sampling period: randomized-block design of 4 soil samples (soil from 3 wheat residue levels and reference) × 3 moisture levels × 5 replicates (soil from the 5 blocks) = 60 experimental units per weed species. However, the term "3 wheat residue levels" here is somewhat misleading since there will likely be more than 3 wheat residue levels.

Seedlings were supplied with an adequate supply of nutrients before transplanting. However depending on the nutrient content of the soil and the growth period, additional nutrients may need to be supplied to the bioassay system. Time allowed for seedling growth may need to be adjusted depending on soil and growth rates of weed seedlings. Soil samples taken from plots without wheat residues were used as reference soils. The term wheat residue, as used here, includes all wheat straw [i.e., stems and leaves], stubble, and root and mycorrhizal fragments (see Sect. 4.3.2). Depending on soil type and rates of evapotranspiration, the predetermined moisture levels and the frequency of simulated-rain water additions may need to be adjusted. See Sect. 4.2.2.1 for soil analyses and environmental measurements. Isolated individual plant soil cores could also be taken at various distances from a plant of interest and tested. For an example of this approach see Wilson and Rice (1968).

Hypothesis If wheat residues on or in the soil release to the soil sufficient amounts of promoters, modifiers, and inhibitors as the residue is decomposed and leached, then the soil with the recognizable wheat residues removed should still stimulate or inhibit seedling biomass of sensitive weed species if the residual concentrations of promoters, modifiers, and/or inhibitors left in soil are sufficient enough to be

effective. Some wheat residue particles, however, will be left behind because very small wheat residue particles are not distinguishable from the organic matter of the soil. Potential allelopathic compounds in the soil may be composed of hydrophilic, hydrophobic, and volatile compounds.

If the soils from different wheat residue treatment plots have similar pH values, organic and inorganic contents, and physical characteristics, then the observed effects on biomass of sensitive weed seedlings by the soil are a product of the organic promoters, modifiers, and inhibitors (subset of all organic compounds) in the soil and subsequently produced by oxidation/reduction and microorganisms.

If weed seedling biomass is stimulated, then promoters dominate the promoter/modifier/inhibitor complex and if inhibited, then inhibitors dominate the complex. (Reminder: Theoretically any given compound may be a promoter, modifier, or inhibitor depending on the circumstances [see Sect. 2.1.3.1]).

If the soils from different wheat residue treatment plots have different pH values, organic and inorganic contents, and physical characteristics in addition to differences in organic promoters, modifiers, and inhibitors (subset of all organic compounds), then the observed effects on biomass of sensitive weed seedlings are a product of the direct or indirect actions and interactions of all the factors in the soil.

Analyses *Step 1:* Do soils with recognizable wheat residues removed from wheat residue field plots stimulate or inhibit the production of weed biomass? Regression analysis may be used to determine the relationship between the aboveground and belowground wheat residues removed from the soil (X, independent variable) and weed seedling biomass (shoot, root, or seedling biomass; Y, dependent variable).

Step 2: What are the potential causative agents? Multiple regression analysis could be used to determine the relationships between soil organic matter, soil moisture, soil pH, NPK, water-soluble F–C reactive compounds, metabolic profiles, etc. and the biomass of roots, shoots, or seedlings.

Comments The resulting data provide some insight regarding the potential presence and potential action of residual promoters and inhibitors left in the soil after recognizable wheat residues have been removed from the soil. However, the reader should be aware that during the entire bioassay, sorption, desorption, oxidation/reduction, and microbial activity have modified the qualitative and quantitative nature of the promoters, modifiers, and inhibitors present in the soil. That potentially includes hydrophilic, hydrophobic, and volatile compounds. Microbial activity can be of a particular concern for nutrient rich bioassay systems. As an initial screening tool for detecting the presence of residual potential allelopathic compounds (e.g., promoters and inhibitors) in soils, such direct soil bioassays are useful but clearly not definitive. See Sect. 4.4.4 for additional comments.

4.4.2 Soil Plus or Minus Activated Carbon

Objective To determine the potential stimulation or inhibition of field soil from which a range of recognizable aboveground and belowground wheat residues have

been removed and to which different amounts of activated carbon have been added. Soil samples taken from plots without wheat residues were used as reference soils.

Materials and Methods The experimental protocol (Option) for Sect. 4.4.1 was modified by mixing a range of activated carbon into soil subsamples from each of the combined and processed soil samples collected. The range of activated carbon to be added to soil was determined by a preliminary experiment. The basic experimental design for each sampling period was: randomized-blocked design of soils from 4 wheat residue levels (3 wheat residue levels and reference) × 3 moisture levels × 3 activated carbon levels × 5 replicates (soil from the 5 blocks) = 180 experimental units per weed species. However, just as for Sect. 4.4.1 the term "3 wheat residue levels" is somewhat misleading since there will likely be more than 3 actual wheat residue levels that were removed from the soil. The actual number of weed species tested was based on their response during the soil study (see Sect. 4.4.1).

Hypotheses See hypotheses for Sect. 4.4.1 for soils without activated carbon.

If activated carbon is mixed into soils and if activated carbon inactivates by sorption (i.e., absorption and/or adsorption) residual allelopathic compounds, then as the amount of activated carbon mixed into the soil increases, the stimulation or inhibition of biomass for sensitive weed seedlings should be reduced until all the residual promoters and inhibitors are sorbed or the activated carbon is saturated.

Analyses The same type of analyses as for Sect. 4.4.1 may be used here except with activated carbon as an additional independent variable.

Comments A decline in the ability of a soil to stimulate or inhibit seedling biomass by increasing additions of activated carbon suggests that the soil being tested may contain promoters, modifiers, and inhibitors that are sorbed to activated carbon. However, care must be taken in interpreting the results from such bioassays since activated carbon sorbs a range of organic and inorganic compounds (Hassler 1963; Smíšek and Černý 1970; Mattson and Mark 1971; Cheremisinoff and Ellerbush 1978; Huang 1978; Bansal and Goyal 2005). What is actually sorbed is determined in large part by the source, type, and surface area of the activated carbon, pH and type of solvent, nature and combination of organic and inorganic compounds present in the solvent and on the activated carbon, ratio of sorbent to solvent, and flow rate of the solvent (Smíšek and Černý 1970; Cheremisinoff and Ellerbush 1978; Bansal and Goyal 2005). However, activated carbon is highly efficient in the sorption of aromatic compounds from water (Bansal and Goyal 2005; Wang et al. 2005). In addition activated carbon can also influence plant and microbial responses independently of allelopathic compounds (see Lau et al. 2008; Wurst et al. 2010). As an initial screening tool for detecting the presence of potential residual promoters, modifiers, and inhibitors in soil, testing soils with the addition of a range of activated carbon is useful but also clearly not definitive.

4.4.3 Soil Extracts

See Sects. 2.1.3.2 and 2.1.3.3 for collecting and using soil extracts. Water extracts of soil were tested in the Petri dish bioassay (see Sect. 4.2.2.2 Option 1 for details).

Comments The primary concern about testing soil extracts in Petri dish bioassays is that in addition to organic compounds the pH, solute potential, and inorganic compounds of the extracts will also vary from soil extract to soil extract. Such variation makes it extremely difficult to identify cause and effect unless extract pH, solute potential, and inorganic content are adjusted to be equivalent or accounted for directly (e.g., including appropriate controls/references) or indirectly (e.g., regression analysis; see Sect. 6.4.2). An additional concern about water extracts of soil is the activity of microorganisms during the extraction procedure and the bioassay. However, soil samples can be sterilized before extraction (see Dalton et al. 1983), soil samples and extracts can be sterilized during extraction (Blum et al. 1991, 1992), or soil extracts can be sterilized after extraction (e.g., filter sterilization). Applications of soil extracts to substrate systems, particularly soil systems, are not recommended because of the high probability of creating anaerobic soil environments. Unlike residue and soil bioassays, soil extract and residue leachate bioassays test primarily for the actions of hydrophilic (water-soluble) compounds.

4.4.4 Significance

What we knows at this point from these bioassays is that soils and their extracts have or do not have the potential to stimulate or inhibit weed seedling growth under the environmental conditions of our bioassays. If that is the only objective, then the researcher needs to go no further. However, if he/she wishes to establish the range of environmental conditions under which these potential effects may be expressed, than additional experiments using a range of environmental conditions will be required. Furthermore, if we wish to establish that the observed stimulation or inhibition by soil and its extracts are due to allelopathic compounds released directly from plant residues or indirectly by oxidation/reduction and the actions of microorganisms in the soil, then the release and roles of such compounds will also have to be demonstrated individually and within the context of soil systems and soil extracts (Willis 1985, 1994, 2007; Blum et al. 1999; Molisch 2001; Blum 2011; also see Sect. 1.1 and Chap. 6). This can be readily achievable if the observed effects are due to a small number of highly active compounds (i.e., the herbicide paradigm) but may not be so readily achievable if the observed effects are due to a large number of compounds with low activity and low concentrations acting in concert (i.e., the more holistic paradigm). In the latter case metabolic profiling (metabolomics) may provide some answers (Summer et al. 2003; Dunn and Ellis 2005; Prince and Pohnert 2010; D'Ambrosca et al. 2013; Duke et al. 2013; see Sect. 6.6).

Assuming that all other factors are constant, soil sample bioassays test for stimulation or inhibition by residual active hydrophilic (water-soluble) compounds and residual active non-water-soluble compounds present in the soil (i.e., hydrophobic

and volatile compounds). Bioassays of properly collected soil extracts (i.e., extracts that do not include irreversibly sorbed compounds) test only for the stimulation or inhibition of the residual active water-soluble compounds. Both soil and soil extract bioassays represent one point in time, i.e., the time when they were collected. In field soils stimulation or inhibition is a product of all (not just the residual) active water-soluble and non-water-soluble compounds that are periodically or continuously released from living plants, plant residues, and residual soil organic matter and those modified by oxidation/reduction and modified and produced by organisms in the soil. Active compounds are defined as compounds that actually interact with seeds, roots, and mycorrhizae.

At this point the reader may be asking why bother if all that is being tested are residual concentrations for soil and soil extracts in laboratory bioassays. Organic compounds (and inorganic compounds) in soil and soil solutions (i.e., residual amounts obtained by soil extracts) constitute the background amounts (frequently a steady state amount) in which seeds germinate and roots and mycorrhizae grow. That background at times can be sufficient to stimulate or inhibit plant processes and provide indirect evidence for potential plant-plant allelopathic interactions. The absence of an effect, however, provides no evidence for or against potential plant-plant allelopathic interactions since the amounts tested (residual amounts) represent only a portion of the entire amounts that may, in a theoretical but real sense, interact with seeds, roots, and mycorrhizae.

4.5 Final Comments

Hopefully the bioassays presented in addition to my previous comments and reflections (see Chap. 3) will suffice to provide the necessary general guidelines for designing bioassays that are as relevant as possible to field environments. However, an initial impression may lead the reader to conclude that the above proposed experimental designs are not all that different from most of the designs already found in the literature. So why are these more relevant?

a. They emphasize the use of promoter/modifier/inhibitor complexes released from living plants and residues instead of individual active compounds in isolation of all other compounds.
b. They attempt to simulate the sources and locations of inputs to soil surfaces or within soils of the field study site.
c. They attempt to simulate natural conditions of soils whenever and wherever possible.
d. They are designed to be more inclusive of the range of environmental conditions found in field environments with two caveats.

The first caveat is that at times they attempt to eliminate or minimize differences (e.g., pH, solute potential, inorganic compounds such as NPK, etc.) other than organic compounds associated with the addition of leaf leachates and “root exudates plus” leachates from living plants, aboveground and belowground residues and

their leachates, and soils and their extracts. The second caveat is that for all the bioassays described in this Chapter, the entire seed, root, and mycorrhizal surface areas have been treated. However, the probability that entire root and mycorrhizal surface areas are in contact with allelopathic compounds under field conditions is low. Adjustments to treatment surface areas in laboratory bioassays are thus appropriate and desirable. See Sect. 6.5 for details on how this can be achieved.

Finally, in this chapter the author has described a limited set of solution and soil culture bioassays for screening the effects of putative allelopathic compounds on germination, radicle growth, or seedling biomass of sensitive species. There are, of course, a whole range of other types of screening bioassays. They use different species, media, containers, treatments, and environments and measure different plant responses. Ultimately, however, they have similar limitations and concerns described in this and the previous chapters (e.g., see Einhellig et al. 1985; Leather and Einhellig 1988; Inderjit and Dakshini 1995; Wu et al. 2001b; Belz and Hurle 2004; Hoagland and Williams 2004; Blum 2011).

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Chapter 5
Effects, Modifiers, and Modes of Action of Allelopathic Compounds Using Phenolic Acids as Model Compounds

Abstract This chapter provides an abridged version of the known effects, physicochemical and biotic factors that modify effects, and the modes of action of allelopathic compounds using phenolic acids as the model compounds.

5.1 Introduction

Previously some ambiguity was expressed about the term allelopathic compound or compounds (see Sects. 1.3 and 2.1.3.1). This ambiguity stemmed from the fact that theoretically any given organic compound can function as a promoter, modifier, inhibitor, or neutral compound. The function of a particular organic compound is determined by the sensitivity of the species, the process being monitored, the concentrations of the compound, the nature of other organic and inorganic compounds present, and the biotic and physicochemical environments (Blum and Rice 1969; Rice 1984, 1986; Blum et al. 1989, 1993; Gerig et al. 1989; Gerig and Blum 1991, 1993; Pandey 1994; Pue et al. 1995; Blum 1996, 2006, 2011; Belz et al. 2005, 2007; Duke et al. 2006; Belz 2008; Gianinazzi et al. 2010; also see Sects. 3.6 and 3.7). Therefore, in an absolute sense there is no such thing as a compound that is always an allelopathic compound. There are only organic compounds that function as promoters or inhibitors under the appropriate physicochemical and biotic conditions. In other words, under the appropriate conditions and at the appropriate concentrations, organic compounds function as allelopathic compounds or agents. Theoretically this is true but from a practical perspective there clearly are organic compounds that at concentrations and environments found in nature readily modify (stimulate or inhibit) plant processes and organic compounds that do not modify plant processes (e.g., inactive because their concentrations are too low or because conditions are not met by the field environment). When it comes to both positive and negative plant-plant allelopathic interactions, logic says that the focus of research should be on the former, i.e., the potential active compounds found in nature. That, however, would ignore the total or summed actions of all the individual compounds making up a complex mixture (e.g., promoter/modifier/inhibitor complexes of leachates, "root exudates plus", and soil solutions) where the concentrations of the individual compounds making up the mixture are well below their individual stimulatory or inhibitory concentrations. Research clearly indicates that given the right conditions,

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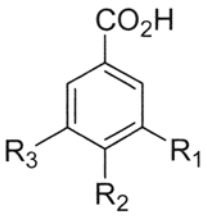
the actions of individual organic compounds at low concentrations (i.e., "apparently inactive") can have similar joint action (synergistic, additive, partially or wholly antagonistic) or have modifying actions in the presence of low concentrations of other organic compounds (see Blum et al. 1993; Pue et al. 1995; Blum 1996, 2011; Sect. 6.3). This would suggest that the research focus should be broadened to include the potential similar joint actions (synergistic, additive, or antagonistic), or modifying actions of "apparently inactive" individual organic compounds within complex mixtures.

The focus of Chaps. 2 (bioassay fundamentals), 3 (challenges), and 4 (standard screening bioassays) has been on how complex mixtures of organic and inorganic compounds (e.g., plant and litter leachates) and identified putative allelopathic compounds individually, in various simple combinations, or in combinations with other organic compounds affect (stimulate or inhibit) seed germination and seedling growth and biomass of sensitive species. The focus on seed germination and seedling growth and biomass was a product of the nature of the original field research described in the first volume (Blum 2011), an emphasis on system responses, and a desire to design laboratory bioassay systems that are more relevant to field systems (Sect. 1.5.2). However, effects on germination, growth, and biomass are far removed from the primary effects of allelopathic compounds and in fact are the end products of a complex series of events that starts at the molecular or cellular levels. In addition, allelopathic compounds may have multiple target sites (Gniazdowska and Bogatek 2005; Duke and Dayan 2006). As a result, there is an extensive, diverse, and confusing literature on the actions of allelopathic compounds ranging from the molecular to the whole plant level with only a limited understanding or appreciation of the modes of action, the individual processes affected, and the final effects expressed at the seed, seedling, or older plant level. The same limited understanding or appreciation extents to how these aspects may be modified by a range of physicochemical and biotic factors. In fact, a clear understanding of all these relationships is still in its infancy. The most promising tool to rectify this lack of understanding is the laboratory bioassay.

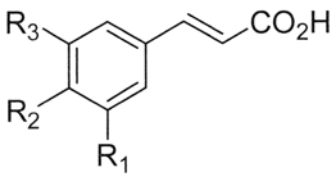
So what is known at this point? To start with what are agents, modes of action, mechanisms, and effects?

a Agents are external forces (e.g., physical agents such as energy) or substances (e.g., water, organic and inorganic compounds) that cause changes to seed, seedling, or plant processes under study. For allelopathic plant-plant interactions the primary agents are organic compounds that function as allelopathic agents (i.e., promoters or inhibitors); the rest are modifying agents (modifiers). Allelopathic compounds include an array of organic compounds that individually or in combination promote or inhibit sensitive species. Any given agent may act on more than one primary site and more than one agent can act on the same primary site. Modifying agents alter the actions of the primary agent or agents of interest.

b. Modes of action refers to the specific physical or biochemical actions or reactions by which an allelopathic compound (i.e., promoter or inhibitor) or modifier produces or modifies an effect at the molecular and cellular levels which leads to functional changes in physiology, anatomy, and morphology at the organismal level.



R_1	R_2	R_3	
H	OH	H	*p*-hydroxybenzoic acid
OH	OH	H	protocatechuic acid
OMe	OH	H	vanillic acid
OMe	OH	OMe	syringic acid



R_1	R_2	R_3	
H	OH	H	*p*-coumaric acid
OH	OH	H	caffeic acid
OMe	OH	H	ferulic acid
OMe	OH	OMe	sinapic acid

Fig. 5.1 Some common simple plant phenolic acids, cinnamic acid derivatives on the *right* and benzoic acid derivatives on the *left*, where H equals hydrogen, OH equals hydroxy, and OMe equals methoxy. (Figure taken from Blum (2011), figure used with permission of Springer Science and Business Media)

c. Mechanisms are fundamental processes involved in or responsible for an action or reaction, i.e., a means or pathway by which effects are produced. Since modes of action and mechanisms are frequently used interchangeably the author will not make a distinction between these terms.
d. Effects are simply the consequence of the action of any agent or combination of agents. Primary effects will normally occur at the molecular or cellular levels but not all molecular and cellular effects constitute primary effects. Primary effects lead to secondary, tertiary, etc. effects but depending on the "buffering capacity" of a plant or its ability to adjust (acclimate), primary effects at the molecular and cellular levels may or may not be expressed at the whole plant level. The effects can be positive or negative depending on the species, the individual (e.g., stage of life cycle, state of health and acclimation, past environments, etc.), type of effects monitored (e.g., primary, secondary, tertiary, etc. effects), the nature of agent or agents tested (e.g., function of organic compounds as promoters, modifiers, or inhibitors and their site or sites of action), and the present physicochemical (e.g., pH, inorganic compounds present, day/night temperatures, light quality and quantity, and nature of substrate) and biotic (e.g., rhizosphere organisms, and presence or absence of symbiotic relationships) environments.

Given these definitions and settings as a benchmark, let us take a closer look at how and under what circumstances one group of putative allelopathic compounds (i.e., agents) frequently cited in the literature, the simple phenolic acids (e.g., cinnamic and benzoic acid derivatives; Fig. 5.1), impact seedling growth and development. For simplicity of this presentation the author will use cucumber seedlings as a representative model species (see Blum 2011).

From a historical perspective the order has been to first identify potential allelopathic plants by testing their leachates, "root exudates", or residues for example in

seed, seedling, or plant bioassays (see Chap. 4), to identify, quantify, and isolate the potential agents responsible for stimulation or inhibition (for example see Waller et al. 1999; Sampietro et al. 2009), to determine how various environmental factors modify allelopathic effects of identified compounds (for example see Einhellig 1987, 1989, 1999), and finally to determine the potential modes of action that may be responsible for the effects observed (for example see Einhellig 1986, 2004; Bais et al. 2003; Macías et al. 2007; D'Abrosca et al. 2013; Duke et al. 2013). That is the sequence of the following presentation but starting with an identified set of putative allelopathic compounds, simple phenolic acids.

5.2 Individual Compounds

Effects of phenolic acids on sensitive seedlings have been found to be stimulatory (positive), neutral (no effect), or inhibitory (negative) to seedling processes (see Li et al. 1993; Belz et al. 2007). Which type of effect (stimulatory or inhibitory) occurs depends on a whole host of factors (see Chaps. 2 and 3). However for a given bioassay system and a given phenolic acid, the effects and the magnitude of effects are determined by the concentration of active phenolic acid molecules that interact with seeds, roots, and mycorrhizae. Frequently when a pattern is found, the pattern for increasing concentration of individual phenolic acids within a given bioassay system is as follows: no effects, stimulatory effects, and finally inhibitory effects (see Blum and Rebbeck 1989; Li et al. 1993; An et al. 2001; Ghareib et al. 2010). However, the stimulatory effects of phenolic acids are frequently not observed for seeds, seedlings, or older plants because they are associated with a narrow range of concentrations that are rarely included in laboratory dose-response studies (An et al. 2001; Duke et al. 2006). At times stimulatory and inhibitory effects can also occur simultaneously for different processes of seeds, seedlings, or plants (see Sect. 5.2.1). Since the primary focus of most researchers, however, has been on the inhibitory actions (effects and modes of action) of allelopathic compounds, there is only a limited amount of information available for the stimulatory effects of simple phenolic acids.

5.2.1 Stimulatory Effects

There are primarily three instances when phenolic acids are or appear to be stimulatory and these are:

1. when phenolic acids stimulate some plant processes while inhibiting other plant processes at the same concentration,
2. when phenolic acids stimulate plant processes at low concentrations and inhibit them at higher concentrations (i.e., hormesis), and
3. when inhibited plant processes overcompensate in the recovery phase after treatment.

There is, however, considerable overlap for the first two instances since the doses associated with hormesis (i.e., stimulation at lower doses and inhibition at higher doses (see Belz et al. 2005, 2007; Duke et al. 2006) will likely vary for different plant processes. The author lists the first two instances separately because most researchers do not run complete dose-response curves and although hormesis is commonly found, it is not always evident or may be absent depending on the species and process under study (Belz et al. 2007).

Examples for Instances 1. and 2 Zanardo et al. (2009) observed that ≥ 0.25 mM *p*-coumaric acid (pH 6.0) stimulated cell-wall-bound peroxidase activity and lignin content in soybean roots but inhibited root growth. They suggested that *p*-coumaric acid is channeled into the phenylpropanoid pathway where it is converted to *p*-coumaryl CoA or to sinapate. Subsequently these metabolites are converted to alcohols which are polymerized into the lignin of cell walls. The strengthened cell walls lead to a restriction of root growth. Similar observations to that of Zanardo et al. have been made for ferulic acid and soybean roots by dos Santos et al. (2004; pH 6.0) and for cucumber seedlings by Shann and Blum (1987b; pH 4.5, 5.0, or 7.0). D'Abrosca et al. (2013; no pH provided) observed a stimulation (0–300 %) or an inhibition (0–100 %) of 16 metabolites and an inhibition (0 to > 50 %) of shoot and root length for wild wheat seedlings (*Aegilops geniculata* Roth also known as *Triticum ovatum* L) grown for a week in Hoagland's nutrient solution containing 10^{-5} or 10^{-3} M ferulic acid, *p*-coumaric acid, catechol, *p*-hydroxybenzoic acid, juglone, or coumarin. Metabolites were determined by NMR metabolomic analysis. They concluded that the metabolome changes in their test plants confirmed the reported modes of action of the putative allelopathic compounds tested.

Ghareib et al. (2010) observed that radicle and plumule length of tomato seedlings were stimulated by ≥ 0.1 ppm vanillic acid (pH 5.6) but inhibited by ≥ 2 ppm of vanillic acid (pH 5.6). They also observed a similar pattern of response for the activities of catalase, peroxidase, polyphenoloxidase, and superoxide dismutase. The pattern, however, was reversed for H_2O_2 content and lipid peroxidation. Blum and Rebbeck (1989) observed an increase (stimulation) in the number of secondary roots in solution culture (pH 5.5) but a reduction (inhibition) of the average root length when cucumber seedlings were treated with ≥ 0.125 mM ferulic acid (30 % increase and 15 % decrease, respectively, at 0.125 mM and 123 % increase and 67 % decrease, respectively, at 0.5 mM). They also observed an inhibition of photosynthesis, photosynthate translocated to roots, leaf expansion, and biomass of cucumber seedlings by ≥ 0.25 mM ferulic acid.

Given such observations, it should be clearly evident that stimulation and inhibition of phenolic acids at the molecular, cellular, organ, tissue, and whole plant levels are convoluted. The following are a few additional examples of stimulatory effects from the literature.

a. Tomato radicle and plumule length were stimulated by vanillic acid in the ≥ 1 ppm to ≤ 2 ppm range (Ghareib 2010; pH 5.6).
b. Germination and/or radicle growth of four common weeds were at times significantly stimulated by gallic acid, ferulic acid, vanillic acid, *p*-coumaric acid,

p-hydroxybenzoic acid, and *p*-vanillin. When the stimulation was significant, it occurred between the 10^{-5} and 10^{-3} M range (Reigosa et al. 1999; pH 6.0).

c. Hypocotyl elongation of etiolated lettuce seedlings was stimulated by caffeic and ferulic acids between 10^{-4} to 10^{-3} M (Li et al. 1993; no pH provided).
d. Dry weight of sorghum seedlings was slightly stimulated by ferulic acid and *p*-coumaric acid at 1.25×10^{-4} M (Rasmussen and Einhellig 1977; pH 3.9).

Examples for Instance 3 Overcompensation of plant processes in the recovery stage (i.e., after concentrations of phenolic acids fall below inhibitory levels or to zero) may at times lead to the false impression of stimulation by phenolic acids. This is of particular concern for bioassay systems that rapidly deplete phenolic acids (e.g., nutrient culture systems). For example, if phenolic acids inhibit cell expansion but not the accumulation of metabolites (Boyer 1970; Acevedo et al. 1971), then when cell expansion is released from the actions of phenolic acids, rapid cell expansion due to the negative water potentials of the cell could lead to excessive (i.e., overcompensation) leaf expansion. A specific example of this type of overcompensation can be found in Sect. 5.2.3.1 under Leaf Expansion.

As an aside, data suggest that the magnitude of the inhibitory effects on germination and seedling growth as well as other plant processes, increase as the pH of the treatment solution is lowered (i.e., made more acidic; Harper and Balke 1981; Blum et al. 1985b, 1989; Lehman and Blum 1999b). Given that, the author would suggest that stimulatory effects for germination and growth are much more likely to occur under slightly acidic and neutral conditions than under very acidic conditions. However since pH and concentration effects are not independent, a given percent stimulation or inhibition can be produced by varying either the concentration or the pH of the treatment solution whenever the effective concentration is above the appropriate threshold level (see Sect. 5.2.3.1). The importance of this relationship should not be underestimated since changes in pH readily occur within the rhizosphere or mycorrhizosphere over time due to the normal actions of roots and microorganisms (see Blum et al. 1985b; Rending and Taylor 1989; Blum and Gerig 2005).

5.2.2 Inhibitory Effects

Because considerable emphasis has been placed on the inhibitory effects of simple phenolic acids, we have a somewhat better grasp of the effects and modes of action of inhibitory concentrations of phenolic acids. One primary effect for sensitive seedlings which has been clearly identified for inhibitory concentrations of simple phenolic acids such as cinnamic acids and benzoic acids, is a perturbation (e.g., depolarization; alteration of permeability) of root cell membranes (Glass 1973, 1974; Glass and Dunlop 1974; Balke 1985; Baziramakenga et al. 1995). Circumstantial evidence, however, suggests that there are very likely additional primary (i.e., direct) effects of phenolic acids at the molecular and cellular levels of roots independent of root membrane perturbation (e.g., cell wall lignifications and products produced by enzymes that utilize phenolic acids as a substrate). The bottom line is

that root cell membrane perturbation in conjunction with other potential primary effects of phenolic acids leads directly or indirectly to:

a. a reduction in net water uptake and transpiration (Blum and Dalton 1985; Lyu and Blum 1990; Holappa and Blum 1991; Blum and Gerig 2005, 2006),
b. a reduction in net nutrient uptake and efflux of nutrients (Glass 1973, 1974, 1975; McClure et al. 1978; Harper and Balke 1981; Balke 1985; Lyu and Blum 1990; Bergmark et al. 1992; Booker et al. 1992; Baziramakenga et al. 1995; Yu and Matsui 1997; Lehman and Blum 1999a, b; Prasad and Devi 2002),
c. a decrease in water potential and turgor pressure, and at times solute potential (Einhellig et al. 1985; Einhellig 1986; Booker et al. 1992; Barkosky and Einhellig 1993),
d. changes in root cell processes such as down-regulation of cell cycle-related genes, endoreduplication, and associated blockage of mitosis (Zhang et al. 2009),
e. increased lignification of cell walls and associated modification of enzyme activity (Shann and Blum 1987b; Politycka 1998; Politycka et al. 2004; Politycka and Mielcarz 2007; Zanardo et al. 2009),
f. reduced rates of photosynthesis and carbon allocation to roots (Einhellig et al 1970; Patterson 1981; Blum and Rebbeck 1989; Zhou and Yu 2006),
g. modification of respiration and associated anabolic and catabolic processes (Demos et al. 1975; Moreland and Novitzky 1987), and
h. a whole host of other secondary, tertiary, etc. effects that together will ultimately slow growth and reduce biomass of sensitive seedlings over time when compared to seedlings growing in the absence of phenolic acids (Prasad and Devi 2002; Einhellig 2004).

Each of the effects listed above could be recognized as an independent effect (i.e., primary effect) for phenolic acids and by inference have been given such status in the literature at various times. In fact a number of these effects are not independent effects of phenolic acids. They are actually secondary, tertiary, etc. effects. At present the way all of these effects actually relate to each other has not been fully established.

What about direct effects of phenolic acids in aboveground tissue? Since phenolic acids are taken up by roots and mycorrhizae (Shann and Blum 1987a; Lehman and Blum 1999b; Blum and Gerig 2005) and then distributed to aboveground tissues (Shann and Blum 1987a), direct and indirect inhibitory effects of phenolic acids on cellular processes (e.g., photosynthesis, respiration, transfer of ions, function of enzymes, replication of genetic materials, cell division, etc.) for aboveground tissues are also possible (see Moreland and Novitzky 1987; Einhellig 2004; Zhou and Yu 2006; Ishii-Iwamoto et al. 2006). However, once phenolic acids are taken up by root cells, they are rapidly inactivated by being stored in vacuoles, metabolized, glucosylated, and utilized in synthesis of lignin among other things (see Harborne 1982; Shann and Blum 1987b; Zanardo et al. 2009). Thus, the amounts of free phenolic acids reaching aboveground tissue tend to be small compared to what is retained within root tissue (Shann and Blum 1987a). For example Shann and Blum (1987b) observed that 20, 13, and 3 % of the radioactivity present in root, shoot, and

leaf tissue, respectively, was in the form of free ferulic acid when cucumber seedling roots were exposed to [U-ring-^{14}C] ferulic acid. The bottom line is that direct effects of phenolic acids in aboveground tissue may be minor compared to direct effects for belowground tissues particularly since phenolic acids are not systemic in their actions but local in their actions (Blum 2011). If one accepts this premise, then effects on water relations and nutrition become the major focal points for researchers studying inhibitory effects on growth. Unfortunately a range of other stressors (e.g., water, nutrient, and temperature stress, presence of other inhibitory organic compounds, etc.) will also affect water relations, nutrition, and some or all of the other effects listed above (see Levitt 1972; Chapin 1991; Gniazdowska and Bogatek 2005; Pedrol et al. 2006). Thus, the effects associated with inhibitory phenolic acid concentrations are not particularly unique or distinguishable from the effects of other environmental stressors.

5.2.3 Modifying Factors

Research literature clearly demonstrates that for a given species the concentrations required for a given percent inhibition (or stimulation) can vary substantially with phenolic acid, pH and solute potential of the treatment solution, frequency and duration of treatment, treatment surface area contacted, time of treatment, time after treatment, the effect monitored, type of growth medium, and the physicochemical (e.g., pretreatment, present, and post treatment environments) and biotic (e.g., presence or absence of microbes and symbiotic relationships and density of bioassay species) environments among other things (see below and Chaps. 2 and 3 and Blum (2011) for references). Such observations lead to two questions.

a. How do modifying factors influence the inhibition (or stimulation) of seed germination or seedling growth?
b. How do modifying factors influence our ability to characterize, formulate, understand, and make generalizations about potential or actual field plant-plant allelopathic interactions?

Due to the large number of potential modifying factors, the complex and sometimes subtle and ephemeral nature of their influence, the substantial literature about modifying factors for inhibitory effects, and the abridged nature of this presentation, the author will attempt to answer these questions by describing the modifying actions of a representative subset of modifying factors (e.g., pH, solute potential, organic compounds, treatment surface area, and microorganisms, among others). Furthermore this presentation will primarily be limited to inhibitory effects of cellular processes, leaf expansion, water utilization, and nutrient uptake and efflux.

5.2.3.1 Single Treatment—Solution Culture

Role of Solution pH

Leaf Expansion Leaf expansion is a product of two primary processes, cell division and cell expansion. Cell division is determined by a complex set of genetic and cellular processes that are beyond the scope of this volume. Cell expansion is determined by cell wall flexibility and the inward diffusion of water which produces the turgor pressure necessary for cell expansion (Kramer 1983). Inward movement of water requires an external water supply and a cell-water potential that is more negative within the cell than on the outside of the cell. Water potential of a cell is determined by the solute (effects of soluble organic and inorganic compounds within the cell), tugor pressure (water content and back pressure by cell walls), and matric potentials (affinity of water to cell colloids, cell walls, etc.) of the cell. Net water uptake (uptake minus loss) determines the water content of a cell. Water content of a seedling is largely determined by the rate of net water uptake (uptake minus loss) by roots and mycorrhizae and the rate of water lost by transpiration.

Figure 5.2 describes the effects of a single two day treatment of ferulic acid or *p*-coumaric acid on leaf growth of cucumbers seedlings (*Cucumis sativus* L., Early Green Cluster) grown in Hoagland's nutrient solution (Hoagland and Arnon 1950; Blum et al. 1985b). Complete nutrient solution changes occurred on alternate days starting with day 2. Water was added daily to compensate for transpiration. Solutions were brought back to the original volume each time water was added. Seedlings were grown at room temperature (21–30 °C) in light banks with a 12-h photoperiod (Photosynthetic Photo Flux Density (PPFD) of 150 μmol/m^2/sec). On day 16, seedlings were treated with 0–1 mM ferulic acid or *p*-coumaric acid. MES (2[N-morpholino]-ethanesulfonic acid) buffer (5 mM) was used to stabilize the pH of these solutions (Hoagland's solution plus or minus phenolic acid). The pH values (5.5, 6.25, and 7.0) of the solutions in the figure are the initial pH values. Treatment solutions were replaced by nutrient solution on day 18. Average absolute and relative rates of seedling leaf expansion were determined just prior to solution changes starting with day 12 and leaf area and dry weight were determined at harvest on day 22. For additional details see Blum et al. (1985b).

To help decipher the patterns of Fig. 5.2, percent inhibition or stimulation for the effects of 0.25 mM of ferulic acid and *p*-coumaric acid were calculated using regression equations from Blum et al. (1985b).

a. Absolute rates of leaf expansion (day 16–18; cm^2/2 days) of cucumber seedlings growing in solution culture under light banks (Blum et al. 1985b) and treated on day 16 with a nutrient solution containing 0.25 mM of ferulic acid or *p*-coumaric acid at three pH values were inhibited (or stimulated) by:

   1. 27 and 20 % at pH 5.5,
   2. 16 and 0 % (actually 8 % stimulation) at pH 6.25, and
   3. 11 and 9 % at pH 7.0, respectively.

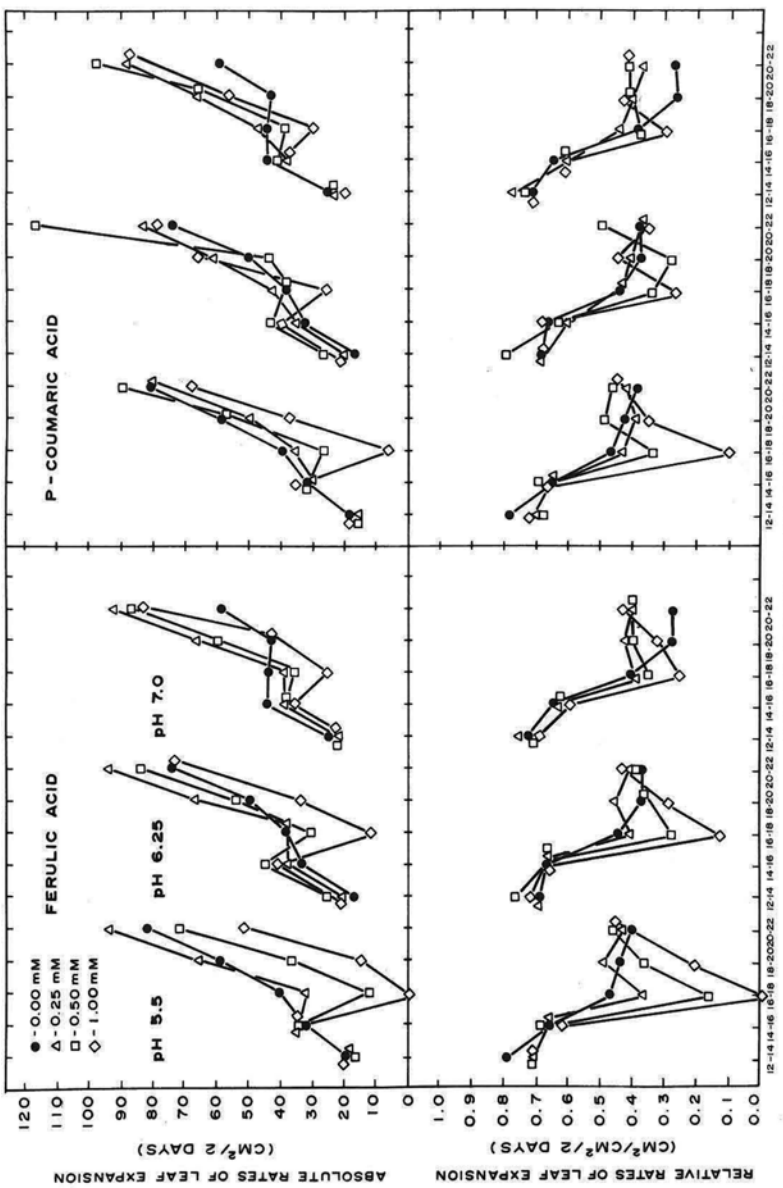


Fig. 5.2 Effects on absolute and relative rates of leaf expansion of cucumber seedlings grown in solution culture given a single 0, 0.25, 0.5 or 1 mM ferulic acid or *p*-coumaric acid treatment on day 16. All treatment solutions were replaced on day 18 with nutrient solutions. Seedlings were grown in Hoagland's nutrient solution plus or minus phenolic acids and 5 mM MES buffer. The pH values of the initial treatment solutions were 5.5, 6.25, or 7.0 ($N=3$). Points are connected only to aid in the visualization of patterns over time. (Figure taken from Blum et al. (1985b). Plenum Publishing Corporation, figure used with permission of Springer Science and Business Media)

Treatment solutions were replaced by nutrient solution on day 18 and the inhibition (or stimulation) of absolute growth rates for day 18–20 were:

1. 18 and 0% at pH 5.5,
2. 0 and 0% at pH 6.25, and
3. 0% (actually 32% stimulation) and 0% (actually 36% stimulation) at pH 7.0, respectively.

b. Relative rates of leaf expansion (day 16–18; cm^2/2 days) of cucumber seedlings growing in solution culture under light banks (Blum et al. 1985b) and treated on day 16 with a nutrient solution containing 0.25 mM of ferulic acid or *p*-coumaric acid at three pH values were inhibited by:

   1. 27 and 9% at pH 5.5,
   2. 18 and 10% at pH 6.25, and
   3. 8 and 7% at pH 7.0, respectively.

Treatment solutions were replaced by nutrient solution on day 18 and the inhibition (or stimulation) of relative growth rates for day 18–20 were:

1. 13 and 0% at pH 5.5,
2. 0 and 0% at pH 6.25, and
3. 0% (actually 39% stimulation) and 0% at pH 7.0, respectively.

Some comments regarding growth rates of leaves are warranted at this point. Leaves will only expand (grow) until they reach their maximum size. In addition the growth rate of each leaf will slowly decline until it reaches its final size (Blum and Dalton 1985; Waters and Blum 1987). The effects of phenolic acids on leaf growth rates are, thus, only detectable for expanding leaves. As seedlings mature, the ratio of fully expanded leaves (i.e., non-response leaves) to expanding leaves (i.e., responsive leaves) increases, and associated with this increase will be a decline in the leaf growth rates of seedlings independent of phenolic acid treatments. Care must, therefore, be taken when comparing leaf growth rates of seedlings for different stages of developing seedlings. A partial way around this dilemma is to calculate the absolute or relative rates of leaf expansion for seedlings. Such growth rates emphasize the effects observed for younger expanding leaves and reduce the dampening effects of the non-responsive leaves. Unless a leaf has reached its maximum size or cell walls have been lignified, the effects of phenolic acids on cell expansion of a leaf is reversed once phenolic acid contact with roots and mycorrhizae ends (Blum and Dalton 1985; Blum and Rebbeck 1989). Effects on cell division, however, may be another matter (see Zhang et al. 2009). Another option around this dilemma is to exclude all fully expanded leaves of a seedling and measure only the expanding leaves (Waters and Blum 1987). For additional information regarding growth and growth analysis see Radford (1967), Evans (1972), and Hunt (1982).

So why use leaf expansion, cucumber leaf expansion in particular, as a gauge for the inhibition of growth by phenolic acids:

1. the morphology of cucumber leaves is symmetrical and very consistent,

2. leaf area of cucumber seedlings can be determined non-destructively at given time intervals,
3. absolute and relative growth rates of individual leaves can be readily calculated from the length and width of a leaf and the regressions required can be derived from length and width of destructively harvested leaves,
4. leaf expansion responds rapidly (within hours) to phenolic acid treatments in solution culture, and
5. leaf area of cucumber seedlings are related to shoot dry weight ($r^2 = 0.92$), root dry weight ($r^2 = 0.62$), root length ($r^2 = 0.67$), number of roots ($r^2 = 0.32$), and average root length ($r^2 = 0.48$). For details regarding procedures and measurements see Blum and Dalton (1985), Blum and Rebbeck (1989), and Blum and Gerig (2005).

c. Cucumber seedlings were harvested on day 22 and leaf areas and plant dry weights were determined. The inhibition (or stimulation) of leaf area (cm^2) and dry weight (g) on day 22 as modified by 0.25 mM ferulic acid supplied to seedlings for day 16–18 were:

   1. 11 and 10% at pH 5.5,
   2. 0% (actually 14% stimulation) and 0% (actually 12% stimulation) at pH 6.25, and
   3. 0 and 0% at pH 7.0, respectively.

The inhibition (or stimulation) of leaf area (cm^2) and dry weight (g) on day 22 as modified by 0.25 mM *p*-coumaric acid supplied to seedlings for day 16–18 were:

1. 6 and 0% at pH 5.5,
2. 0% (actually 19% stimulation) and 0% at pH 6.25, and
3. 0 and 0% at pH 7.0, respectively.

Both phenolic acids, ferulic acid (4 hydroxy-3-methoxy cinnamic acid) and *p*-coumaric acid (*p*-hydroxycinnamic acid), are similar in structure and differ only by a methoxy group. Yet even with this slight difference, the magnitude of effects on the primary sites, e.g., cell membranes, and subsequent secondary, tertiary, etc. effects (e.g., leaf growth and biomass) are of a different magnitude for the two phenolic acids, i.e., the potency of ferulic acid is greater. Both, however, appear to act on the same primary site (or sites). That they act on the same primary site (or sites) is supported by the fact that in mixtures their individual effects are additive at low concentrations and partially antagonistic at higher concentrations (see Sect. 5.3 for details). Furthermore, the inhibitory effects of both compounds on seedling leaf growth rates by short term phenolic acid treatments are reversible. Once phenolic acid solutions are replaced by nutrient solution, growth rates rebound rapidly. Recovery rates after phenolic acid treatment are also different for the two phenolic acids, i.e., more rapid for *p*-coumaric acid. The very rapid recovery may be due to a tendency for metabolites to accumulate during periods when cell expansion is inhibited (Boyer 1970; Acevedo et al. 1971). Once such cells are released from water stress (i.e., in this case phenolic acid contact is eliminated), they tend to expand very rapidly. However, prolonged or more severe water stress generated by phenolic acid treatments results in cell walls that become

too rigid for the resumption of cell expansion (Shann and Blum 1987b; Politycka 1998; Politycka et al. 2004; Politycka and Mielcarz 2007; Zanardo et al. 2009) and, therefore, also for the recovery of leaf expansion (Boyer 1970). There may be an additional explanation. Although mitosis of cucumber radicles is known to be inhibited by phenolic acids under very acidic conditions (Zhang et al. 2009), Blum and Dalton (1985) observed in a preliminary study that short term treatments with slightly acidic ferulic acid solutions resulted in an increased cell number per unit leaf area of cucumber seedlings but a reduced total leaf area. Thus, the rapid recovery after phenolic acid treatment could also have been partly due to expansion of a higher number of cells per unit area for phenolic acid treated leaves.

There are two hypotheses to explain how increasing pH can modify (i.e., reduce) the inhibition of simple phenolic acids such as ferulic and *p*-coumaric acids. Effects of simple phenolic acids occur primarily under acidic conditions. The pK_a of ferulic acid and *p*-coumaric acid both fall between 4.4 and 4.6 (Blum et al. 1999b). Under neutral and basic conditions ferulic acid and *p*-coumaric acid molecules in aqueous solutions have a negative charge and, thus, are thought not to interact and to be taken up by roots or mycorrhizae which also tend to be negatively charged (Harper and Balke 1981; Balke 1985; Blum et al. 1985b; Shann and Blum 1987a; Lehman and Blum 1999b). According to this hypothesis, root and mycorrhizal contact and uptake of phenolic acids occurs more readily in a protonated state which occurs under acidic conditions. In 2002 Ohno et al. (2002) proposed an additional hypothesis for salicylic acid (2-hydroxy benzoic acid) which has a pK_a of 2.97 and that was that decreasing pH protonates the functional –OH groups on cell walls to form positive charged $-OH_2^+$ groups. Decreasing pH could, thus, lead to an increasing electrostatic binding of salicylic acid to cell walls, an associated modification of the chemistry of cell walls, and a change in the solutions reaching root cell membranes. The actual mechanism of how such changes in cell wall chemistry may lead to increasing inhibition has not been determined (Ohno, personal communication).

Water Utilization If the inhibition and recovery of leaf expansion observed for cucumber seedlings (see Fig. 5.2) given a short term phenolic acid treatment (day 16–18) are largely driven by phenolic acids modifying the water potentials (e.g., net uptake [i.e., uptake-loss], and turgor pressure) of seedlings, then a close relationship between leaf expansion (Fig. 5.2) and water utilization (ml/cm^2/2 days) of cucumber seedlings might be expected (Fig. 5.3).

Seedling growth conditions and treatments were identical to those of Fig. 5.2. As can be observed in Fig. 5.3, the patterns of effects of ferulic acid and *p*-coumaric acid on water utilization of seedlings were indeed similar to those of absolute and relative rates of leaf expansion. In other words for both water utilization and leaf expansion:

a. inhibition increased with increasing concentrations of phenolic acids and decreasing pH,
b. inhibition by ferulic acid was greater than the inhibition by *p*-coumaric acid, and
c. recovery occurred very rapidly after the phenolic acid solutions were replaced by nutrient solution.

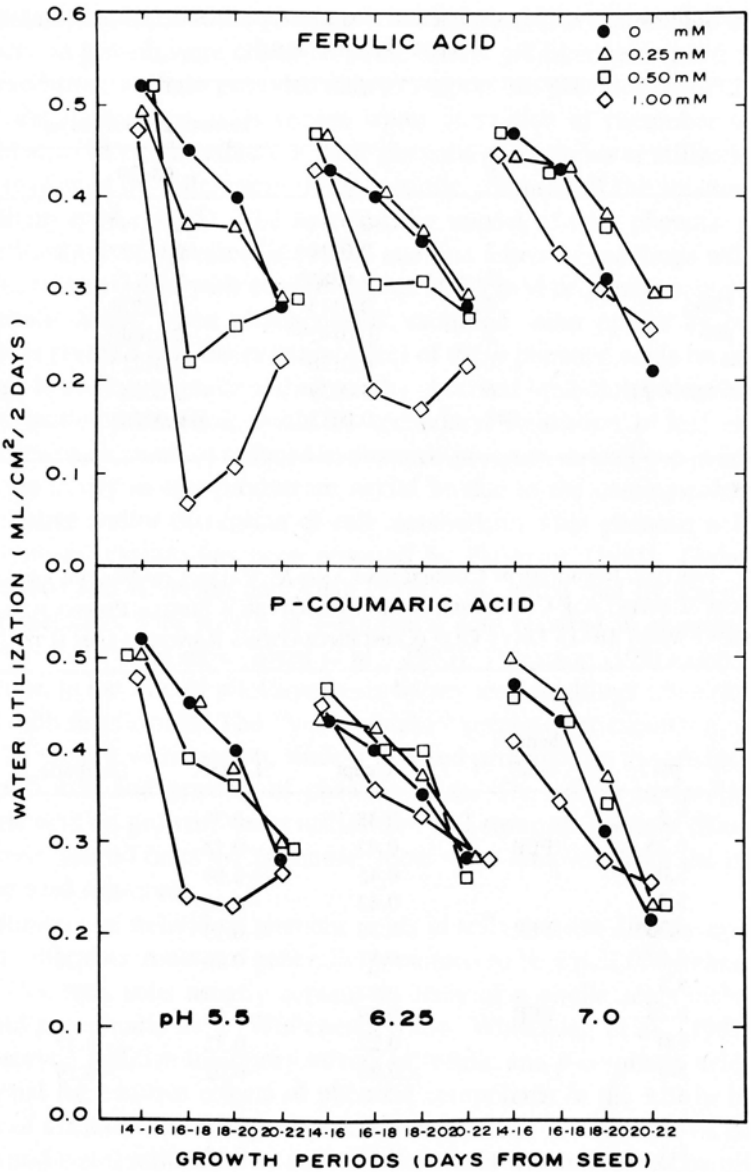


Fig. 5.3 Effects on water utilization of cucumber seedlings grown in solution culture given a single 0, 0.25, 0.5 or 1 mM ferulic acid or *p*-coumaric acid treatment on day 16. All treatment solutions were replaced on day 18 with nutrient solutions. Seedlings were grown in Hoagland's nutrient solution plus or minus phenolic acids and 5 mM MES buffer. The pH values of the initial treatment solutions were 5.5, 6.25, or 7.0 ($N=3$). Points are connected only to aid in the visualization of patterns over time. (Figure taken from Blum et al. (1985b). Plenum Publishing Corporation, figure used with permission of Springer Science and Business Media)

The close relationships of the patterns for water utilization and leaf expansion of cucumber seedlings would tend to support the hypothesis stated at the beginning of this section that phenolic acid effects on leaf growth are driven by water potentials, i.e. changes in turgor pressure. Since NaOH and Na salt was used to adjust the pH values of the phenolic acid treatment solutions (phenolic acid, full-strength Hoagland's solution, and 5 mM MES buffer), one might expect different solute potentials for the phenolic acid concentrations and pH treatment solutions. If those differences turned out to be sufficiently large enough, then the experimental design would include an unaccounted treatment factor, i.e., the solute potentials of the treatment solutions. Unfortunately the solute potentials of the treatment solutions were not determined for this experiment. However, in another study Blum and Dalton (1985) observed that the milliosmoles of a pH 5.8 full-strength Hoagland's solution, a full-strength Hoagland's solution plus 2 mM ferulic acid, and a full-strength Hoagland's solution plus polyethylene glycol (6000 mol wt) were 22, 33, and 27, respectively. Cucumber leaf expansion (day 14–16) for seedlings treated on day 14 was completely inhibited by the Hoagland's solution plus 2 mM ferulic acid. Leaf expansion for the full-strength Hoagland's solution and the Hoagland's solution plus polyethylene glycol treatments were not significantly different. These observations by Blum and Dalton (1985) suggest that solute potential of the treatment solutions may not have been a significant modifying factor.

Nutrient Uptake and Efflux Changes in nutrient uptake and losses can impact a substantial number of functions and aspects of seedlings (Hambidge 1941; Hewitt and Smith 1974; Glass 1989; van Beusichem 1990; Jones 1998). These include all kinds of catabolic and anabolic processes, cell division, water relations, nature and function of cell structures such as membranes and cell walls, and growth to name a few and thus anatomy and morphology. However, a number of these changes take some time to develop since internal available pools of nutrients, for example, must first become limiting. Changes in anatomy and morphology take even longer since they require the formation of new tissues. Here the focus is on short term effect, i.e., effects that may be observed after a single phenolic acid treatment. The following are some potential examples of such short term effects.

a. Changes in the cell content of soluble inorganic and organic compounds will modify the solute potential of cells and, therefore, their water potential (osmotic regulations/adjustments and turgor regulation; Kramer 1983; Glass 1989; Rendig and Taylor 1989).
b. Changes in ion uptake and losses (efflux) can modify the pH of the nutrient solutions surrounding roots and mycorrhizae (Rending and Taylor 1989). Proton efflux or the exchange of protons with entering cations tends to decrease the pH and uptake of anions in conjunction with an efflux of OH^- or HCO_3^- tends to increase the pH. Changes in pH of the surrounding solution can occur fairly rapidly. For example, Blum et al. (1985b) observed that 6–12-day old cucumber seedlings growing in Hoagland's nutrient solutions without buffers and an initial pH of 5.5 and 7 increased and decreased, respectively, the pH of the nutrient solutions by approximately 0.5 pH units within 48 h. In the presence of 5 mM

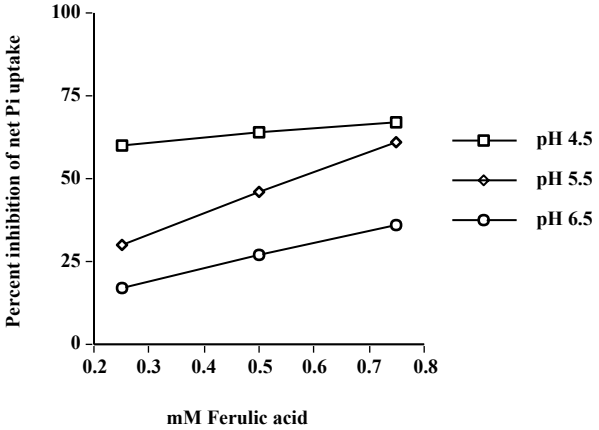


Fig. 5.4 Percent inhibition of net phosphorus (Pi) uptake by cucumber roots given a 5 h treatment of ferulic acid in solution culture when 12 days old. Treatment solutions contained ferulic acid (0.25, 0.5 or 0.75 mM), 0.5 mM $CaSO_4$, 5 mM MES buffer, and 0.5 mM KH_2PO_4. Regression models: pH 4.5 = not significant, pH 5.5 $r^2 = 0.71$, and pH 6.5 $r^2 = 0.45$. Based on figure from Lehman and Blum (1999b). (Plenum Publishing Corporation, data derived from figure used with permission of Springer Science and Business Media)

MES (2[N-morpholino]-ethanesulfonic acid) buffer these changes were on the order of < 0.1 and 0.25 pH unit for the pH 5.5 and 7 solutions, respectively.

c. Changes in ion uptake and ion losses can modify catabolic and anabolic functions of cells (see Glass 1989; Jones 1998).

Treatment of cells, root sections or seedlings with phenolic acids, such as cinnamic and benzoic acids, has been shown to modify net nutrient uptake and efflux. A net reduction in the uptake of ammonium, nitrate, potassium, and phosphorus among others have been reported in the literature (Glass 1973, 1974, 1975; Glass and Dunlop 1974; McClure et al. 1978; Harper and Balke 1981; Balke 1985; Lyu and Blum 1990; Bergmark et al. 1992; Booker et al. 1992; Yu and Matsui 1997; Lehman and Blum 1999a, b; Prasad and Devi 2002). An efflux of potassium, phosphorus, and chloride has also been reported (Glass 1973; Glass and Dunlop 1974; Booker et al. 1992; Baziramakenga et al. 1995). Both inhibition of net uptake and increasing efflux of ions by root cells have been shown to be dependent on phenolic acid concentration and pH, i.e., effects increase with increasing concentration and decreasing pH of the solution surrounding roots (Harper and Balke 1981; Lehman and Blum 1999b; Prasad and Devi 2002). Finally, effects (i.e., inhibition of uptake and increased efflux) are reversible once phenolic acid contact is eliminated (Glass 1974, 1975; Prasad and Devi 2002).

The following is an example of how modifying conditions such as pH can influence the uptake of nutrients by seedlings. Figure 5.4 describes the effects of a single 5-h ferulic acid treatment on phosphorus uptake by cucumbers seedlings (*Cucumis sativus* L., Early Green Cluster) grown in Hoagland's nutrient solution (pH 5.5; Hoagland and Arnon 1950; Lehman and Blum 1999b). Seedlings were grown in a

growth chamber with a 12 hr photoperiod (PPFD of 400 µmol/m^2/sec; NCSU Phytotron, Downs and Thomas (1991)) and a 26/22 °C day/night temperature. On day 11 after seeding, nutrient solutions were replaced with a 0.5 mM $CaSO_4$ solution. On day 12 this solution was replaced with a solution containing 0.5 mM $CaSO_4$, 5 mM MES (2[N-morpholino]-ethanesulfonic acid) buffer, 0.5 mM KH_2PO_4, and 0–0.75 mM ferulic acid adjusted to three different initial pH values (4.5, 5.5, or 6.5). Treatment solutions were aerated. Phosphorus depletion from the treatment solutions was determined after 5 h. For additional details see Lehman and Blum (1999b).

The net phosphorus (Pi) uptake (4.29+0.15 mg/g root dry weight) for the reference solution (0 mM ferulic aid) was not significantly different for the 4.5, 5.5 and 6.5 pH treatments (Lehman and Blum 1999b). The regression model for 0.25, 0.5, and 0.75 mM ferulic acid on the inhibition of net Pi uptake was not significant for the pH 4.5 treatment. At that pH all three concentrations inhibited net uptake of Pi by approximately the same amount. For example inhibition at 0.5 mM ferulic acid for the pH 4.5 treatment was 69.10±5.69%. Regression models, however, were significant for the 5.5 and 6.5 pH treatments. For both models inhibition increased in a linear manner with increasing ferulic acid concentration. Inhibition for the 5.5 pH treatment ranged from 27% at 0.25 mM to 60% at 0.75 mM ferulic acid. Inhibition for the 6.5 pH treatment ranged from 16% at 0.25 mM ferulic acid to 35% at 0.75 mM ferulic acid. Percent inhibition for the three pH treatments declined with increasing pH. For example for 0.5 mM ferulic acid the inhibition was 69.10±5.69% at pH 4.5, 46.76±4.25% at pH 5.5, and 28.61±5.21% at pH 6.5.

The most commonly cited hypothesis for the way in which increasing pH may reduce the inhibition of ferulic acid is as follows. Effects of simple phenolic acids occur primarily under acidic conditions. The pK_a of ferulic acid is approximately 4.5 (Blum et al. 1999b). Under neutral and basic conditions ferulic acid has a negative charge and, thus, is thought not to interact and be taken up by roots or mycorrhizae which also tend to be negatively charged (Harper and Balke 1981; Balke 1985; Blum et al. 1985b; Shann and Blum 1987a; Lehman and Blum 1999b). According to this hypothesis, the root and mycorrhizal contact and uptake of ferulic acids occurs more readily in a protonated state which occurs under acidic condition. At pH 4.5 50% of the ferulic acid molecules were protonated, at pH 5.5 10% of the ferulic acid molecules were protonated, and at pH 6.5 only 1% of the ferulic acid molecules were protonated. This may also suggest an explanation for why there was an identical percent inhibition of net Pi uptake (69%) at pH 4.5 for all the ferulic acid concentrations used. At pH 4.5 all the primary sites for ferulic acid were saturated even at the lowest concentration used. For an additional hypothesis on the way pH may modify the inhibition of phenolic acids, see the previous section on leaf expansion.

Cell Processes As stated previously once phenolic acids are taken up by root cells, they can be transported to the rest of the seedling or older plant and, given sufficient concentrations and an appropriate environment, can directly or indirectly impact cell processes such as lignification of cell walls, cell division, photosynthesis, and

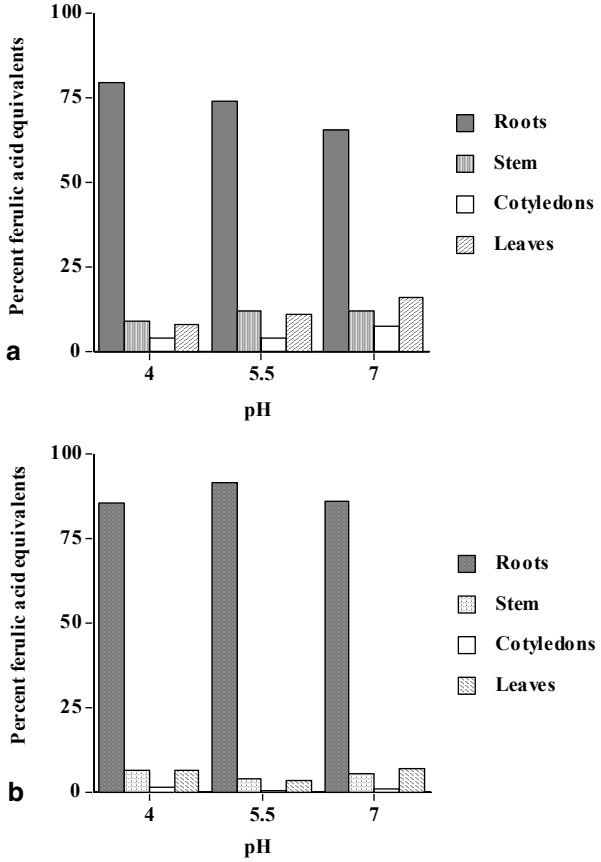


Fig. 5.5 Percent distribution of total ferulic acid equivalents in cucumber seedlings exposed for 5 h to 5 mM plus [U-ring ^{14}C] labeled ferulic acid. Equivalents were based on the specific activity of the treatment solutions. For details regarding treatment solutions see text. Mean seedling dry weights for 8 day-old seedlings were (**a**) roots = 30 mg, stem = 20 mg, cotyledons = 40 mg, and leaves = 110 mg and for 18 day old seedlings (**b**) roots = 90 mg, stem = 50 mg, cotyledons = 50 mg, and leaves = 200 mg. (Figures based on data from Shann and Blum (1987b). Pergamom Journals Ltd., data used with permission of Elsevier B.V.)

respiration (see Sect. 5.2.2). The author is not aware of studies on how the effects of phenolic acid on internal cell processes may be regulated by modifiers such as pH. It has been hypothesized that cell membrane contact and cell uptake of phenolic acids takes place more readily under acidic conditions when phenolic acids are protonated. Such pH related uptake of phenolic acids has been observed for root segments and seedlings (Harper and Balke 1981; Shann and Blum 1987a). However, for seedlings a substantial portion of this uptake is retained in the roots and only a small portion is distributed to the shoots (Fig. 5.5; Shann and Blum 1987b). Thus, direct and indirect effects of phenolic acids at the cellular level are more likely to occur in roots than in shoots.

For Fig. 5.5 cucumber seedlings were grown in aerated Hoagland's nutrient solution under a light bank (Photosynthetic Photo Flux Density (PPFD) of 176 μmol/m^2/sec) at room temperature (21–30 °C) with a 12 hr photoperiod. Seedling treatment solutions consisted of Hoagland's nutrient solution, 5 mM MES buffer, 0.5 mM ferulic acid, and approximately 0.001 μCi/ml of [U-ring-^{14}C] labeled ferulic acid. Treatment solutions were adjusted to pH 4.0, 5.5, or 7.0. Seedlings were treated for 5 h when they were 8 or 18 days old. For additional details see Shann and Blum (1987b).

All indications are that a sizable portion of the phenolic acids reaching seedling roots ended up being bound by various types of bonds to cell walls of the roots (Ohno et al. 2002; also see Sect. 5.2.2). Upon contacting cell membranes, phenolic acids depolarize the membranes (Glass and Dunlop 1974) leading to a whole host of secondary, tertiary, etc. effects (see Sect. 5.2.2). Those molecules that cross root cell membranes and enter the cytoplasm are rapidly detoxified (e.g., conjugated with glucose and amino acids, utilized in lignin synthesis, and metabolized) or stored in vacuoles as glycosides. The remainder are distributed throughout the plant where they can interact with organelles such as mitochondria and chloroplasts and modify various cell functions (Harborne 1982; see Sect. 5.2.2). For example Shann and Blum (1987b) observed that 20, 13, and 3 % of the radioactivity present in root, shoot, and leaf tissue, respectively, was in the form of free ferulic acid when cucumber seedling roots were exposed to [U-ring-^{14}C] ferulic acid. In the process phenolic acids encounter a varied range of pH environments:

a. The pH values of the apoplast (area between cells) of both roots and leaves are typically acidic, ranging from 4.8 to 5.5 for roots and 4.6–6.7 for leaves (Felle 2001).
b. The pH values of the cytoplasm are slightly alkaline (pH of 7.1–7.6) and fairly stable (Kurkdjian and Guern 1989; Felle 1988, 2001).
c. The pH values of vacuoles tend typically to be acidic ranging from 4.6 to 5.9 but the pH values of vacuoles can also be highly variable (Felle 1988; Kurkdjian and Guern 1989). For example CAM plants range from 6 in the light and 3.5 in the dark (Lüttge and Ball 1979; Lüttge et al. 1981).
d. The pH values for chloroplasts in light are typically 8 in the stroma and 4–5 inside the thylakoids (Kurkdjian and Guern 1989).

Furthermore, pH values at these various locations can change with ion uptake, anaerobic stress, drought, etc. (Sakano et al. 1992; Felle 1988, 2001, 2005; Gout et al. 2001). However with the exception of the apoplast and vacuoles, changes in pH usually tend to be less than 1 pH unit. In spite of that, such fluctuations are extremely important to cell processes since even small pH fluctuations can function as signals or messengers for changes in light intensity, drought, lack of oxygen, the presence of symbiotic partners, or microbial attack (Felle 2001). How or if phenolic acids (i.e., negatively charged at neutral and alkaline pH values) can influence cytoplasmic pH has not been determined. The rapid detoxification of phenolic acids and short term pH regulation by the cytoplasm (e.g., physicochemical buffering, metabolic adjustments, and proton export) suggest that the impact of phenolic acids

on cytoplasm pH is very likely to be small although possibly still important (Felle 1987, 1988, 2001).

In summary, what basic insight does the literature provide us regarding cells, pH, and phenolic acids?

a. Inhibition of net uptake of nutrient ions, phenolic acids, and water and the efflux (losses) of nutrient ions by roots increased as the pH of the phenolic acid treatment solution was made more acidic (Harper and Balke 1981; Balke 1985; Shann and Blum 1987a; Booker et al. 1992).
b. Inhibition of net phosphorus uptake by phenolic acids was more frequently and more consistently related to the concentrations contacting roots (i.e., initial concentrations in which roots were immersed) than the uptake of phenolic acids (based on depletion from solution; Lehman and Blum 1999b).
c. Cell membrane depolarization by phenolic acids occurred under neutral to slightly alkaline conditions (pH 7.2; Glass 1974).
d. Once phenolic acids enter the cytoplasm, they may be rapidly detoxified (e.g., conjugated with glucose and amino acids, utilized in lignin synthesis, and metabolized) and stored in vacuoles as glycosides (Harborne 1982)
e. The activities of a variety of cell enzymes (e.g., β-glucosidase; peroxidase, phenylalanine ammonium-lyase,) were modified (increased or decreased) by phenolic acids under a range of pH (6–8) conditions (Shann and Blum 1987b; Politycka 1998; Zanardo et al. 2009; Li et al. 2010).
f. Phenolic acids (no pH values provided) change root cell processes such as down-regulation of cell cycle-related genes, endoreduplication, and associated blockage of mitosis (Zhang et al. 2009). (Note: The phenolic acids were added to distilled water. Therfore, pH values of these solutions would have been very acidic, i.e., somewhere between 3 and 4.)
g. CO_2-dependent oxygen evolution was inhibited in chloroplasts and electron transport was inhibited in mitochondria and chloroplasts when these organelles were placed in a solution containing inhibitory concentrations of phenolic acids at neutral to slightly alkaline pH (Moreland and Huber 1979; Moreland and Novitsky 1987).
h. Oxidation of malate, succinate, and NADH by mitochondria was inhibited and this inhibition appeared to be the result of an alteration and perturbation of the inner membrane of mitochondria when mitochondria were placed in a solution containing inhibitory concentrations of phenolic acids at neutral to slightly alkaline pH (Moreland and Novitsky 1987).

The bottom line is that effects of phenolic acids on and in cells can occur under a broad range of pH values, i.e., from acidic to slightly alkaline. However, that still leaves a lot of questions unanswered regarding how and in what state phenolic acids enter root cells, the role of cell walls in root membrane contact, the state of active phenolic acids in the cytoplasm and cell organelles, the types of potential direct effects of phenolic acids once they enter a cell, and under what conditions stimulatory or inhibitory concentrations of phenolic acids are transported in the xylem to shoot cells. Clearly this is an area that deserves a lot more attention.

Role of Solute Potential

Solute potential is a modifying factor that has frequently been neglected in bioassays. When attempts have been made to determine the effects of solute potential, particularly for plant extracts, it was found that allelopathic effects were at times overestimated (Stowe 1979; Wardle et al. 1992). Mannitol (Bell 1974; McPherson et al. 1971; Chou and Chung 1974; Stowe 1979; Duke et al. 1983; Leather 1983; Wardle et al 1992, 1993; Hu and Jones 1997), sucrose (Bell 1974), polyethylene glycol (Blum et al. 1992; Haugland and Brandsaeter 1996; Hu and Jones 1997), inorganic compounds such as $NaNO_3$ and KCl (Bell 1974); or Hoagland's nutrient (Bell 1974; Blum et al. 1992) solutions with a range of solute potentials have been used to determine and predict the effects of solute potential on seeds or seedlings (e.g., use 1; determined and predicted values were added to control or reference values), used to adjust the solute potential of control or reference solutions (use 2), or used in factorial bioassays to determine effects and potentially interactive effects of solute potential and phenolic acids (use 3). The first two uses assume that there are no interactions between the actions of the allelopathic agents (e.g., compounds, extracts, leachates, etc.) and the solute potentials. This assumption is generally supported by the observations of Duke et al. (1983) for the inhibition of lettuce seed germination and Blum et al. (1992) for the inhibition of radicle and hypocotyl length of clover. Duke et al. found no significant interactions for the effects (actions) of mannitol and phenolic acids. In fact they concluded based on probit analysis that the modes of action for the two were similar. Blum et al. (1992) found that the inhibitory effects of wheat soil extracts on clover radicle and hypocotyl lengths could be explained by either solute potential or total phenolic acid content of the soil extracts. They observed no significant interactions between the effects (actions) of the two treatments. They also did not observe interactions for the effects (actions) of a dilution series of a mixture of phenolic acids and solute potentials generated by different strengths of Hoagland's solution (see Fig. 5.6). Interactions were observed, however, when polyethylene glycol was substituted for Hoagland's solution as the osmoticum. As the milliosmoles of the polyethylene glycol solution increased, the effects (actions) of the phenolic acid mixture on radicle and hypocotyl lengths of clover declined (see Fig. 5.6). The reasons for this interaction are not clear but may be related to molecular weight of the polyethylene glycol used (6000) or impurities within the polyethylene glycol solutions (Lawlor 1970; McClendon 1981; Steuter et al. 1981; Plaut and Federman 1985).

For Fig. 5.6 Twenty clover seeds (*Trifolium incarnatum* L.) inoculated with *Rhizobium* were germinated in 9-cm-diameter Petri dishes containing Whatman No. 1 filter paper and 5 ml of the solution (pH 5.0) to be tested (Blum et al. 1992). Petri dishes were incubated at 30 °C for 48 h in the dark before radicle and hypocotyl lengths were determined. The phenolic acid solution mixture used was composed of 7 phenolic acids at concentration ratios observed in soil extracts from no-till wheat/soybean plots. The range of phenolic acid concentrations for the bioassay was created by a dilution series. Polyethylene glycol 6000 and various strengths of Hoagland's solution were used to create the different milliosmoles (i.e., solute

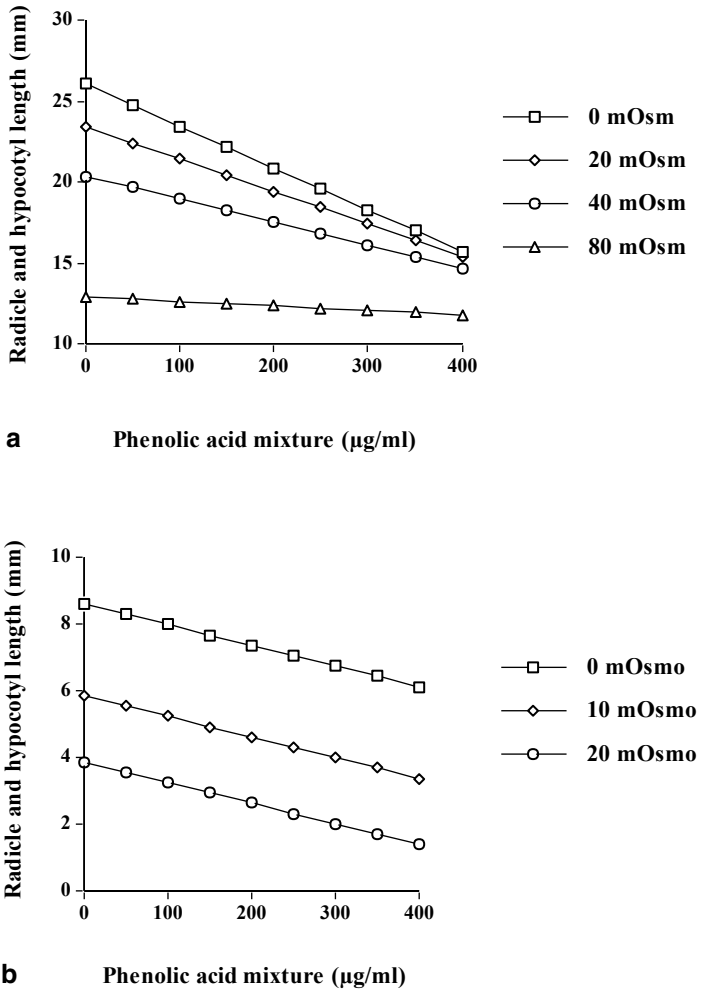


Fig. 5.6 Effects of a 7-phenolic acid mixture modeled after phenolic acids found in no-till wheat-soybean soil extracts (pH 5) on radicle and hypocotyl lengths of crimson clover as modified by polyethylene glycol (a; $r^2 = 0.61$) and Hoagland's solution (b; $r^2 = 0.37$) based on freezing-point depression (milliosmoles, mOsm) of solutions. The 7-phenolic acid mixture was composed of 10 % caffeic acid, 9 % ferulic acid, 35 % *p*-coumaric acid, 15 % *p*-hydroxybenzoic acid, 4 % sinapic acid, 10 % syringic acid, and 17 % vanillic acid. Figures originally based on regressions from Blum et al. (1992). Plenum Publishing Corporation, regressions used with permission of Springer Science and Business Media. (Figure taken from Blum (2011), figure used with permission of Springer Science and Business Media)

potentials) for the solutions. For additional details see Blum et al. (1992). The mode of action by which increasing concentrations of polyethylene glycol and phenolic acids interacted was not determined. What role individual nutrients in the Hoagland's nutrient solution might have had was also not determined. Given the observations by Duke et al. (1983) for mannitol and Blum et al. (1992) for Hoagland's

solution and soil extracts (i.e., no interactions), the interactions observed for polyethylene glycol may not be entirely representative of what occurs in field systems. Clearly the role of solute potential as a modifier of the actions of promoters, inhibitors, or as an independent factor in bioassays deserves much more attention.

For basic information on the use of osmotica see the following references: mannitol (CRC Handbook of Chemistry and Physics under Concentrative Properties of Aqueous Solutions; recent versions have values for freezing-point depression while older versions have values for freezing-point depression and osmolality), sucrose (Bell 1974), polyethylene glycol solutions (Lawlor 1970; McClendon 1981; Steuter et al. 1981; Plaut and Federman 1985), $NaNO_3$ and KCl (Bell 1974), NaCl (Lang 1967), and Hoagland's nutrient solution (Bell 1974; Blum et al. 1992). Mannitol and sucrose are best used under sterile conditions.

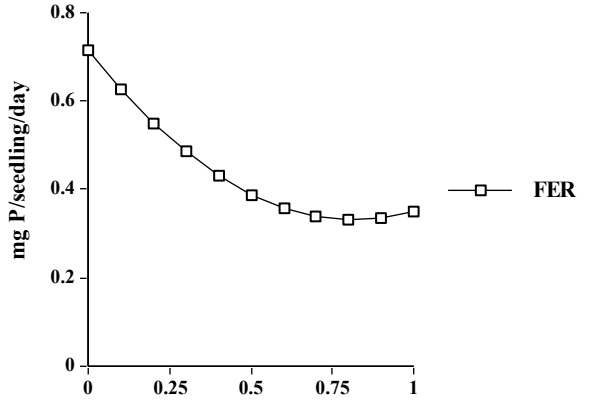
Role of Treatment Surface Area

The surface areas of roots and mycorrhizae are not really modifiers but the distribution (or placement) of roots and mycorrhizae in relationship to the distribution (or placement) of allelopathic compounds (i.e., promoters and inhibitors) within a container or among containers (e.g., split-root technique) will determine the magnitude of effects observed. Therefore, distribution or placement of roots, mycorrhizae, and allelopathic compounds within a container or among containers of a bioassay system functions as the actual modifier.

The proportion of root surface area that is in contact with phenolic acids determines the magnitude of inhibition observed (Klein and Blum 1990a; Lyu and Blum 1990; Lehman et al 1994; Lehman and Blum 1999a). For example, Fig. 5.7 describes the effects of 0.5 mM ferulic acid or 0.5 mM *p*-coumaric acid on net phosphorus uptake, net water uptake, and absolute rates of leaf expansion of cucumber seedlings with their root systems split between two containers. Seedlings were grown and treated under light banks with a 13 or 12 h photoperiod (Photosynthetic Photo Flux Density (PPFD) of 150 or 140 μmol/m^2/sec) at room temperature (21–30 °C). Prior to treatment, seedlings were grown in Hoagland's nutrient solution. Seedling treatments were initiated when seedlings were 15 (Lyu and Blum 1990) and 13 days old (Lehman et al. 1994). Data were collected 24 h after treatment. For additional details see references and Sect. 6.5.

For net phosphorus uptake and water utilization (Lyu and Blum 1990) roots were aerated and 24 h prior to initiating phenolic acid treatments the Hoagland's solutions were replaced with 0.5 mM $CaSO_4$ solutions. Treatment combinations used were:

a. One container was filled with 100 ml of 0.5 mM ferulic acid, KH_2PO_4, and $CaSO_4$ (pH 5.5) and the other with 100 ml of 0.5 mM KH_2PO_4 and $CaSO_4$ (pH 5.5). Roots were split between containers (a range of root contact with ferulic acid).



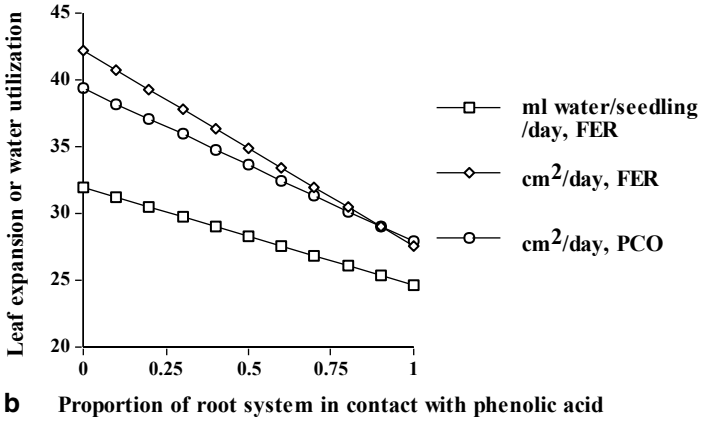


Fig. 5.7 Changes in net phosphorus uptake (**a**; $r^2 = 0.52$), net water uptake (**b**; $r^2 = 0.19$), and absolute growth rates of leaf expansion (**b**; r^2 for FER = 0.76 and PCO = 0.58) of 13–15 day-old cucumber seedlings grown in nutrient culture as the proportion of the root systems in contact with a phenolic acid was increased, where FER equals 0.5 mM ferulic acid and PCO equals 0.5 mM *p*-coumaric acid. Figures originally based on regressions from Lyu and Blum (1990; **a**, net phosphorus uptake; **b**, water utilization) and Lehman et al. (1994; **b**, leaf expansion). Plenum Publishing Corporation, regressions used with permission of Springer Science and Business Media. (Figure taken from Blum (2011), figure used with permission of Springer Science and Business Media)

b. Both containers were filled with 100 ml of 0.5 mM KH_2PO_4 and $CaSO_4$ (pH 5.5). Roots were split between containers (no root contact with ferulic acid).
c. Both containers were filled with 100 ml of 0.5 mM ferulic acid, KH_2PO_4, and $CaSO_4$ (pH 5.5). Roots were split between containers (100% root contact with ferulic acid).

For leaf expansion (Lehman et al. 1994) the following combinations used were:

a. One container was filled with 100 ml of full-strength Hoagland's solution plus 0.2–0.8 mM ferulic acid or *p*-coumaric acid (pH 5.5) and the other with 100 ml of full-strength Hoagland's solution (pH 5.5.). Roots were split between containers (a range of root contact with ferulic acid or *p*-coumaric acid).
b. One container was filled with 100 ml of full-strength Hoagland's solution (pH 5.5). Roots had no contact with ferulic acid or *p*-coumaric acid.
c. One container was filled with 100 ml full-strength Hoagland's solution plus 0.2–0.8 mM ferulic acid or *p*-coumaric acid (pH 5.5). Roots had 100 % contact with ferulic acid or *p*-coumaric acid.

For both b. and c. only one container was used since the nutrients supplied by the 100 ml of Hoagland's solution were more than adequate for normal growth (Blum and Dalton 1985). Preliminary experiments had shown that the use of one to two containers did not modify leaf area expansion over the 24-h time interval.

Net phosphorus uptake, net water uptake, and absolute rates of leaf expansion of cucumber seedlings all declined (were increasingly inhibited) as the percentage of root systems treated with phenolic acid was increased. Increasing inhibition with increasing percent root system contact suggests that effects of simple phenolic acids are local, and not systemic, in nature. When we add that the concentration of ferulic acid in solutions surrounding roots (i.e., contact) was more frequently and consistently related to the inhibition of net phosphorus uptake than the uptake of ferulic acid by roots (Lehman and Blum 1999b) and that one of the major primary sites of action for phenolic acids are root cell membranes (Glass 1973, 1974; Glass and Dunlop 1974; Balke 1985; Baziramakenga et al. 1995), we may safely conclude that root contact (very likely root membrane contact) is largely responsible for the short term inhibitory effects and very likely responsible for a large part of the subsequent long term inhibitory effects of phenolic acids.

Role of Microorganisms

A range of microorganisms (i.e., bacteria, fungi, and actinomycetes; also see Sect. 3.7) associated with seeds, roots, and mycorrhizae can readily transform phenolic acids and utilize phenolic acids as a carbon source as long as other environmental resources are not limiting (Evans 1963; Gibson 1968; Dagley 1971; Martin and Haider 1976; Hartley and Whitehead 1985; Blum and Shafer 1988; Blum 1998, 2004; Blum et al. 1999a;). For example, ferulic acid is initially transformed to caffeic acid and vanillic acid and then to protocatechuic acid before eventually being broken down to CO_2 and water (Evans 1963; Dagley 1971; Martin and Haider 1976). The initial breakdown product of *p*-coumaric acid is *p*-hydroxybenzoic acid (Blum 1998). Preliminary data suggest that broad-spectrum transformation and utilization of phenolic acids by phenolic acid-utilizing microorganisms may be more common (Blum et al. 2000) than species specific microbial transformation and utilization of

phenolic acids. All of these studies have been carried out with microorganisms from pure cultures or from soil samples.

The source of microbial populations in solution-culture systems is primarily from seed or root surfaces unless the culture system has been inoculated with microorganisms. Therefore, the diversity and the initial number of microorganisms are much smaller than found in most soils (see Sects. 3.6 and 3.7). Upon phenolic acid treatment, microbial diversity will very likely decline even further but the total number of phenolic acid-utilizing microorganisms will increase dramatically over time (Note: This has been observed for both solution-culture and soil-culture systems (Blum and Shafer 1988; Blum et al. 1999a, b, 2000; Blum 2011)). The activities or actions of these microbial populations will lead to a number of modifications for treatment solutions and, thus, modifications for the effects of phenolic acids on seed germination and seedling growth. For example:

a. the concentration of the phenolic acid applied to the system (treatment) will decline over time,
b. microbial transformation will produce, at least initially, other phenolic acids (e.g., ferulic acid to vanillic or caffeic acid),
c. microbial breakdown products other than phenolic acids will be produced,
d. microbial breakdown products can accumulate or subsequently be utilized to produce other breakdown products,
e. breakdown products may be more or less inhibitory than the original phenolic acid,
f. utilization of phenolic acids by microorganisms can lead to the production of unique compounds such as antibiotics, etc. which can be more or less inhibitory than the original phenolic acid,
g. the creation of mixtures of phenolic acids and other carbon compounds can lead to differential utilization of carbon sources by the microorganisms (i.e., more readily available organic compounds will be utilized first),
h. the actions or activities of microorganisms can lead to the production of an organic promoter/modifier/inhibitor complex within the treatment solution,
i. the pH of the solution culture will change rapidly unless it is highly buffered (Note: Non-toxic buffers only slow the rate of change.),
j. available oxygen in the solution culture will decline unless the solution culture is aerated, and
k. nutrients such as nitrogen may become limiting for seedling growth.

Many of the changes for treatment solutions, however, are not entirely due to the action of microorganisms alone since seeds, seedling roots, and mycorrhizae can also bring about some of these same changes (e.g., decline in phenolic acid concentration due to uptake of phenolic acids, addition of carbon sources by root and hyphal exudation, secretion, etc., modification of pH due to differential uptake and efflux of ions, and reduction of available oxygen due to respiration). The bottom line is that the presence of microorganisms adds a considerable amount of complexity to solution cultures when it comes to identifying cause and effects. In general the actions of microorganisms have been associated with a decline in inhibition

(Vaughan et al. 1983, 1993; Williamson and Weidenhamer 1990; Blum 1998; Blum et al. 2000) and, given sufficient time, the recovery of inhibited seed, seedling, and older plant processes (see Blum et al. 1984; Blum and Dalton 1985; Blum and Gerig 2005; Blum 2011).

In addition to the functions just described for solution cultures, active microorganisms in substrates (e.g., soil) have an additional range of functions which also modify the effects of allelopathic compounds (i.e., promoters and inhibitors). Among them are:

a. decomposition of plant residues and release of organic compounds including potential allelopathic compounds and inorganic compounds,
b. formation of soil aggregates that modify substrate aeration and mobility of water, organic compounds including allelopathic compounds, and inorganic compounds including nutrients,
c. carbon and mineral cycling,
d. formation of symbiotic relationships, and
e. diseases (see Blum 1998, 2004, 2006; Demain and Fang 2000; see Sect. 3.7.5 for additional references).

There are, therefore, a large number of ways by which microorganisms in solutions and substrate culture can modify and regulate plant-plant allelopathic interactions. Accounting for all these actions is difficult but achievable for at least some of them (see Blum 1998, 2004, 2006; Blum et al. 1999a).

Some Additional Modifiers

In addition to pH, solute potential, treatment surface area (actually distribution of roots and allelopathic compounds), and microorganisms, there are a number of other modifiers that have been identified which will increase or decrease the inhibition of growth, nutrient uptake and efflux, water uptake, and transpiration by phenolic acids. The following are some examples:

a. Pretreatment of cucumber seedlings with phenolic acids, drought stress, or nutrient stress before treating seedlings with inhibitory concentrations of phenolic acids reduced or eliminated the inhibitory effects of phenolic acid on net phosphorus uptake suggesting that pretreatment stresses can increase the tolerance of seedlings to phenolic acids (pH 5.5; Lehman and Blum 1999a).
b. Inhibition of sorghum and soybean seedlings by phenolic acids varied with the temperature of the growth environment. Greater weight reductions were observed at 37 °C than at 29 °C for sorghum and at 34 °C than at 18 °C for soybean (Einhelling and Eckrich 1984).
c. Inhibition of barley seedling dry weight by phenolic acids was modified by nutrient concentration when seedlings were grown in sand culture (essentially a solution culture since sand is inert). The greatest and most consistent inhibition occurred at low N and P concentrations. For example, 50 ppm of vanillic acid in

complete nutrient solution had no effect but inhibited dry weight by 38 % when phosphorus was reduced to 1 ppm (Stowe and Osborn 1980).

d. The modification by seedling roots of active phenolic acid concentrations, pH, microbial populations, etc. surrounding roots suggests that seedling roots can directly influence the magnitude of primary and secondary, tertiary, etc. effects of phenolic acids through feedback regulation (Blum and Gerig 2005).

There are obviously many more modifiers. For some additional modifiers, see Rice (1984), Einhellig (1987, 1989, and 1999), Pedrol et al. (2006), and Gawronska and Golisz (2006). What needs to be said here is that simple dose-response studies in the laboratory, although extremely useful in defining and characterizing potential causes and effects, can provide only a very limited perspective, if that, about what is actually happening under field conditions on an hour by hour or day by day basis.

5.2.3.2 Multiple Treatments—Solution Culture

Uptake by seedlings and microbial utilization rapidly deplete phenolic acids in solution culture (Shann and Blum 1987a; Lehman and Blum 1999b; Blum and Gerig 2005; Blum 2011). For example, a 25 % depletion of *p*-coumaric acid by 13 day-old cucumber seedlings growing in nutrient culture (initial pH 5.0 and a volume of 110 ml) took 8.5, 12, 19.5, 25, and 29.5 hr for 0.125, 0.25, 0.5, 0.75, and 1 mM treatments, respectively (Blum and Gerig 2005). The authors estimated that for every 0.1 mM decline of *p*-coumaric acid there was a 12–14 % decline in the inhibition (i.e., recovery) of leaf area expansion of 12-day old cucumber seedlings (pH 5.0; also Sect. 5.2.3.1). Therefore, continuous or frequent additions of phenolic acids to solution cultures are required to maintain the initial treatment concentrations. Unless such additions are made, the potential effects of a given treatment concentration will be underestimated. Underestimates would be the greatest for the lowest concentrations and would decrease with increasing concentrations. However, since the decline of phenolic acids in nutrient culture is frequently not predictable, it is easier to make complete solution changes at given time intervals instead of direct additions of phenolic acids to solution cultures. An additional benefit of changing solutions is that nutrients, pH values, and solute potentials for the solution culture system are brought back to their initial states and microbial populations are kept in check. The bottom line is that multiple treatments can reduce or minimize the impact of some of the inherent, but confounding, modifying factors of laboratory bioassay systems.

Another benefit of multiple treatments, if done correctly, is that the experimental period can be extended. Seedling development now becomes a modifying factor that must be accounted for when evaluating concentration effects. For example, cucumber seedlings were grown in Hoagland's nutrient solution and treated with 0–1 mM ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, or vanillic acid (initial solution pH 5.0; no MES buffer) every other day starting with day 6 and ending with day 10 (Blum and Gerig 2005). Growth conditions and water additions were

Table 5.1 Relative potencies and standard errors for effects of *p*-coumaric acid, *p*-hydroxybenzoic acid, and vanillic acid compared to ferulic acid for transpiration, water utilization, leaf area, absolute and relative rates of leaf expansion, and shoot and root dry weights of cucumber seedlings growing in nutrient culture (pH 5.2)[a]

Variables	Ferulic acid[b]	*p*-Coumaric acid[b]	Vanillic acid	*p*-Hydroxybenzoic acid
Transpiration	1.000±0.000	1.004±0.085	0.415±0.063	0.331±0.059
Water utilization	1.000±0.000	0.778±0.060	0.318±0.026	0.212±0.022
Leaf area	1.000±0.000	0.760±0.048	0.335±0.021	0.173±0.016
Absolute rates of leaf expansion	1.000±0.000	0.826±0.076	0.332±0.033	0.181±0.027
Relative rates of leaf expansion	1.000±0.000	0.823±0.079	0.314±0.049	0.100±0.042
Shoot dry weight	1.000±0.000	0.804±0.180	0.390±0.084	0.228±0.064
Root dry weight	1.000±0.000	0.978±0.186	0.340±0.181	0.298±0.165

[a] Seedlings were treated to phenolic acid (0–1 mM) on day 6, 8, and 10. Dry weights were determined on day 12. Relative potencies and standard errors are from Blum and Gerig (2005). Data used with permission of Springer Science Business Media

[b] Initial breakdown products of ferulic acid and *p*-coumaric acid (cinnamic acid derivatives) are vanillic acid and *p*-hydroxybenzoic acid (benzoic acid derivatives), respectively (Evans 1963; Dagley 1971; Martin and Haider 1976; Blum 1998)

similar to those of the experiment in Sect. 5.2.3.1 under leaf expansion with the exception that treatment solutions were completely changed every other day starting with day 6. On day 12 seedlings were harvested to determine root and shoot dry weights. For additional details see Blum and Gerig (2005). The effects of the three phenolic acids were similar, only the magnitudes of their effects were different. The cinnamic acid derivatives, ferulic acid and *p*-coumaric acid, were more inhibitory to cucumber seedlings than the benzoic acid derivatives, vanillic acid and *p*-hydroxybenzoic acid. The average percent inhibitions of *p*-coumaric acid, vanillic acid, and *p*-hydroxybenzoic acid were approximately 85, 35, and 22 %, respectively, of ferulic acid (Table 5.1). To simplify matters only data for *p*-coumaric acid are provided here (see Fig. 5.8, 5.9, and 5.10) because *p*-coumaric acid was found to be the dominant phenolic acid in wheat residues and the soil from no-till wheat/soybean systems (see Blum 2011).

Increasing concentrations of *p*-coumaric acid led to increasing percent inhibition for all variables measured including dry weights on day 12 (Fig. 5.8 and 5.10). The magnitude of percent inhibition, however, did vary over time for some variables. Although actual transpiration and water utilization varied (see reference treatment; Fig. 5.9), the values for percent inhibition over the range of *p*-coumaric acid concentrations tested were fairly consistent over time (Fig. 5.8). Variation in transpiration and water utilization is to be expected since the temperature and relative humidity under the light banks in the laboratory varied over time. Total seedling leaf area increased in a linear manner (see reference treatment; Fig. 5.9). Percent inhibition of leaf area, however, took approximately four days to fully develop and stabilize (Fig. 5.8). Some variation in the absolute rates of leaf expansion (cm^2/seedling/unit time) for seedlings is also to be expected (see reference treatment; Fig. 5.9) since the rates are determined by the timing of the formation of individual leaf primordia,

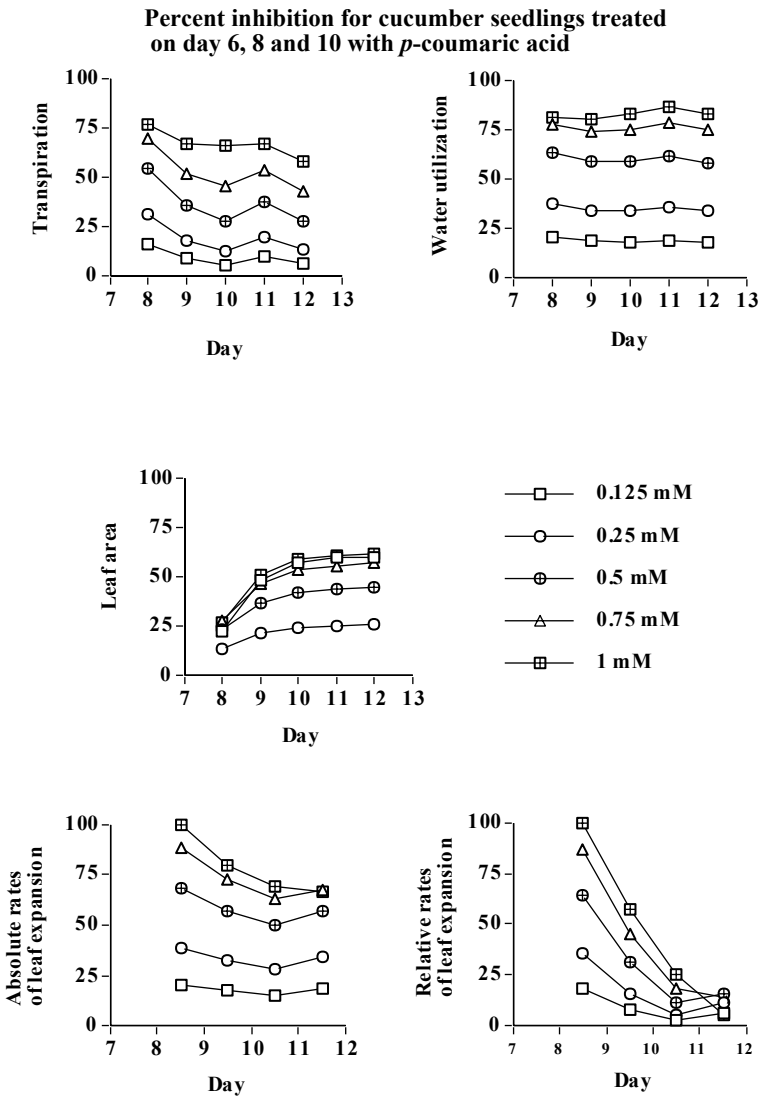


Fig. 5.8 Percent inhibition of *p*-coumaric acid treatments (day 6, 8, and 10) on transpiration, water utilization, leaf area, and absolute and relative rates of leaf expansion of cucumber seedlings growing in nutrient culture (pH 5.0). The reference (or baseline) values for untreated seedlings are presented in Fig. 5.9. (Figures taken from Blum and Gerig (2005). Figures used with permission of Springer Science and Business Media)

the expansion rates of individual leaves, and the nature of the environment. Again in spite of that, the percent inhibition for absolute rates of leaf expansion was fairly consistent over time although a decline in percent inhibition was observed at the higher treatment concentrations.

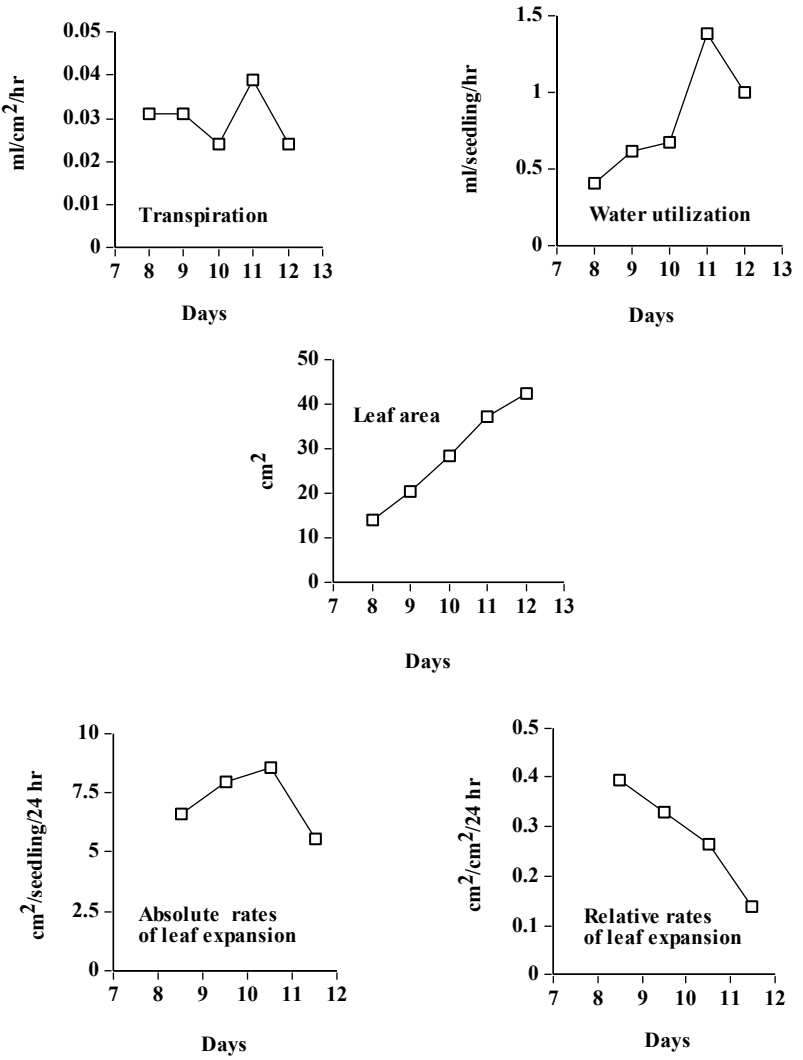


Fig. 5.9 Transpiration (ml/cm^2/hr), water utilization (ml/seedling/hr), leaf area (cm^2), and absolute (cm^2/seedling/24 h) and relative (cm^2/cm^2/24 h) rates of leaf expansion of cucumber seedlings growing in nutrient culture (pH 5.0) in the absence of phenolic acids. Means and standard errors in the figures were derived from parsimonious models of Blum and Gerig (2005). Absence of standard error bars indicates that error bars are smaller than the symbols representing the means. Means and standard errors used with permission of Springer Science and Business Media

Relative rates of leaf expansion normally decline over time as the ratio of non-expanding (i.e., fully expanded leaves) to expanding leaf tissues (i.e., immature leaves) increases (see reference treatment; Fig. 5.9). As a result, the percent inhibition by phenolic acids will also decline for relative rates of leaf expansion (Fig. 5.8). Shoot and root dry weights were determined only on day 12 for the cucumber seedlings (Fig. 5.10). However since shoot ($r^2 = 0.92$) and root ($r^2 = 0.62$) dry

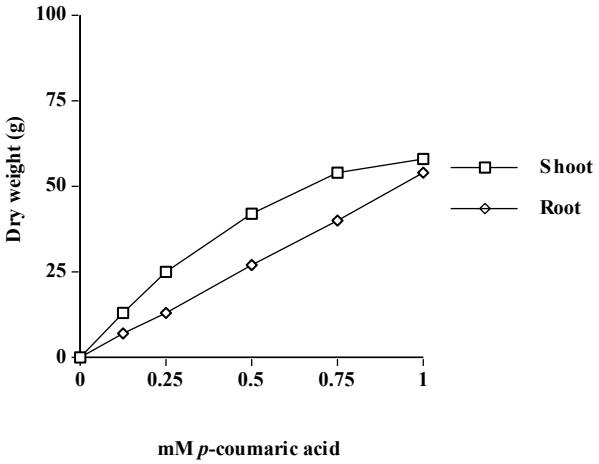


Fig. 5.10 Percent inhibition of *p*-coumaric acid treatments (day 6, 8, and 10) on shoot and root dry weights (g) of 12 day-old cucumber seedlings grown in Hoagland's nutrient culture (pH 5.0). Shoot and root dry weights of untreated seedlings were 0.123 ± 0.006 and 0.031 ± 0.002 g, respectively. Percent inhibition in figure for shoot ($r^2 = 0.66$) and root ($r^2 = 0.47$) dry weight was derived from parsimonious models of Blum and Gerig (2005). Models were used with permission of Springer Science and Business Media

weights have been shown to be significantly correlated with total leaf area (Blum and Rebbeck 1989), it would be anticipated that the pattern for percent inhibition of shoot and root dry weights for other days would be similar to that of seedling leaf area (Fig. 5.8). The bottom line is that comparisons of the magnitude of effects for a range of secondary, tertiary, etc. effects such as growth rates, water utilization, etc. must be done with care since the magnitude of effects, at least in terms of absolute values, varies not only with the environment but also with the stage of development of a seedling.

5.2.3.3 Single and Multiple Treatments—Substrate Culture

Inert substrates such as clean quartz sand generally have only a minimal impact on phenolic acid concentrations. Sand cultures function to large extent as solution cultures in that regard. Sand cultures, however, when compared to solution cultures have:

a. root and mycorrhizal systems with modified anatomy and morphology, and reduced growth rates,
b. generally greater levels of aeration and lower availability of water and nutrients,
c. reduced mobility and distribution for allelopathic compounds such as phenolic acids in the substrate, and
d. different types and populations of microorganisms within the rhizoplane, rhizosphere, mycorrhizosphere, and bulk sand.

These differences tend to reduce the observed inhibition for a given phenolic acid treatment and to slow the response time of seedlings to phenolic acid treatments. The magnitude of these differences from solution culture varies with the particle size of the sand (Barber and Gunn 1974; Mozafar 1991; Engelaar et al. 1993; Gordon et al. 1993).

Substrates such as soils composed of clays, silt, sand and organic matter, etc. have additional modifying factors and processes, particularly, reversible and fixed (non-reversible) sorption and polymerization (see Dalton 1999; Tharayil et al. 2008; Blum 2011; Sect. 1.4). For the potential range of sorption mechanisms that occur in soils see Burchill and Haynes (1981), Stevenson (1982), Sawhney and Brown (1989), and Blum (2006, 2011). Most soils also contain water-soluble organic and inorganic compounds that can directly or indirectly modify seedling responses to phenolic acids (see Sects 5.2 and 5.3; also see Tharayil et al. 2008). For a more detailed comparison of solution and soil cultures see Sect. 7.2. Essentially, substrates and their associated processes function as modifying factors that change seedling root and mycorrhizal characteristics and growth, the frequency and duration of contact of roots and mycorrhizae with phenolic acids, the concentrations required for an effect, and the response time of observed effects.

For simplicity the focus of the following discussion is on roots. How do characteristics of roots grown in sand or soil culture differ from those grown in nutrient culture and more importantly do these differences matter when it comes to the inhibitory activity of phenolic acids? Since the greatest differences are to be found between solution and soil cultures, roots from these two systems will be compared. The anatomy, morphology, growth rates, distribution, and function of roots in soil are altered by mechanical impedance (resistance) generated by soil (see Taylor 1974; Bennie 1996; also see references for sand above). In general the roots in soils are thicker, shorter, and more irregularly shaped than the thinner, longer, and regularly shaped fibrous roots found in solution culture. Lateral roots frequently are stimulated but may also be substantially reduced or absent in soils with very high mechanical impedances. Root hairs are frequently absent in solution culture. The greater the mechanical impedance of a soil, the greater is the change in root structure, growth, distribution, and function (Carson 1974; Waisel et al. 1996). In addition, roots in soil cultures are much more likely to form symbiotic relationships (e.g., nodules and mycorrhizae) than roots in solution culture adding an additional potential structural and functional difference for roots growing in soil culture (see Sects. 3.6 and 3.7).

Before we attempt to address the second part of the question, it is important to remind the reader that the treatment procedures for solution and soil systems are different. For example, complete treatment solution changes to maintain initial conditions (e.g., pH, solute potential, inorganic compounds [e.g., nutrients], phenolic acid concentrations, etc.) are not possible for soil systems. Thus, compensation for phenolic acid and nutrient depletion over time, for example, are much more complex and more difficult to achieve in soil systems. Multiple additions or continuous additions by drip irrigation of phenolic acids and nutrients must be used instead (see for example Blum et al. 1987, 1999a; Blum and Shafer 1988; Blum and

Gerig 2006). Balancing inputs with losses, particularly when losses are unknown, is extremely difficult to achieve. In addition keeping rhizoplane, rhizosphere, and bulk-soil microbial populations in check (i.e., at normal levels in relationship to field systems) under even the best of circumstances is extremely difficult to achieve and in most cases impossible to achieve without dramatically modifying the system (e.g., sterilizing system components, etc.). Finally, observed modifications and effects will vary with soil type and the overall nature of the soil system.

As far as the author is aware, there has not been a coherent attempt to answer the second part of the question. What we do know is that seedling shoots and roots and their associated microorganisms are known to adjust and acclimate to all kinds of environmental factors and situations and in the process seedlings become more or less susceptible or vulnerable to other environmental stresses including phenolic acids (Duke et al. 1983; Einhellig and Eckrich 1984; Einhellig 1987, 1989, 1999; Chapin 1991; Lehman and Blum 1999a). It is, however, not an all or nothing proposition. That means that there appears to be some theoretical threshold at which changes for shoots and roots and their microorganisms start to change their susceptibility or vulnerability to any particular environmental stress, including phenolic acids. These thresholds, however, have not been adequately defined. Clearly this is an area that deserves much more attention.

In regard to contact and concentrations, reversible and fixed sorption by mineral and organic particles in the soil, polymerization to soil particles, conversion and utilization by microorganisms, and uptake by roots all tend to modify the horizontal and vertical distributions of available phenolic acids in soil and in general reduce the concentrations of available and active phenolic acids added to soil systems, sometimes in a very dramatic fashion (Dalton et al. 1983, 1989; Blum 1998; Blum et al. 1994; Dalton 1999). Applications of phenolic acid solutions to the surface of soils can also lead to vertical concentration gradients particularly for lower treatment concentrations (Blum et al. 1987; Klein and Blum 1990b). Variation in horizontal distribution patterns can also occur if solutions are not evenly applied to the total surface area of soils. Since inhibitory effects of phenolic acids on seedlings depend on the proportion of the root system in contact with active phenolic acids (Lyu and Blum 1990; Klein and Blum 1990a; Lehman et al. 1994; Blum 2011), such modifications of available (active and inactive) phenolic acid concentrations and their distributions by soil processes necessitates higher treatment concentrations for soil cultures than solution cultures. For example, Blum (2011) calculated the *p*-coumaric acid required for a 20% inhibition of absolute rates of leaf expansion for 12-day old cucumber seedlings treated on day 6, 8, and 10 in solution and Cecil A_p soil cultures. It was concluded that a 20% inhibition in soil culture required 29 times more *p*-coumaric acid than in solution culture (based on data from Blum and Gerig 2005, 2006). In addition Blum and Gerig observed that the time required for initial detection of inhibitory effects by *p*-coumaric acid required 1 and 3 days for absolute rates of leaf expansion and evapotranspiration (primarily transpiration) in soil culture, respectively (Blum and Gerig 2006). Maximum development of inhibition required 1 and 6 additional days, respectively. Recovery after maximum expression required another 2–5 days. In nutrient culture maximum expression and

subsequent recovery required less than 4 days (Blum and Dalton 1985; Blum et al. 1985a; Blum and Rebbeck 1989).

When solution cultures were compared to soil cultures, the following was found:

a. Response times for seedling effects by phenolic acid treatments are slower in soil culture than in solution culture.
b. Concentrations of phenolic acids required for similar levels of inhibition for seedling processes are considerably greater in soil culture than in solution culture.
c. Induction and selection of phenolic acid-utilizing microorganisms is considerably greater in solution culture than in soil culture given the complexity of the rhizosphere, mycorrhizosphere, and bulk soil.
d. Mobility of phenolic acids in soil culture is much slower than in solution culture.
e. Horizontal and vertical distributions of available phenolic acids in soil culture are much more variable than in solution culture.
f. Available phenolic acid concentrations decline fairly rapidly in soil cultures and continued inhibition requires multiple or continuous additions of phenolic acids. The decline of phenolic acids in solution culture is also fairly rapid. The difference is that in solution culture the whole solution system is depleted by roots and microbes at the same time (mixing is essentially instantaneous) while the depletion in soil systems is much more heterogeneous depending on phenolic acid, root, microbial, clay, and organic matter distributions, capillary movement, transpirational pull, etc.

5.2.4 Summary Comments

What do all these observations tell us about the effects of simple phenolic acids in laboratory bioassay systems?

a. Phenolic acids may have multiple primary target sites leading to a complex of interacting plant processes, modes of action, and observable effects.
b. Actions and effects of phenolic acids are local and not systemic.
c. A frequent pattern for increasing concentrations of an individual phenolic acid is: no effect at very low concentrations, a region of stimulatory effects (e.g., hormesis), and finally a region of inhibitory effects. Other patterns are also possible.
d. Stimulatory and inhibitory effects may occur simultaneously for different processes of seeds, seedlings, or older plants. Stimulatory effects can also be observed during the recovery phase after inhibitory concentrations of phenolic acids have been reduced or eliminated from the media.
e. Plant processes can recover after phenolic acid contact is lost or eliminated. Anatomical and morphological features once set are not reversible.
f. Effects of simple phenolic acids at the molecular, cellular, organ, seedling or older plant levels, and the types (i.e., stimulatory or inhibitory) and magnitude of effects observed for sensitive seeds, seedlings, and older plants are regulated by a whole host of modifying factors (e.g., media, pH, solute potential, nutrition,

temperature, pre and post stress, etc.). Effects of phenolic acids do not occur in isolation.

g. Sensitivity of seedlings and older plants to phenolic acids varies with the stage of development and the past and present environments.
h. Effects of phenolic acids are not particularly unique or distinguishable from the effects of other environmental stressors.

5.3 Simple Mixtures

5.3.1 Stimulation

There is presently little information regarding the stimulatory effects of simple mixtures of phenolic acids although attempts have been made to predict with some success the concentration range and the amplitude of hormesis for binary mixtures composed of a range of organic compounds (see Belz et al. 2008).

5.3.2 Inhibition

All indications are that the types of effects from inhibitory concentrations of simple mixtures of phenolic acids appear to be identical to those of individual phenolic acids (see Sect. 5.2) as long as the total concentration of the mixture is inhibitory to the processes under study. However for mixtures, the concentrations of individual phenolic acids making up a mixture may or may not be inhibitory. The actual concentration required for each individual phenolic acid within a mixture depends on the relative potencies of the individual phenolic acids making up the mixture, the actions of the individual phenolic acids in that mixture (e.g., whether their actions are additive or antagonistic to other phenolic acids present), and the total concentration of the mixture required for an inhibitory effect (Table 5.1 and 5.2; Blum et al. 1984, 1985a, 1989; Lyu et al. 1990; Gerig and Blum 1991; Lehman et al. 1994; Pue et al. 1995; Blum 1996; Inderjit et al. 2002). Theoretically the actions of individual phenolic acids in a mixture of phenolic acids may be synergistic, additive, or antagonistic. The total concentration of a mixture of phenolic acids required for an inhibitory effect is determined by the sensitivity of the bioassay species and the physicochemical and biotic environments of the bioassay system (see Sect. 5.2.3 for details).

Synergistic effects have been reported (Rasmussen and Einhellig 1977; Einhellig and Rasmussen 1978; Einhellig et al. 1982; Blum et al. 1985a). However, the data for synergistic effects of individual phenolic acids in mixtures of phenolic acids are not very convincing (Inderjit et al. 2002; Belz et al. 2007). Some would even argue that synergistic effects are unlikely to occur since the actions and sites of action for simple phenolic acids are identical at least when it comes to root membranes.

Table 5.2 Relative potencies of sinapic acid, *p*-coumaric acid, vanillic acid, syringic acid, caffeic acid, *p*-hydroxybenzoic acid, and protocatechuic acid compared to ferulic acid for inhibition of absolute rates of leaf expansion of cucumber seedlings grown in Portsmouth soil B_1 soil materials (pH 5.2)[a]

Phenolic acids	Absolute rates of leaf expansion
Ferulic acid (4 hydroxy-3-methoxycinnamic acid)	1.00 a[b]
Sinapic acid (4 hydroxy-3,5 dimethoxy-cinnamic acid)	0.70 ab
p-Coumaric acid (*p*-hydroxycinnamic acid)	0.67 b
Vanillic acid (4 hydroxy-3-methoxybenzoic acid)	0.67 b
Syringic acid (4 hydroxy-3,5-dimethoxybenzoic acid)	0.59 bc
Caffeic acid (3,4 dihydroxycinnamic acid)	0.38 cd
p-Hydroxybenzoic acid (*p*-hydroxybenzoic acid)	0.35 d
Protocatechuic acid (3,4 dihydroxybenzoic acid)	0.13 e

[a] Cucumber seedlings were treated with 0–0.5 *u*mol/g soil of phenolic acid every other day starting with day 8 and ending with day 16. Nutrient solution was supplied to the soil sytems on alternate days. For additional details see Gerig and Blum (1991). Plenum Publishing Corporation, data used with permission of Springer Science Business Media

[b] Potencies of compounds followed by the same letter do not differ at the 0.5 level of significance

Additive effects of individual phenolic acids in a mixture are most frequently observed at lower concentrations and/or higher (neutral or basic) pH values for treatment solutions and culture systems. Partially antagonistic effects are most frequently observed at higher concentrations and/or lower (acidic) pH values for treatment solutions and culture systems (see Blum et al. 1989; Gerig and Blum 1991; Blum 1996, 2011; Inderjit et al. 2002). Additive and partially antagonistic effects of individual phenolic acids in mixtures have been observed for the uptake of phenolic acids and nutrients (Lyu et al. 1990), leaf expansion (Blum et al. 1985a, 1989; Gerig and Blum 1993; Lehman et al. 1994), and radicle and root length (Blum et al. 1984; Inderjit et al. 2002). The bottom line is that as the number of phenolic acids in a treatment solution was increased, the concentration of each phenolic acid in the mixture had to be reduced in order to maintain a given percent inhibition (Fig. 5.11). For example, an approximate 30% inhibition of absolute rates of leaf expansion for 8–18 day-old cucumber seedlings growing in Portsmouth B soil (pH 5.2) was produced by multiple treatments (5 treatments on alternate days starting with day 8) of 0.23 μmol/g soil of ferulic acid or 0.35 μmol/g soil of vanillic acid, however when the two phenolic acids were combined, only 0.16 μmol/g soil of ferulic acid and 0.15 μmol/g soil of vanillic acid was required (see Fig. 5.11; Blum and Gerig 1991; Blum 1996). Examples of complete antagonism have been few. Li et al. (1993) observed for etiolated seedlings of lettuce that caffeic and ferulic acids at 5×10^{-4} M completely reversed growth inhibition of hypocotyl elongation by 5×10^{-4} or 10^{-3} M *trans*-cinnamic and *o*-, *m*-, *p*-coumaric acids, but did not reverse the inhibition of radicle elongation.

Additions of organic compounds other than phenolic acids (e.g., abscisic acid, methionine, glucose, benzoxazione derivatives, polygodial, and herbicides) at inhibitory or non-inhibitory concentrations have also been found to modify the inhibitory effects of phenolic acids (Fig. 5.12). The actions of such individual organic compounds have been found to be independent, similar joint action (synergistic,

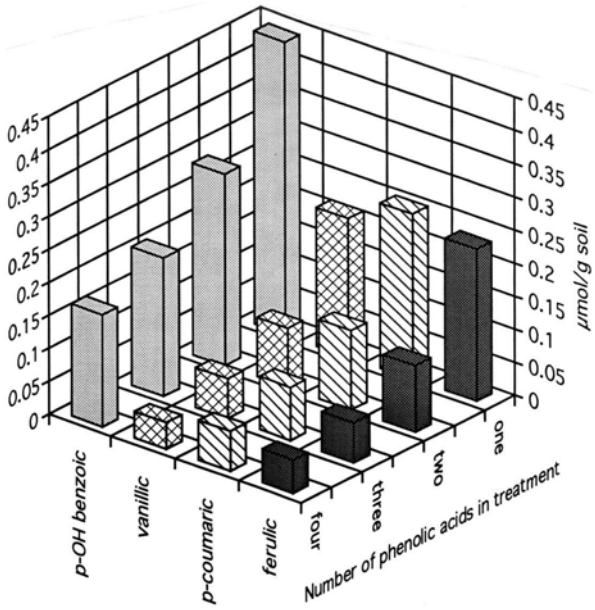


Fig. 5.11 Concentrations for each phenolic acid (*one*), any combination of two and three phenolic acids, and a combination of all four phenolic acids required for an approximate 30% inhibition of absolute rates of leaf expansion for 8–18 day-old cucumber seedlings grown in Portsmouth B soil materials. (Figure taken from Blum (1996), figure used with permission of Society of Nematologists)

additive, or antagonistic), or modifying to the actions of phenolic acids (Morse 1978; Blum et al. 1993; Gerig and Blum 1993; Li et al. 1993; Pue et al. 1995; Einhellig 1996; Fujita and Kubo 2003; Jia et al. 2006). The modifying actions of organic compounds presently appear to be predominately indirect. They can enhance or reduce the effects of phenolic acids by influencing microbial activity. Enhancement occurs when microorganisms preferentially utilize organic compounds other than phenolic acids (Blum et al. 1993; Pue et al. 1995). Reductions occur when microorganisms preferentially utilize phenolic acids over other organic compounds.

Figure 5.12 presents inhibition isolines for combinations of *p*-coumaric acid and methionine (*p*C-M) and *p*-coumaric acid and glucose (*p*C-G) which will inhibit the dry weight of morningglory seedlings grown in Portsmouth A (pH 6.6) and Cecil B (pH 5.4) soils, respectively. Seedlings were given multiple treatments (treatment solution pH was 5) on alternate days ranging from day 6–16 for *p*C-M (Blum et al. 1993) and from day 8–18 for *p*C-G (Pue et al. 1995). For additional experimental details see references. The concentrations for *p*-coumaric acid and methionine were inhibitory and the concentrations for glucose were not inhibitory. The actions of *p*C-M were best described by a multiplicative model (i.e., independent actions) and actions of *p*C-G were best described by a modification model (i.e., modifying action of glucose). The impact of glucose was indirect in that microbial utilization of *p*-coumaric acid was reduced in the presence of glucose (Pue et al. 1995). Such differ-

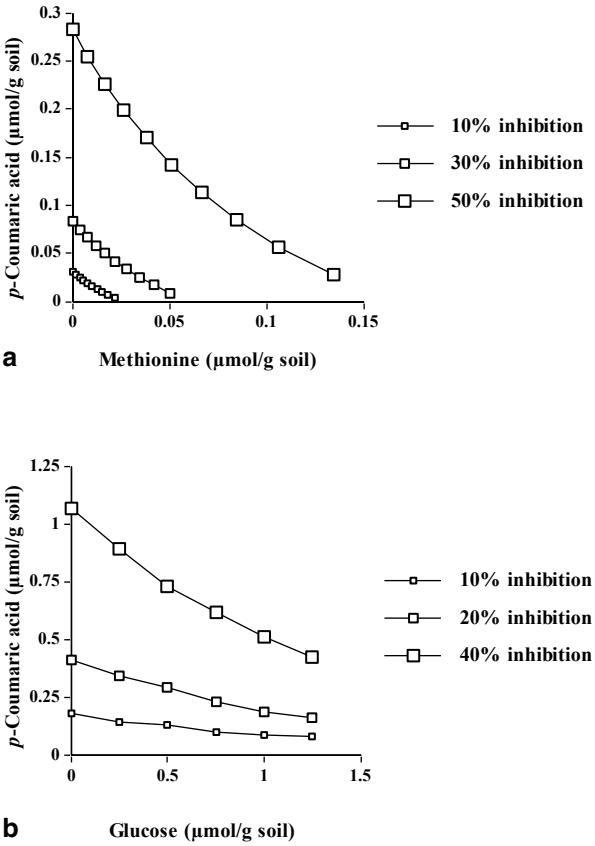


Fig. 5.12 Concentrations of *p*-coumaric acid and methionine (**a**), and *p*-coumaric acid and glucose (**b**) required to inhibit dry weight of morningglory seedlings growing in Portsmouth B and Cecil B soils, respectively, by 10–50 %. Figures adapted/replicated from (**a**) Blum et al. (1993) and (**b**) Pue et al. (1995). Plenum Publishing Corporation, figures used with permission of Springer Science and Business Media. (Figure taken from Blum (2011), figure used with permission of Springer Science and Business Media)

ential utilization of organic compounds by microorganisms is fairly common (Harder and Dijkhuizen 1982; Papanastasiou 1982). Pue et al. (1995) also observed that microbial utilization of *p*-coumaric acid was reduced in the presence of phenylalanine and *p*-hydroxybenzoic acid but not in the presence of methionine. In addition increasing the levels of nitrate (e.g., potentially stimulating microbial utilization) applied to a morningglory bioassay system treated with a mixture of *p*-coumaric acid and methionine lowered the methionine and increased the *p*-coumaric acid concentrations required for a given percent inhibition of seedling dry weight (Blum et al. 1993). This observation suggested that the microbial breakdown products of methionine inhibited morning glory seedlings more than methionine alone.

5.3.3 Summary Comments

What do all these observations and those of Sect. 5.2 say about the effects of simple mixtures of phenolic acids and simple mixtures of phenolic acids and other organic compounds in laboratory bioassay systems?

5.3.3.1 Effects of Simple Mixtures of Phenolic Acids on Seeds, Seedlings, or Older Plants

a. The actions of active cinnamic and benzoic acids are local (e.g., on root cell membranes) not systemic.
b. Active cinnamic and benzoic acids appear to act on similar or identical primary sites and, therefore, compete with each other for primary sites when concentrations exceed the primary sites available.
c. Active concentrations of individual compounds are modified by a variety of environmental factors such as pH, nutrition, media, distribution within media, and actions of microorganisms (for details see Sect. 5.2.3).
d. Concentrations of phenolic acids in bioassay systems are as follows: total (available and bound), available (active and inactive), and active (molecules that actually interact with seeds, root, and mycorrhizae).
e. The actions of active individual phenolic acids in phenolic acid mixtures have been found to be additive, partially antagonistic, or completely antagonistic to other phenolic acids present depending on the phenolic acid mixture, the sensitivity of the species, and the physicochemical and biotic environments of the bioassay system.
f. The additive actions of active individual phenolic acids within a treatment mixture can be converted to partially antagonistic actions by modifying the environment such as reducing (making more acidic) the pH of the treatment solution and bioassay system or by increasing the concentrations of the individual phenolic acids within a treatment mixture (Blum et al. 1989).
g. In order to maintain a constant percent inhibition for a particular process under study, the concentrations of the individual active phenolic acids in the treatment solution must be reduced as the numbers of active phenolic acids are increased. The amount of the reductions will depend on whether the actions of individual phenolic acids are additive or partially antagonistic.
h. Dose response bioassays utilizing individual phenolic acids can be used to study the effects of active phenolic acids but cannot be used to determine the minimum concentrations required for inhibition by phenolic acids in field soils since field soils contain multiple active phenolic acids as well as other organic compounds.

5.3.3.2 Effects of Phenolic Acids and Other Organic Compounds on Seeds, Seedlings, or Older Plants

a. The addition (or presence) of active organic compounds other than phenolic acids can directly or indirectly influence the effects of active phenolic acids. The actions of such organic compounds have been found to be independent, similar joint action (synergistic, additive, or antagonistic), or modifying to the actions of phenolic acids depending on the nature of the mixture, the sensitivity of the species, and the physicochemical and biotic environments of the bioassay system.
b. The addition (or presence) of organic modifiers (i.e., organic compounds other than phenolic acids; although phenolic acids can also act as modifiers) will indirectly influence the effects of phenolic acids depending on the preferential utilization of compounds by microorganisms. When other organic compounds are preferentially utilized by microorganisms, then the effects of phenolic acid concentrations are enhanced. When phenolic acids are preferentially utilized, then the effects of phenolic acid concentrations are reduced. The indirect role of microorganisms, however, can be more complex than that. For example, increasing the levels of nitrate applied to a morningglory-bioassay system treated with *p*-coumaric acid and methionine mixtures lowered the methionine and increased the *p*-coumaric acid concentration required for a given percent inhibition of seedling dry weight (Blum et al. 1993).
c. In order to maintain a constant percent inhibition for a particular process under study, the concentrations of the individual active phenolic acid and/or the other active organic compound within the treatment solution must be reduced. The amount of the reduction depends on whether the actions of the individual compounds are independent, additive, partially antagonistic, or modifying.
d. Dose response studies utilizing individual phenolic acids or other organic compounds can be used to study the effects of these compounds but cannot be used to determine the minimum concentrations required for inhibition in field soils since the complex nature of active organic compounds in field soils cannot presently be duplicated in the laboratory.

5.4 Complex Mixtures

Leachates from living and dead plant tissues, “root exudates plus”, and soil solutions are composed of various concentrations of both organic and inorganic compounds. Numerous examples of the effects of such complex mixtures are to be found in the literature (see Chap. 4). What is lacking in essentially all instances, however, are the specific causes (modes of action) by which such complex mixtures affect plant processes. In the past the assumption has been that a small group of compounds within such mixtures were responsible for the observed effects, i.e., what has previously been referred to as the “herbicide paradigm” (Chap. 1; Blum 2011). The

observations described in this volume regarding allelopathic compounds and environmental modifiers suggest that the effects are actually determined by a complex set of interacting organic promoters, modifiers, and inhibitors and that the ultimate effects observed depend on which agents (i.e., promoters or inhibitors) dominate the mixture and on how various organic modifiers and other modifiers (i.e., environmental factors and processes) influence the outcome (see Chap. 1; Blum 2011). In other words, the answers to our questions regarding field plant-plant allelopathic interactions lie somewhere among the complex actions and interactions that occur in plant-microflora and fauna-soil systems.

5.5 Modes of Action

Since phenolic acids are taken up by root cells and translocated to the rest of the plant, it should not be surprising that phenolic acids can potentially affect a large number of potential sites within plants although all indications are that only a small fraction of the phenolic acids taken up by roots actually ends up in the shoots (see Shann and Blum 1987a). Alleged sites of action for phenolic acids include gene expression, chloroplast photosystem I, II, and photophosphorylation, translocation, mitochondrial respiration, protein synthesis, cell wall lignification, and membrane functions among others (for details see Moreland and Novitzky 1987; Blum and Rebbeck 1989; Bais et al. 2003; Einhellig 2004; Duke and Dayan 2006; Duke et al. 2008; Li et al. 2011; D'Abrosca et al. 2013). The number of alleged sites of action and their associated secondary, tertiary, etc. effects for phenolic acids makes it extremely difficult to sort out specific modes of action for phenolic acids in plants. To demonstrate this conundrum, let us look at one cell process, cell expansion.

Phenolic acid treatments have been shown to inhibit cell expansion by depolarizing membranes (Glass and Dunlop 1974; Baziramakenga et al. 1995). Membrane depolarization leads to a reduction in ion uptake, an increase in efflux of some inorganic ions (Glass 1973, 1974, 1975; McClure et al. 1978; Harper and Balke 1981; Balke 1985; Lyu and Blum 1990; Bergmark et al. 1992; Booker et al. 1992; Baziramakenga et al. 1995; Yu and Matsui 1997; Lehman and Blum 1999ab; Prasad and Devi 2002), and an associated decrease in water potential, turgor pressure, and at times, solute potential (Einhellig et al. 1985; Einhellig 1986; Booker et al. 1992; Barkosky and Einhellig 1993). Phenolic acid treatments also increase lignification of cell walls and modify associated enzyme activities (Shann and Blum 1987b; Politycka 1998; Politycka et al. 2004; Politycka and Mielcarz 2007; Zanardo et al. 2009). The reductions in water potential, turgor pressure, and at times, solute potential and the increase in lignification (i.e., reduced flexibility) of cell walls will reduce the rates of cell expansion. However, phenolic acids interact not only with cell walls and membranes but also enter cells where they are either detoxified, stored in the vacuoles, used as a substrate, modify cell functions such as transcription, protein synthesis, respiration, and block mitosis, etc. (Demos et al. 1975; Harborne 1982; Moreland and Novitzky 1987; Shann and Blum 1987a; D'Abrosca et al. 2003;

Einhellig 2004; Duke and Dayan 2006; Zhang et al. 2009). All of these primary and/or secondary, tertiary, etc. effects are interrelated and have the potential to reduce the growth of roots and shoots by reducing, not only cell expansion but also, cell division. The interactions of phenolic acids with multiple sites and the resulting complexity of the interacting secondary, tertiary, etc. effects suggest that a different and much more comprehensive approach is required to identify the actual processes that determine cell expansion.

In recent years new tools and techniques have been introduced to do just that. Included among them are the use of omics methods (genomics, proteomics, metabolomics, and physionomics; see Macías et al. 2007; Duke et al. 2008, 2013; Wallenstein and Weintraub 2008; Chen et al. 2009; Leão et al. 2009; D'Ambrosca et al. 2013; also see Sect. 6.6), the use of silenced transgenic plants (Inderjit et al. 2009), and the use of mutant lines (Xu et al. 2012). It is hoped that once such methods and techniques, as well as others, are fully implemented, that considerable progress will be made in identifying and characterizing the modes of action by which allelopathic compounds such as phenolic acids impact cell expansion and cell division, as well as other plant processes, which ultimately determine plant growth, development, and reproduction (also see Sect. 6.6).

5.6 Final Comments

Although the focus of this chapter has been on hydrophilic compounds such as simple phenolic acids, the presence and actions of other compounds such as hydrophobic and volatile compounds cannot be ignored. These add additional dimensions to understanding the roles of plant-plant allelopathic interactions within natural and managed systems. Compared to hydrophilic compounds such as phenolic acids, hydrophobic and volatile compounds will have different pathways of inputs and losses, solubilities, mechanisms of transport, modes of action, and responses to environmental factors.

A hydrophilic compound (molecule) typically has an affinity for water, is usually charged or has polar side groups, and is capable of hydrogen bonding enabling it to more readily adsorb/absorb water and dissolve in water or other polar solvents. Some hydrophilic compounds such as calloids do not dissolve. A hydrophobic compound (molecule) typically is repelled by water (insoluble), tends to be electrically neutral (no charge) and non-polar enabling it to more readily dissolve in neutral or non-polar solvents or molecular environments. A single molecule can have both hydrophilic and hydrophobic sites. Fatty acids, for example, which are amphipathic have a hydrophilic head and a hydrophobic tail allowing them to dissolve in both polar (e.g., water) and non-polar solvents (e.g., oils). A volatile organic compound has a high vapor pressure, a low boiling point, tends to be hydrophobic, and evaporates or sublimates from liquids or solids at room temperature. The level of volatility for organic compounds varies from low to high. See the following references for additional details (Gauthier 1997; McNaught and Wilkinson 1997). In regard to

solubility, however, the distinction between hydrophilic, hydrophobic, and volatile compounds may become somewhat less distinct when and if we begin to consider solubility, not in terms of absolute solubility but in term of relative concentrations required for biological activity (Weidenhamer et al. 1993). This may also provide a useful context for the study of the independent actions, joint actions (synergistic, additive, and antagonistic), and modifying actions of all these different types of compounds.

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Chapter 6
Hypothetical Cause and Effect Bioassays

Abstract This chapter describes a number of standard seed and seedling laboratory bioassays for identifying and characterizing the effects and causes by which identified putative allelopathic compounds, mixtures of allelopathic compounds, mixtures of organic and/or inorganic compounds, and residues may stimulate or inhibit sensitive weed species. Five different approaches will be described: (a) a bioassay for simple mixtures of identified putative allelopathic compounds, (b) a bioassay for residue leachates plus or minus XAD-4 resin, (c) the application of regression analysis to data from residue and soil extract bioassays, (d) a bioassay for determining the role of treatment surface area, and (e) using omics methods as tools to determine modes of action.

6.1 Introduction

One line of attack, although not a practical one, would be to separate all the available water-soluble organic compounds from all the available water-soluble inorganic compounds present in stimulatory or inhibitory leachates (e.g., leaf, "root exudates plus" or residue leachates) and soil solutions (e.g., soil extracts) and to determine the effects of these water-soluble organic and inorganic fractions separately and in various combinations on germination, seedling emergence, growth, and biomass in light bank, growth chamber or greenhouse bioassays. Taking such an approach could eventually aid in the identification of organic promoter/modifier/inhibitor complexes with potential allelopathic activity, in explaining how organic and inorganic complexes affect, interact and modify each other's activities, and in explaining the modes of action by which these complexes modify plant processes such as germination, seedling emergence, and growth. With additional work (e.g., determining effects of active isolated compounds on molecular and cellular levels) the potential modes of action for all active organic and inorganic compounds in complex mixtures could be determined. Given the impracticality of this approach, researchers studying plant-plant allelopathic interactions have used various other types of strategies. What follows are several examples.

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Using chemical partitioning, paper, thin layer, and column chromatography, high performance liquid chromatography, molecular sieves, anion or cation columns, etc. researchers have isolated and then identified specific organic compounds with allelopathic potential within and released from plants and residues and in soils. Once identified, putative allelopathic compounds have been tested individually or in simple mixtures in laboratory bioassays (see Sect. 6.2; for examples see Rice 1984). Due to the nature of this methodology, however, the focus is essentially only on the highly active compounds within and released from plants and residues and in soils. The benefits of taking such an approach are that one can determine:

a. the range of concentrations of specific organic compounds or simple mixtures of organic compounds for stimulation or inhibition under a given set of conditions for a species of interest,
b. the potential modes of action by which individual organic compounds or simple mixture of organic compounds affect species of interest,
c. whether the action of an individual organic compound within a simple mixture of organic compounds is neutral, independent, synergistic, additive, or antagonistic, or whether the compound functions as a modifier of the actions of other compounds present in the mixture when seeds, seedlings or plants are stimulated or inhibited,
d. how various soil physicochemical (e.g., pH, solute potential, or inorganic compounds) and biotic (e.g., microorganisms and seed or seedling density) factors modify allelopathic effects of individual organic compounds or simple mixtures of organic compounds, and
e. how a range of physicochemical and biotic factors can influence the sensitivity of bioassay species to individual organic compounds or simple mixtures of organic compounds.

The down side of taking such an approach is that:

a. depending on the environment and the total concentration chosen, an organic compound or a simple mixture of organic compounds may be stimulatory, neutral, or inhibitory to the process or processes measured at least theoretically,
b. the lack of synergistic, additive, antagonistic or modifying effects of all the other organic and inorganic compounds present in leachates, extracts, and residues completely distort the actual (real time) concentrations of organic compounds required for allelopathic effects (i.e., stimulation or inhibition),
c. treatments (enrichments) of bioassay systems with single organic compounds or simple mixtures of organic compounds can lead to atypical rates of soil system processes such as selection and induction of microbial populations, microbial utilization, soil sorption, contact with and uptake by roots and mycorrhizae, etc., and
d. by default it presupposes that allelopathic interactions are driven only by highly active organic compounds when in reality that is unlikely to be the case.

Another approach has been the use of agents that sorb and remove organic compounds from leachates (see Sects. 4.2.3.2 and 6.3) and then testing the treated and

untreated leachates in bioassays. Such sorbing agents have also been used to quantify allelopathic compounds. Agents that have been used are polyvinylporrolidone (PVP), polyvinylpolyporrolidone (PVPP), activated carbon, and XAD-4 resin but there are others that could also be explored (PVP and PVPP (Frömming et al. 1981; Song et al. 1991; Makkar et al. 1993; Lehman and Blum 1997; Toth and Pavia 2001; Magalhães et al. 2010), activated carbon (Hassler 1963; Smíšek and Černý 1970; Mattson and Mark 1971; Cheremisinoff and Ellerbush 1978), XAD-4 resin (Tang and Young 1982; Tang 1986; Tang et al. 1989; Tomás-Barberán et al. 1992; Vaughan et al. 1993; Lara and Thomas 1994; Kim et al. 2005; Hao et al. 2007; Liu et al. 2008; Zeng et al. 2011), others (Ringhand et al. 1987; Tomás-Barberán et al. 1992; Lara and Thomas 1994; Maurice et al. 2002; Lee et al. 2004; Kim et al. 2005; Weidenhamer et al. 2009; Zeng et al. 2011)).

PVP and PVPP have been used to sorb tannins and phenolic acids in order to quantify or remove these compounds from solutions (Anderson and Todd 1968; Makkar et al. 1993; Lehman and Blum 1997; Toth and Pavia 2001; Magalhães et al. 2010). However, other organic compounds with diverse chemical structures and some inorganic compounds are also sorbed by PVP and PVPP (Frömming et al. 1981; Song et al. 1991). As far as the author has been able to determine, the potential use of PVP or PVPP in plant-plant allelopathic bioassays has not been explored. Both activated carbon (Mahall and Callaway 1992; Nilsson 1994; Wu et al. 2000; Ridenour and Callaway 2001; Bertin et al. 2009) and XAD-4 resin (Tang and Young 1982; Tang 1986; Tang et al. 1989; Vaughan et al. 1993; Hao et al. 2007; Liu et al. 2008) have been used in plant-plant allelopathic bioassays. Similar to PVP and PVPP, activated carbon and XAD-4 resin also sorb a range of organic and inorganic compounds (activated carbon (Hassler 1963; Smíšek and Černý 1970; Mattson and Mark 1971; Cheremisinoff and Ellerbush 1978; Huang 1978; Bansal and Goyal 2005), XAD-4 resin (Mills 1982; Ben-Poorat et al. 1986; Lee et al. 2004; Kim et al. 2005; Zeng et al. 2011)). What is actually sorbed is determined in large part by the source, type, and surface area of the sorbing agent, pH and type of solvent, nature and combination of organic and inorganic compounds present in the solvent and on the sorbent, ratio of sorbent to solvent, and flow rate of the solvent (activated carbon (Smíšek and Černý 1970; Cheremisinoff and Ellerbush 1978; Bansal and Goyal 2005), XAD-4 resin (Vaughan et al. 1993)). Both agents, however, are highly efficient in the sorption of aromatic compounds from water (Bansal and Goyal 2005; Wang et al. 2005). In the case of XAD-4 resin the sorbed compounds can also be readily desorbed for analysis (Tang and Young 1982; Tang 1986; Tang et al. 1989; Paik et al. 2004; Liu et al. 2008; Vaughan et al. 1993). The benefits of using such an approach are that:

a. the bioassays of treated and untreated (with and without sorbent additions, respectively) solutions (e.g., leaf, "root exudates plus" and residue leachates or soil extracts) can be tested for changes in stimulation or inhibition of bioassay species,
b. the bioassays from a. represent tests of promoter/modifier/inhibitor complexes, not just individual organic compounds or simple mixtures of organic compounds,

c. many of the organic and inorganic compounds bound to the sorbing agents, such as XAD-4 resin, can be released from the sorbing agents, identified, quantified, and tested individually or in various combinations for stimulation or inhibition of bioassay species, and
d. since sorption of non-metallic salts by these sorbents appears to be limited (Cheremisinoff and Ellerbush 1978; Mills 1982), the impact on mineral nutrition in bioassays should be minimal.

The down side of taking such an approach is that:

a. the exact composition of the organic and inorganic compounds sorbed to activated carbon, XAD-4 resin, and other potential sorbents is an unknown or at best difficult to determine,
b. the sorption and desorption efficiencies vary with the physicochemical environment, and
c. the sorbents change promoter/modifier/inhibitor complexes in unknown ways.

Therefore, even though such an approach can provide considerable insight regarding potential cause and effect, there are still significant uncertainties associated with such an approach (see Lau et al. 2008, Wurst et al. 2010). Please note that sorbents such as activated carbon can also be mixed into soil directly (see Sect. 4.4.2).

Still another approach has been to use regression analyses to determine the relationships between physical and chemical characteristics of residues, soil extracts, or simulated-soil extracts and seed or seedling responses (see Sect. 6.4; Hall et al. 1983; Blum et al. 1992; Lehman and Blum 1997). The author is not aware of any studies of this nature for leaf, "root exudates plus" or residue leachates. However, there is no obvious reason why this approach could not also be used for them since this approach eliminates some of the down side associated with testing individual organic compounds or simple mixtures of organic compounds but comes with its own set of challenges principally:

a. the collection of authentic and real time leachates (e.g., leaf, "root exudates plus" or residue leachates), residues, and soil solutions,
b. the simulation of relevant physicochemical environments for bioassay systems,
c. the determination and implementation of the appropriate addition(s) or application(s) of leaf, "root exudates plus" or residue leachates, soil solutions, and residues (e.g., times, rates, concentrations, location, etc.) to bioassay systems,
d. the identification and quantification of available and active organic allelopathic compounds (i.e., promoters and inhibitors) and organic, inorganic, physicochemical and biotic modifiers present in bioassay systems, and
e. the sorting out of co-linearities and other types of interactions between active and modifying factors.
f. The bottom line is that these challenges in various forms also turn out to be challenges for the other approaches (i.e., identified putative allelopathic (IPA) compounds and sorbents).

Finally in the last decade a range of molecular methods such as genomics, proteomics, metabolomics, and physionomics have been developed (Bais et al. 2003;

Summer et al. 2003; Gidman et al. 2003; Dunn and Ellis 2005; Macías et al. 2007; Duke et al. 2008, 2013; Chen et al. 2009; Leão et al. 2009; Metz 2011; also see Sect. 6.6). These methods have enormous potential in that they allow researchers to identify phenotypic responses at the molecular level and to identify modes of action for a range of stressors including allelopathic compounds. Of these methods, metabolic fingerprinting and profiling (metabolomics) have been used extensively by researchers to study the nature, behavior, and relationships of plants and microorganisms (Johnson et al. 2003; Narasimham et al. 2003; Grossmann 2005; Wallenstein and Weintraub 2008; Wallenstein et al. 2010; Liebeke et al. 2009; Leão et al. 2009; Bertram et al. 2010; Prince and Pohnert 2010; Kim et al. 2011; Sardans et al. 2011; Carlsen et al. 2012; Grossmann et al. 2012; Nguyen et al. 2012; D'Abrosca et al. 2013; Marti et al. 2013). Some potential benefits of using metabolomics as a tool:

a. unstable compounds are more likely to be detected than when individual compounds are isolated by conventional methods,
b. identification and quantification of individual organic compounds can be achieved without the need for excessive purification,
c. metabolic fingerprinting or profiling can help to identify molecules that act as signals for physiological responses of organisms,
d. provide a starting point for identifying potential modes of action by which a range of environmental factors including individual organic compounds or complex mixture of organic compounds modify plant and microbial behavior,
e. detect potential independent or joint actions (synergistic, additive, or antagonistic) of organic compounds within complex mixtures,
f. provide actual functional status of an organism, and
g. identify a range of individual organic compounds and their concentrations within tissues and litter leachates, soil solutions, etc.

The down side of using metabolomics as a tool:

a. the considerable overlap of metabolic profiles,
b. the difficulty of establishing baseline metabolism and detecting what constitutes significant metabolic changes due to the inherent noise of living organisms (e.g., genetic variability and phenotypic plasticity) and their environments,
c. the difficulty of establishing relationships between significant changes in metabolites and the functional responses or behaviors of seeds, seedlings, plants, and microorganisms,
d. the large number of organic compounds that are present in plant, microorganism, and soil samples and the present lack of available data bases and platforms to identify many of these organic compounds, and
e. the complex equipment and analytical tools required (Summer et al. 2003; Fiehn 2001; Dunn and Ellis 2005; Barofsky et al. 2009; Bundy et al. 2009; Prince and Pohnert 2010; Metz 2011; Sardans et al. 2011; Duke et al. 2013).

There are clearly a whole variety of ways that potential cause and effect of leaf, "root exudates plus" or residue leachates, residues, and soil solutions can be determined. Both a reductionist and a more holistic approach can be taken (see Sect. 1.5). Below the reader will find a set of hypothetical laboratory bioassays that it is hoped

will provide some guidance for designing cause and effect bioassays which are consistent with the tenets of Chap. 3 and ultimately relevant to plant-plant allelopathic interactions in the field. For continuity the sources of materials to be tested and the bioassay species used in these examples are the same as in Chap. 4. For simplicity and convenience let us assume that all the options for the hypothetical experiments have been completed, the data have been collected, and that the data are now ready to be analyzed. However since these are hypothetical experimental designs, the reader will find at times only general design details and a number of appended notes describing cautions, adjustments, modifications, and improvements to the experimental designs.

6.2 Identified Putative Allelopathic (IPA) Organic Compounds

Petri dish, solution and soil bioassays have been the principal means by which potential allelopathic effects of purchased and isolated organic compounds have been determined. Typical experimental designs might look like this:

a. a range of concentrations of an organic compound × replicates × species of interest × a given environment or a range of environmental conditions, or
b. a range of concentrations for individual organic compounds and simple mixtures of organic compounds × replicates × species of interest × a given environment or a range of environmental conditions.

In regard to b. the following simple mixtures of organic compounds with allelopathic potential have been tested in Petri dish, solution or soil bioassays:

a. phenolic acid mixtures (Rasmussen and Einhellig 1977; Einhellig and Rasmussen 1978; Einhellig et al. 1982; Williams and Hoagland 1982; Blum et al. 1984, 1985, 1989; Lyu et al. 1990; Gerig and Blum 1991; Lehman et al. 1994; Pue et al. 1995; Blum 1996; Reigosa et al. 1999; Inderjit et al. 2002),
b. phenolic acids and coumarins (Einhellig 1996),
c. phenolic acids and rutin (Einhellig 1996),
d. phenolic acids and amino acids, carbohydrates, or nitrogen (Blum et al. 1993; Pue et al. 1995),
e. phenolic acids and abscisic acid (Li et al. 1993),
f. phenolic acids and herbicides (Einhellig 1996),
g. phenolic acids and volatile compounds (Inderjit et al. 1997), and
h. mixtures of volatile compounds (Bradow and Connick 1988a, b; Weidenhamer et al. 1994).

Objective To determine the effects of increasing concentrations of individual organic compounds (e.g., three phenolic acids) and a series of equal-molar mixtures of these compounds on weed germination in Petri dishes, weed biomass in solution culture, and weed seedling emergence in soil. More specifically the effects of increasing concentrations of individual phenolic acids, the effects of individual

phenolic acids within mixtures of phenolic acids, and the relative potencies of the individual phenolic acids.

Materials and Methods *Option 1 (germination without soil):* Three compounds, ferulic acid (FA), *p*-coumaric acid (PCO) and vanillic (VA) acid identified in soil solutions of wheat cover crop residue plots (Blum et al. 1991, 1992) were applied in various combinations to a Petri dish (see Fig. 4.2; 100 mm × 25 mm) system containing # 1Whatman filter paper and weed seeds (morning glory [*Ipomoea hederacea* L.], pigweed [*Amaranthus retroflexus* L.], or prickly sida [*Sida spinosa* L.]). Petri dishes and filter papers were sterilized by autoclaving. Weed seeds were not surface sterilized. Number of weed seeds per Petri dish was constant for each species and based on seed size (see Sect. 3.2.2.6). All treatment and reference solutions were made up with simulated-rain water and adjusted so that their pH values, solute potentials, and nutrient content were identical (see Sects. 4.2.1, 4.3.3, and 5.2.3.1; Blum et al. 1992). All solutions were filter-sterilized (0.2 μm membrane filters). Volume of treatment solutions or simulated-rain water (reference) added to Petri dishes was also based on seed size but was identical for all experimental units of a given weed species. Bioassays were carried out at 30 °C in a dark growth chamber with a relative humidity of 70 %. Germination (number of seeds with radicles) and radicle length were determined after several days; the number of days was determined by the weed species. Treatments were as follows:

a. reference (simulated-rain water)
b. 0.03125, 0.0625, 0.09375, 0.125, 0.15625, 0.1875, 0.21875, 0.25, 0.375, and 0.5 mM for FA, PCO, and Van, individually
c. all 0.03125 mM two-way combinations (total concentrations 0.0625mM)
d. a 0.03125 mM three-way combination (total concentration 0.09375 mM)
e. all 0.0625 mM two-way combinations (total concentrations 0.125 mM)
f. a 0.0625 mM three-way combination (total concentration 0.1875 mM)
g. all 0.09375 mM two-way combinations (total concentrations 0.1875 mM)
h. a 0.09375 mM three-way combination (total concentration 0.28125 mM)
i. etc.

All two and three way combinations were made until the total concentration for each reached 0.5 mM or slightly above to avoid excessive extrapolation of data (i.e., estimating values that fall outside the range of known values for individual compounds). The basic experimental design was as follows:

$$51 \text{ treatments } (50 \text{ treatment solutions and reference solution}) \times 3 \text{ replicates} = 153 \text{ experimental units per weed species.}$$

Seeds can be surface sterilized. However, obtaining seed lots in which all seed surfaces are sterile is extremely cumbersome and time consuming. In addition species sterilization procedures can impact germination and radicle growth (e.g., see Blum et al. 1984) and surface sterilization will not eliminate microorganisms within seeds (Leben 1961; Mundt and Hinkle 1976). Solutions added to Petri dishes should be at room temperature. After imbibitions a small amount of free solution should still be

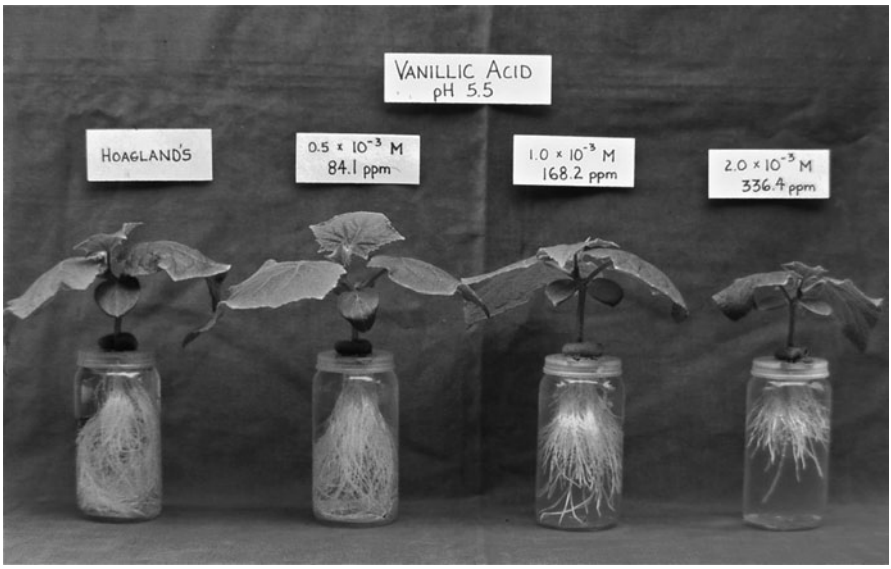

Fig. 6.1 An example of a nutrient-solution culture system. For this example, cucumber seedlings were treated with different concentrations of vanillic acid which were given 4 times on alternate days starting with day 8

present in the Petri dish. Multiple additions of treatment solutions and simulated-rain water should be avoided if at all possible since such additions change the concentrations of organic and inorganic compounds, the water potentials, and/or the pH of the treatment solutions. Relative humidity should be kept as high as possible to minimize evaporation from the Petri dishes. Petri dishes may also be sealed to minimize evaporation but anaerobic conditions may be a concern.

To determine the contribution of individual organic compounds within mixtures to stimulation or inhibition of a particular biological processes, requires, at least theoretically, that all individual compounds, all 2-way, 3-way, etc. combinations of compounds be tested with an appropriate overlapping range of concentrations. However, all possible combinations may not actually be required since regression analysis can at times be used to extrapolate (i.e., estimate) effects between concentrations. A delay in seed germination due to leachate treatments will impact radicle length (i.e., estimated growth rates for radicles). Thus if radicle growth rates are to be determined independently of germination time, then pre-germinated seeds with roughly equal radicle lengths would need to be utilized in the leachate bioassays.

Option 2 (seedling biomass without soil): Morning glory (*Ipomoea hederacea* L.), pigweed (*Amaranthus retroflexus* L.), or prickly sida (*Sida spinosa* L.) seeds were germinated at 30 °C in a dark growth chamber for 24–48 h in trays containing vermiculite moistened with Hoagland's nutrient solution (Hoagland and Arnon 1950). Seeds were not surface sterilized. Seedlings were then placed under a light bank (140–180 μmol/cm^2/sec; 12 h photoperiod) in the laboratory until sufficiently large enough to be transferred to glass bottles (4.5 × 9 cm, 110 ml, sterilized) con-

Fig. 6.2 Light banks with cucumber seedlings growing in nutrient-solution culture. (Figure taken from Blum (2011), figure used with permission of Springer Science and Business Media)

taining Hoagland's nutrient solution (pH 5.5). (Note: Solutions added to bottles should be at room temperature.) Seedlings were then suspended by foam collars inserted through plastic caps located on top of the glass bottles (Fig. 6.1) and bottles were covered with aluminum foil or placed into wooden racks to prevent light from reaching roots. One hundred ml of a given filter-sterilized (0.2 μm membrane filter) treatment solution containing individual or mixtures of phenolic acids (ferulic acid [FA], *p*-coumaric acid [PCO] and/or vanillic [VA] acid in nutrient solution) or nutrient solution (reference) were added to the sterile glass bottles (Blum et al. 1985). All treatment solutions and reference solutions were adjusted initially so that their pH values, solute potentials, and nutrient content were identical (see Sects. 4.2.1, 4.3.3, and 5.2.3.1; Blum et al. 1992). Filter-sterilized distilled water was added to each bottle twice a day (morning and evening) to compensate for evapotranspiration (i.e., bottles were brought back to the initial volume each time). Solutions were completely replaced every other day so that phenolic acid and nutrient concentrations, solute potentials, and pH could be brought back to the initial levels and microbial populations would be kept in check. Bioassays were carried out in light banks (Fig. 6.2) with light intensities of 140–180 μmol/cm^2/sec, 12-h photoperiods, and the normal range of laboratory temperatures. Phenolic acid concentrations and combinations used were the same as in Option 1a. Minimum and maximum air temperatures and water utilization were recorded daily for 6 days. Dry weights of roots and shoots were determined at the end of the experimental period. The basic experimental design was as follows:

$$40 \text{ treatments } (39 \text{ treatment solutions and reference solution}) \times 3 \text{ replicates} = 120 \text{ experimental units per weed species.}$$

Since formation of mycorrhizae by weed seedlings is unlikely to occur given the time interval required for the formation of mycorrhizae, roots were not inoculated with mycorrhizal fungi (see Sects. 3.6 and 3.7). These bioassays could be improved by metabolic fingerprinting or profiling of seedlings for the individual and various mixtures of phenolic acid treatments. This could go a long way towards identifying and confirming the modes of action by which individual and mixtures of phenolic acids modify seedling biomass (see Sects. 6.3 and 6.6). Fingerprints or an index for profiles or profile subsets could also be used to determine if any correlations exist between fingerprints and profiles and seedling biomass.

Option 3 (seedling emergence in soil): Solutions containing individual or various combinations of three compounds, ferulic acid (FA), *p*-coumaric acid (PCO) and vanillic (VA) acid were applied to soil (15 cm pots with a 1767 cm^3 volume and no drainage holes) containing weed seeds (morning glory [*Ipomoea hederacea* L.], pigweed [*Amaranthus retroflexus* L.], or prickly sida [*Sida spinosa* L.]) located 1 cm below the soil surface. Number of seeds per pot was constant for each weed species and based on seed size. Seeds were located roughly equidistant from each other in each pot to minimize competition (see Sect. 3.2.2.6). All treatment solutions were made up with simulated-rain water (see Sect. 4.3.3) and adjusted so that their pH values, solute potentials, and nutrient content were identical (see Sects. 4.2.1, 4.3.3, and 5.2.3.1; Blum et al. 1992). Similar adjustments were also made for the simulated-rain water reference solutions. All solutions added to soil systems were filter-sterilized (0.2 µm membrane filter) and at room temperature. The total amount of treatment solution added to each experimental unit was identical. Treatment solutions were added every other day and simulated-rain water was supplied daily as needed.

The compositions of the treatment solutions were identical to Option 1 except that the solutions added were much more concentrated. For phenolic acid solutions, 15 mM can be readily achieved at pH values of 5.0 and above (Blum 2011). The more concentrated solutions were used to keep the volume of treatment solution added to the soil systems to a minimum while at the same time providing a means by which sufficient amounts could be added to the soil to achieve the desired µmol/g soil concentrations. Solution volumes of soil systems were kept below field capacity. (Note: Soils at field capacity or above for very short time intervals right after the addition of solutions may be acceptable for some systems. Timing and frequency of solutions additions may need to be adjusted depending on the rates of evapotranspiration.) The volume and concentration of treatment solutions in mM added every other day to soil systems were sufficient to produce the desired concentrations. Treatment concentrations tested were: 0, 0.03125, 0.0625, 0.09375, 0.125, 0.15625, 0.1875, 0.21875, and 0.25 µmol/g soil for individual phenolic acids and all 2 and 3-way combinations were similar to Option 1. The total solution volume of soil systems was chosen to meet and slightly exceed the daily minimum require-

ments for normal germination and growth. Pots were brought to a constant weight (i.e., the initial chosen weight) each time solutions were added.

Bioassays were carried out in a greenhouse. Emerging seedlings were counted daily. Weed seedlings were removed after each count to minimize competition. When removing weed seedlings, soil disturbance was minimized as much as possible. Daily averages, total numbers at the end of the experimental period, and slopes based on cumulative numbers of weed seedlings per day were determined for seedling emergence (Note: Daily emergence or cumulative daily numbers could also be used for time series studies). The number of days for experimental periods were determined by when seedling emergence ceased for the reference treatment of a given weed species. Light intensity at pot level, temperature, and relative humidity were monitored during the experimental period. The basic experimental design was as follows:

$$51 \text{ treatments (50 treatment solutions and reference solution)} \times 3 \text{ replicates} = 153 \text{ experimental units per weed species.}$$

Depending on soil, the addition of sand may be required for adequate percolation and aeration. The final g/pot of soil or soil-sand mixture should be identical for all pots. Multiple daily additions of treatment solutions should be avoided if at all possible. Since phenolic acids in the soil are rapidly sorbed, oxidized/reduced, polymerized and utilized by microorganisms, metabolic profiles of soil solutions (e.g., soil extracts) taken at various time intervals before and after phenolic acid treatments could be used to determine the nature and composition of the organic compounds in the soil surrounding seeds and seedling roots (see Sects. 6.4.1 and 6.6). Metabolic profiles can also provide insight regarding breakdown products of phenolic acids and the synthesis of new and unique compounds produced by microorganisms in seedling- microorganism-soil bioassay systems. For environmental measurements see Pearcy et al. (1989).

Hypotheses If treatment solutions of individual and various mixtures of phenolic acids have the same pH, nutrient content, and solute potential but are composed of a range of overlapping increasing concentrations of phenolic acids, then their observed effects (stimulation or inhibition) on weed seed germination, weed seedling biomass, and weed seedling emergence will be due to the action of individual phenolic acids or the product of the actions and interactions of the various combinations of phenolic acids.

If the first hypothesis is true for Options 1, 2, and 3, then by using the proper statistical tools it will be possible to determine:

a. the concentrations required for a specific percent stimulation or inhibition of individual compounds or combination of compounds,
b. the effective dose [e.g., ED_{25}, ED_{50}, etc.] and percent stimulation or inhibition isolines,
c. whether the effects of individual phenolic acids in a mixture were independent, synergistic, additive, or antagonistic, and
d. the relative potencies of individual compounds.

Analyses A variety of modeling approaches have been used to examine and evaluate the resulting data of such factorial experiments. Since with minor exceptions the resulting models will be nonlinear, analytical tools such as SAS/NLIN (Gerig and Blum 1993; SAS Institute Inc. 1999) will be required. For effects of individual compounds within mixtures of compounds, the following types of modeling may be used.

a. The multiplicative model can be used if all the compounds are independent in action.
b. The similar joint action model can be used for two or more compounds that show reciprocity in their actions. This model allows for the determination of synergistic, additive and antagonistic actions of individual compounds within a mixture.
c. The modification model can be used if a second compound is not in itself stimulatory or inhibitory but changes the effective concentration of a stimulatory or inhibitory compound.

For all three models one can calculate effective treatment doses for specific percent stimulation or inhibition (e.g., ED_{50}; Inderjit et al. 2002; Fujita and Kubo 2003), relative potencies (Gerig and Blum 1991), and percent stimulation or inhibition isolines (Blum et al. 1993; Pue et al. 1995). These can be used to compare the effectiveness of individual or mixtures of compounds. However, none of the three models (i.e., a., b., or c.) can be used when two or more compounds interact and form a new compound or compounds (Gerig et al. 1989; Gerig and Blum 1991, 1993; Inderjit et al. 2002). Of the three models the similar joint action model (a) is the most appropriate for phenolic acid mixtures since the modes of action for simple phenolic acids such as cinnamic and benzoic acid derivatives appear to be identical (see Sect. 5.3).

Comments In the literature as well as for Options 1, 2, and 3, equal-molar concentrations have been used. However, solutions mimicking concentrations found in leachates or soil solutions are much more meaningful. Therefore instead of using equal-molar mixtures, one could, for example, use the phenolic acid concentrations found in soil solutions. A range of concentrations with the correct proportion of organic compounds simulating soil solutions could then be applied to the system described in Options 1, 2, and 3. For example, a hypothetical analysis of soil solutions soil extracts from wheat residue plots resulted in the following average phenolic acid content: 1 *u*mol/g soil of ferulic acid, 2 *u*mol/g soil of *p*-coumaric acid, and 0.5 *u*mol/g soil of vanillic acid. (Note: These are unrealistic values chosen here to simplify this presentation. See Blum et al. (1991) for some actual values for phenolic acids in wheat residues and soils.) Given these as starting points, the basic experimental design could be as follows for the soil bioassay system (Option 2):

a. 0, 0.125, 0.25, 0.5, 0.75 and 1 *u*mol/g soil ferulic acid (FA),
b. 0, 0.25, 0.5, 1, 1.5, and 2 *u*mol/g soil *p*-coumaric (PCO),
c. 0, 0.0625, 0.125, 0.25, and 0.5 *u*mol/g vanillic acid (VAN),
d. FA and PCO combinations: 0.125 FA & 0.25 PCO, 0.25 FA & 0.5 PCO, etc.,
e. FA and VAN combination: 0.125 FA & 0.0655 VAN, 0.25 FA & 0.125 VAN, etc.,

f. PCO and VAN combination: 0.25 PCO & 0.0625 VAN, 0.5 PCO & 0.125 VAN, etc., and
g. FA, PCO and VAN combinations: 0.125 FA & 0.25 PCO & 0.0625 VAN, 0.25 FA & 0.5 PCO & 0.125 VAN, etc.

In this example the total concentration of any 3-way mixture should not exceed beyond 3.5–4 *u*mol/g. The same proportions of phenolic acids could be used for all three Options but the solution bioassay values for Options 1 and 2 would be in μM or mM and for Option 3 in *u*mol/g. However, all of these cause and effect bioassays are still hypothetical or theoretical in nature and, therefore, provide little, if any, actual insight as to what might actually be happening under field conditions, since:

a. Options 1 and 2: the potential effects and modifications of a whole range of organic and inorganic compounds normally present in soil solutions and soil processes were absent,
b. Options 1, 2 and 3: equal-molar concentrations are not representative of soil solutions, (Note: The addition of mixtures simulating real time leachates or soil solutions would be an improvement.)
c. Options 1, 2 and 3: the concentration ranges of phenolic acids used in the bioassays are simulations,
d. Options 1, 2 and 3: the usual dynamics and range of physical and chemical characteristics found in field soils were absent, and
e. Option 3: the application of treatment solutions to the soil surface will produce a declining concentration gradient where the highest concentrations will occur near the surface of the soil. The extent to which this gradient is typical or atypical will be determined by the treatment concentrations added to the soil surface.

Furthermore, the experimental designs for Options 1, 2, and 3 imply that the phenolic acids function either as promoters or inhibitors. Actually in the presence of microorganisms, phenolic acids could also function as modifying agents since differential preferences for the utilization of phenolic acids by microorganisms have been demonstrated (Pue et al. 1995). However, given the nature and complexity of these bioassay systems, that would be extremely difficult to demonstrate. What is really needed at this point is to develop and implement bioassays in which the effects of specific organic compounds with allelopathic potential can be quantified in the presence of a representative mixture of promoters, modifiers, and inhibitors found in soil solutions under typical field environmental conditions.

6.3 Complex Solutions

Leachates (e.g., leaf, residue or "root exudates plus" leachates) and soil solutions contain complex mixtures of organic and inorganic compounds (see Sect. 2.1.3). Stimulation or inhibition of sensitive species by these solutions in bioassay systems is a product of the actions and interactions of the active organic and inorganic

compounds making up these solutions. Activity of an individual compound within a mixture of compounds is not only determined by its concentration but also by the physical and chemical nature of the solution (e.g., pH, solute potential, and presence of promoters, modifiers, and inhibitors) and the nature of the bioassay system (e.g., root and mycorrhizal surface area, type of substrate and microorganisms present). Therefore, the treatment of bioassay systems with a potentially active compound is only a first step in understanding its role in the overall observed stimulation or inhibition of a mixture of compounds. To truly understand the role of an individual compound and its required effective concentration within a particular bioassay system, the treatment compound has to be tested with its normal complement of associated organic and inorganic compounds. How could that be done?

A first step toward achieving that goal might be to identify the presence of additional organic compounds in complex solutions such as leachates, etc. (see Comments Sect. 4.2.3.2; Sects. 6.4.1 and 6.6) and to test the original active organic compound in various combinations with the other organic compounds identified to determine how its stimulation or inhibition is modified by the presence of the other organic compounds (see Sect. 6.2). The same thing could be done for various combinations of organic and inorganic compounds (for an example see Blum et al. 1993). The benefit of such an approach is that it is a very logical and orderly process which will allow for the determination of effective concentrations and the independent, synergistic, additive, antagonistic, or modifying actions of each compound being tested in a mixture of compounds. The down side is that there are practical limits to how many combinations of organic and inorganic compounds can be tested.

A potentially more holistic, but more uncertain, approach has been the use of sorbents such as XAD resins and activated carbon which have been used to sorb organic and some inorganic compounds from solutions and various substrates. Solutions and substrates before and after treatment with sorbents have then been tested to determine differences in stimulatory or inhibitory effects in bioassays. When differences have been observed, attempts have been made to identify the causative agents. Such an approach has been taken for "root exudates plus" leachates (Tang and Young 1982; Tang 1986; Tang et al. 1989; Vaughan et al. 1993; Hao et al. 2007) and a variety of substrates (e.g., soil, sand, agar, and Metro-mix) (Mahall and Callaway 1992; Nilsson 1994; Wu et al. 2000; Ridenour and Callaway 2001; Bertin et al. 2009).

To the knowledge of the author, there has not been a systematic attempt to manipulate the concentrations of individual compounds identified as potential causative agents in leachates or soil solutions in order to determine how the presence of the other organic and inorganic compounds in these solutions may modify the concentrations required for stimulation or inhibition. This is an area that deserves to be explored even if the attempts at this point are rudimentary. For example, it may be possible to use specific amounts of polyvinylporrolidone (PVP), polyvinylpolyporrolidone (PVPP), activated carbon, XAD resins, or other sorbents (also see Sects. 4.2.3.2 and 4.4.2) to sorb organic (e.g., phenolic acids) and inorganic compounds in such solutions, determine what has been removed, manipulate the solutions by incrementally adding back removed compounds, and testing how the

resulting solutions affect sensitive species in the bioassay systems. For information regarding what is preferentially sorbed by PVP, PVPP, activated carbon, and XAD resins see Mills (1982), Ben-Poorat et al. (1986), Ringhand et al. (1987), Tomás-Barberán et al. (1992), Lara and Thomas (1994), Maurice et al. (2002), Lee et al. (2004), Paik et al. (2004), and Liu et al. (2008). It would seem that considerable insight could be gained from such bioassays regarding the importance of individual promoters, modifiers, and inhibitors in complex mixtures of organic and inorganic compounds. For convenience and simplicity let us use wheat residue leachates as an example and an experimental design which is much more hypothetical than the previous and following experimental designs.

Objective To determine how various specific organic (and inorganic) compounds and combinations of organic (and inorganic) compounds at concentrations present in wheat residue leachates can potentially influence the germination and radicle lengths of weed seeds in the absence of soil.

Materials and Methods For the collection, processing, and storage of residue leachates prior to testing see Sects. 2.1.3.2 and 4.3.5. Samples of wheat residue leachates were passed through columns with glass beads (GB) or columns with XAD-4 resin (XAD). In preliminary bioassays, the leachates from columns with (XAD) and (GB) were tested by Petri dish weed germination bioassays using simulated-rain water ® as reference (see Option 1 Sect. 4.2.2.2). Mean comparisons were used to test for significant differences between GB, XAD and R solutions. For simplicity of this presentation it will be assumed that Petri dishes with GB leachate had a significantly lower number of germinated weed seeds than Petri dishes with reference solution R. Then theoretically, depending on the preference and effectiveness of the XAD-4 columns in reducing active compounds (e.g., promoters, modifiers, and/or inhibitors) from the leachate (initial leachate and GB leachate should theoretically be identical), the number of germinated weed seeds in the Petri dishes with treated XAD leachate could be higher than the reference solution R (promoters now dominate), could be intermediate between GB and R solutions (partial reduction of inhibitors), or could be identical to reference solution R (complete removal of promoters and/or inhibitors). Other scenarios are also possible. The actual enhancement or moderation of promoters or inhibitors by other potential modifiers would be difficult to determine in this instance.

The next step would be either to determine the differences in organic and inorganic compounds for GB and XAD leachates or to determine the sorbed organic and inorganic compounds released from the XAD-4 resin by solvents such as methanol (Tang and Young 1982; Tang 1986; Tang et al. 1989; Paik et al. 2004; Liu et al. 2008; Vaughan et al. 1993). Once compounds of interest have been identified and quantified, they may be added back incrementally to XAD leachates and tested in any number of bioassay systems. For example, testing wheat residue leachates (Option 1 Sect. 6.2) on morning glory (*Ipomoea hederacea* L.), pigweed (*Amaranthus retroflexus* L.) or prickly sida (*Sida spinosa* L.) seeds in a Petri dish bioassay would require the following treatment solutions:

a. R solution,
b. GB leachate,
c. XAD leachate,
d. XAD leachate plus incremental additions of IPA organic and inorganic compounds consistent with the quantitative analyses of GB (see Sect. 6.2 for guidance regarding potential experimental designs), and
e. IPA compound (s).

The final experimental design would depend on the various types and concentrations of organic and inorganic compounds added back to the XAD leachate. Initially the design should be as simple as possible. For example, manipulating one or two organic compounds would be a good place to start. The basic experimental design for one compound might be as follows: 11 treatments (R solution, GB leachate, XAD leachate, XAD leachate plus IPA compound at 4 concentrations, and 4 concentrations of IPA compound) × 3 replicates = 33 experimental units per weed species. For two compounds: 23 treatments (R solution, GB leachate, XAD leachate, XAD leachate plus IPA compound 1 at 4 concentrations, IPA compound 2 at 4 concentrations, mixtures of both IPA compounds at 4 concentrations, 4 concentrations of IPA compound 1, and 4 concentrations of IPA compound 2) × 3 replicates = 69 experimental units per weed species (see Sect. 6.2 for types of combinations to use). If this turns out to be satisfactory, even more complex designs could be tested in a range of bioassay systems.

The pH values and solute potentials of test solutions may be different for the different test solutions and, therefore should be monitored and accounted for or should be adjusted to be identical. XAD-4 resin should be cleaned and tested for available nutrients (e.g., NPK) and organic compounds prior to use. For details regarding cleaning and reactivation of XAD-4 resins see Tang and Young (1982). The volume/mass of resin to be used in the columns will vary with the nature of the solution. A preliminary bioassay should be used to determine the correct volume/mass of resin required. In this example the author has chosen to test IPA compounds but any identified compound could be tested. For procedures regarding isolation, identification, purification, and collection of allelopathic compounds, the reader may wish to consult Waller et al. (1999), Yu and Dahlgren (2000), and/or Sampietro et al. (2009) or for metabolic profiling consult Summer et al. (2003), Dunn and Ellis (2005), Xi et al. (2008), Prince and Pohnert (2010), Sardans et al. (2011), and Sect. 6.6.

Hypotheses If wheat residue leachates contain promoter/modifier/inhibitor complexes that stimulate or inhibit weed germination and radicle length, then the reintroduction of various combinations of organic and inorganic compounds removed by XAD-4 resin (or other sorbents) will provide a means to determine:

a. whether the individual IPA compounds are stimulatory, neutral, or inhibitory in the absence of other compounds present in GB solutions,
b. whether the IPA compounds are stimulatory, neutral, or inhibitory in the presence of other compounds in the GB solution, and

c. the identity of potential primary, secondary, etc. attributes (e.g., organic modifiers, pH, solute potential, and substrates) of residue leachates that may modify and regulate plant-plant allelopathic interactions.

Analyses Means comparisons and regression analyses could be used.

Comments The complexity of such bioassays can be overwhelming but that does not have to be the case if a step by step approach is taken. The proposed experimental approach is a step in the right direction in determining the potential roles and effects of specific compounds within complex mixtures of organic and inorganic compounds found in leachates and soil solutions. Clearly we are still a long way away from determining what happens in the field but such an approach is much more insightful regarding the potential roles and effects of individual compounds found in leachates and soil solutions than testing individual organic compounds in isolation.

6.4 Using Regression Analyses to Relate Potential Causes with Effects

Hall et al. (1983) found that ground freeze-dried sunflower tissue mixed into soil inhibited pigweed seedling dry weight. It was possible to partially simulate this inhibition with chlorogenic acid, a phenolic acid in sunflower. However, total phenolic acid in chlorogenic acid equivalents of sunflower tissues did not account for a significant amount of the inhibition of pigweed seedling dry weight unless total N, P, and K of the sunflower tissue was also included in the regression model. The effects of total phenolic acids and total N were negative whereas P and K were positive (P and K were determined for dry-ashed plant samples). These observations suggest that such an approach has possibilities particularly if more detailed analyses of tissues, residues, and soils are included in the regression analysis. Blum et al. (1992) proposed that testing soil extracts (surrogates for soil solutions) for biological activity (slope based on serial dilutions of extracts i.e., dose response) and then determining the relationships between initial soil extract characteristics (e.g., total phenolic acid, pH, water potential [based on freezing-point depression], and inorganic compounds [NO_3^-, NH_4^+, K and P]) and the biological activity of these extracts by regression analysis was a means by which potential cause and effect could be determined. They observed that biological activity, the slopes for radicle and hypocotyl lengths of clover seeds, became more negative with increasing total phenolic acid, pH, and water potential of wheat-no-till soil extracts. Effects were independent in this instance. Other soil extract characteristics such as NO_3^-, NH_4^+, K and P were not found to make a significant contribution to the biological response. The observed effects for higher pH values for wheat-no till soil extracts were counter to what one might expect given that phenolic acids require an acidic environment to be inhibitory (see Blum et al. (1992) and Blum (2011) for

details). The authors further suggested that the ratio of extracts with and without biological activity could be used as an indicator of allelopathic potential in field plots as long as field plots were adequately sampled. It would appear that the more holistic approaches taken by Hall et al. (1983) and Blum et al. (1992) provide another potential approach for identifying how various physical and chemical factors of tissues, residues, leachates (e.g., throughfall, fog drip, "root exudates plus", and residue leachates), and soil solutions influence weed seedling germination, emergence, and growth.

6.4.1 Residues

Objectives To characterize the chemical and physical characteristics of the different aged (weathered, fragmented, and decomposed) aboveground and belowground residue samples of wheat straw, stubble, root, and mycorrhizae.

To characterize how a natural combination of aboveground and belowground residues on and in the soil will modify soil characteristics when soil-residue systems are given different simulated-rain events.

To determine how weed seedling emergence may relate to the different chemical and physical characteristics of the residues added to the soil and the average chemical and physical characteristics of soil-residue systems given different rain events.

Materials and Methods A basic experimental design similar to the one described under *Residue Bioassays* Option 1 Sect. 4.3.4 was used with the following modifications:

a. Monthly samples of wheat straw, stubble, root and mycorrhizae residues were collected from the field, processed, and stored (see Sect. 4.3.2). Subsamples were analyzed for various chemical and physical characteristics (e.g., hydroxamic acids, phenolic acids, F-C reactive compounds, various organic acids, metabolic fingerprints or profiles, nutrients [NO_3^-, NH_4^+, K and P, etc.], C/N ration, state of fragmentation, etc.).
b. The most sensitive weed species based on preliminary bioassays was chosen for this study.
c. The amount and ratio of aboveground and belowground residues for the youngest (i.e., least aged) wheat residue samples collected in the field which clearly stimulated or inhibited weed seedling emergence were identified by preliminary bioassays.
d. The amount of residue identified in c. was added to all experimental units. Thus, the amount of aboveground and belowground residue added to all soil systems was identical but the chemical and physical characteristics of the different aged (weathered) residues added to the soil systems were different.
e. During the bioassay environmental factors of the soil-residue systems (e.g., light penetration to the soil surface, soil temperature, redox potential, moisture content, and pH, etc.) were monitored as frequently as possible.

The basic experimental design was as follows: 5 monthly residue samples ×1 residue amount ×1 weed species ×4 rain events ×5 replicates (blocks) =100 experimental units. Based on the results from previous bioassays (for example, see Sect. 4.3.4), some of the older non-active residues could be eliminated from the bioassay to reduce the number of experimental units. However, if monitoring of soil characteristics requires destructive sampling, then the number of experimental units would have to be increased.

The carbon or organic matter and F-C reactive compounds are surrogates for the promoter/modifier/inhibitor complex in leachates. The Folin–Ciocalteau reagent is reduced by substances with phenolic hydroxyl groups (e.g., cinnamic acids, hydroxybenzoic acids, gallic acid, tannic acids, and tyrosine), some substances without phenolic hydroxyl groups (e.g., pyridines, purines, alanine, cysteine, tryptophan, ascorbic acid, fructose, and proteins), some inorganic substances (e.g., iron sulphate, manganese sulphate, and potassium nitrate), and some other compounds (Anderson and Todd 1968; McAllister 1969; Box 1983; Ikawa et al. 1988; Blum et al. 1991, 1992). F-C equivalent values could be expressed in terms of any of these compounds although substances with phenolic hydroxyl groups have been the primary reference compound used in the literature. Metabolic profiles of the soil solutions (e.g., soil extracts) before and various times after the different residues were added to the soil could also go a long way toward identifying the range of organic compounds actually interacting with seeds and seedling roots in the soil for the different residue types and rain events. See Summer et al. (2003), Dunn and Ellis (2005), Xi et al. (2008), Prince and Pohnert (2010), and Sardans et al. (2011), and Sect. 6.6 for metabolic profiling. See Robinson (1980), Harborne (1984), Allen (1989), Pearcy et al. (1989), Walinga et al. (1995), Kalra (1998), Waller et al. (1999), Yu and Dahlgren (2000), and Sampietro et al. (2009) for residue analyses. See Bear (1964), Black (1965a, b), Page (1982), Klute (1986); Allen (1989), Carter (1993), Sparks (1996); Tan (1996), and Margesin and Schinner (2005) for soil analyses. See Pearcy et al. (1989) for environmental measurements.

Hypothesis If chemical and physical characteristics of different weathered, fragmented, and decomposing residues and soil-residue systems given different rain events are related in some quantitative way to the magnitude of stimulation or inhibition of weed seedling emergence, then it should be possible to identify specific residue and soil-residue system characteristics that are potentially responsible for the observed stimulation or inhibition of weed seedling emergence.

Analyses *Step 1:* How does the same weight of wheat residue at different stages of weathering, fragmentation, and decomposition treated with increasing volumes of simulated-rain multiple times affect weed seedling emergence for the experimental period?

Step 2: How do the chemical and physical characteristics of the residues and the average chemical and physical characteristics of soil systems relate to the stimulation or inhibition of weed seedling emergence?

Multiple regression analysis can be used to answer both these questions. For more complex statistical analysis, such as time series, modeling, etc. the reader is

advised to consult a statistician for proper statistical and analytical tools and techniques.

6.4.2 Soil Extracts

Objective To determine the biological activity of soil extracts from wheat residue and reference plots at monthly intervals by monitoring germination and radicle length of weeds.

To determine the relationships between biological activities of soil extracts and the full-strength extract characteristics of soil extracts obtained from soils taken from wheat residue and reference plots at monthly intervals.

Materials and Methods *Step 1 (Soil Extract and Extract Analysis):* Five soil samples were collected from each wheat residue and reference plot (see Sects. 4.3.1 and 4.3.2). Soil samples were taken to a depth of 10 cm with a 15 cm diameter core sampler at random locations within each plot between the weed subplots. Soil samples were taken at zero (right after), one, and two months after the wheat was harvested. Surface residues for the soil cores from each plot were removed and combined. Soil cores in each plot were combined, mixed, and sieved (0.25 mm) to remove rocks and belowground plant residues. Soil samples from each plot were then quickly frozen in liquid nitrogen, and stored in sealed containers in a −20 °C freezer. Aboveground and belowground residues were freeze dried, weighed, and stored in a desiccator in the dark for potential future use.

Soil samples were defrosted in a 5 °C refrigerator and extracted by the water-autoclave method (Blum et al. 1991, 1992; Blum 2011). Fresh soil and water ratio used for extracts were equivalent to 50 g dry weight of soil per 100 ml water (Note: This ratio may require adjustment depending on the biological activity of the extracts). Extracts were filtered with sterile funnels containing sterile Whatman #1filter paper to remove soil particles and stored in the refrigerator at 5 °C in sterilized containers. Storage time of extracts was short, i.e. no longer then a day. Subsamples of the extracts were analyzed for organic compounds (e.g., specific compounds such as phenolic acids, hydroxamic acids, various organic acids, FC-reactive compounds, and metabolic fingerprints or profiles (see Sects. 6.2 and 6.6)), inorganic compounds (e.g., NO_3^-, NH_4^+, K and P), pH, solute potential, C/N ratio, etc.. The final total number of soil extracts =60 (4 treatments [3 seeding levels plus reference] ×3 soil sample times ×5 blocks). See Rodier (1975), Allen (1989), Buurman et al. (1996), and/or Nollet (2000) for solution analyses. See Sect. 6.4.1 for other analyses.

Step 2 (Biological activity of soil extracts): Serial dilutions (units of extract/unit of simulated-rain water (see Sect. 4.3.3); full, three quarter, half, and quarter strength dilutions) of each soil extract were added to sterile Petri dishes containing sterile #1 Whatman filter paper and weed seeds (morning glory [*Ipomoea hederacea* L.], pigweed [*Amaranthus retroflexus* L.], or prickly sida [*Sida spinosa* L.]). Petri dishes and filter paper were sterilized by autoclaving (121 °C, 1.2 kg.cm^2, for

15 min) and simulated-rain water was filter sterilized (0.2 μm membrane filter) but seeds were not surface sterilized. Volumes of extracts added to Petri dishes and weed seeds per Petri dish were based on seed size but was identical for all experimental units for a given weed species (see Sect. 3.2.2.6). Germination and radicle lengths were measured after several days; the number of days was determined by the weed species. Germination and radicle growth were carried out in a growth chamber with a relative humidity of 70 % and a photoperiod and day/night temperature mimicking the times when residues were collected. For additional experimental details see Sect. 4.2.2.2. The basic experimental design was as follows: 20 treatments (15 [3 seeding rates × 5 blocks] wheat residue extracts and 5 reference plot extracts) × 3 soil sample times × 4 dilutions = 240 experimental units for each species. In order to make the bioassay more manageable, the number of sampling times could be reduced, the number of weed species could be reduced to the most sensitive species, and/or the number of reference soil extracts could be reduced.

The serial dilution series selected for biologically active extracts must produce a linear response slope; otherwise the serial dilution series should be readjusted. Based on preliminary bioassays, the initial dilution of soil extracts can be determined and insensitive weed species can be eliminated to reduce the number of experimental units. As stated in Chap. 3 Sect. 3.2.5, serial dilutions of soil extracts are artificial when viewed from field soil perspective but here the only interest is in the biological activity (an index) of a given individual soil extract. Only the chemical and physical characteristics of the full-strength (initial undiluted) soil extracts were used in the subsequent regression analysis. Seeds may be surface sterilized. However, obtaining seed lots in which all the seed surfaces are sterile is extremely cumbersome and time consuming. In addition depending on species, sterilization procedures can impact germination and radicle growth (e.g., see Blum et al. 1984). Surface sterilization will not eliminate microorganisms within seeds (Leben 1961; Mundt and Hinkle 1976). Solutions added to Petri dishes should be at room temperature. After imbibitions a small amount of free solution should still be present in the Petri dish. Multiple additions of treatment solutions and simulated-rain water should be avoided if at all possible since such additions change the concentrations of organic and inorganic compounds, the water potentials, and/or the pH of the treatment solutions. Relative humidity should be kept as high as possible to minimize evaporation from the Petri dishes. Petri dishes may also be sealed to minimize evaporation but anaerobic conditions may be a concern.

Step 3 (Multiple Regression analysis): The final step is to determine the relationships between biological activities (i.e., linear slopes) and full-strength soil extract characteristics.

Hypothesis If germination and radicle length of weed seeds treated with serial dilutions of a soil extract have a significant linear positive or negative slope, then the slope may be used as a surrogate for the biological activity of that soil extract (see Step 2).

If soil extracts are representative of soil solutions and biological activities (i.e., slopes for weed germination and radicle length) and effects of soil extracts on seed

germination and radicle length are determined by their chemical and physical characteristics, then significant relationships between biological activity (slopes for germination and radicle length) and full strength soil-extract characteristics should provide insight regarding the potential direct or indirect effects of water-soluble compounds released from wheat residues into the soil (see Step 3).

If the previous hypothesis is true, if each treatment plot and its replicates have been adequately sampled, and if biological activity is related to the active water-soluble compounds (i.e., promoters, modifiers, and/or inhibitors) in extracts and not related solely to the physical characteristics of extracts, then the frequency of significant biological activity for the soil samples taken from each treatment plot and its replicates may be used as an index of the potential importance of plant-plant allelopathic interactions in plots (see Step 3).

Analyses For Step 2 use regression analysis with germination or radicle length as dependent variable and dilution of soil extract as independent variable. For Step 3 multiple regressions may be used to determine significant relationships between biological activities (i.e., significant linear regression slopes) of soil extracts and full-strength soil extract characteristics (e.g., hydroxamic acids, phenolic acids, various other organic acids, FC- reactive compounds, metabolic fingerprints or profiles, nutrients, pH, water potential, C/N ratio, etc. (see Sects. 6.4.1 and 6.6)). Soil extracts without biological activity (i.e., non-significant slopes for serial dilutions) should not be included in the multiple regression analysis. In order to determine the potential importance of plant-plant allelopathic interactions for wheat treatments, the frequencies and patterns of significant and non-significant extract slopes for the various treatment plots over time can be used as an indication of the potential importance of plant-plant allelopathic interactions for each treatment plot.

Comments The actual merit of this approach depends on a variety of assumptions which may or may not be valid. Among these assumptions are the following:

1. That chemical and physical characteristics of soils and their soil-water extracts are modified by increasing or decreasing amounts of aboveground and belowground wheat residue biomass.
2. That the modifications of aboveground and belowground wheat residues on soil chemical and physical characteristics and their soil-water extracts are moderated by weathering, fragmentation, and decomposition of wheat residues.
3. That physical and chemical characteristics of soil samples and their soil-water extracts are consistently related to each other.
4. That the initial soil-water extracts are actually representative of soil solutions, although they are diluted versions of soil solutions. (Note: The initial dilution is determined by the water/soil ratio selected and used. What is recovered will be determined by the extraction procedure used. In wet or mesic soils, soil solutions can be collected by centrifugation or with lysimeters and compared to soil extracts to determine similarities and differences between the two.)
5. That the water-soluble compounds recovered by the soil extraction procedure used are representative of residual water-soluble compounds in soil solutions

and that the functional characteristics of the water-soluble compounds can be identified and quantified.
6. That dose responses determined by serial dilutions of soil extracts actually represent biological activity.
7. That co-linearities and complex interactions, for example, do not hide the roles of causative agents.

If we assume that all these assumptions are valid, then the results from this series of procedures can provide considerable insight about potential causes and effects for observed stimulation or inhibition of weed germination and radicle growth by soil solutions in wheat-residue soil systems (see Sect. 4.4.4 for the limitations of testing soil extracts).

6.5 Treatment Surface Areas

Presently there are still a number of important field factors (e.g., relevant densities, symbiotic relationships, treatment concentrations, treatment surface areas, microbial populations, and physicochemical environments) that continue to be poorly represented in laboratory bioassays (see Chaps. 2 and 3). The factor to be discussed here is the treatment surface area. Given the small surface areas of most seeds, this probably is not a major concern for seeds. It is also probably not a major concern for germinating seeds and seedlings right after their initial emergence since their radicle and root surface areas, respectively, will still be small. It is, however, an important issue for root and mycorrhizal systems with sizable surface areas and extensive distributions particularly when the allelopathic compounds (i.e., promoters or inhibitors) are not systemic in their actions and when the promoters, modifiers, and inhibitors are unevenly distributed in the soil (also see Sect. 2.1.1.4). Unfortunately, for the majority of seedling and older plant bioassay studies in the literature the potential modifying factor of treatment surface area has been ignored, i.e., as a general rule, the entire root or mycorrhizal system has simply been treated.

One way of determining the potential role of treatment surface area is to treat only parts of root and mycorrhizal systems with IPA compounds (Lyu and Blum 1990; Klein and Blum 1990; Lehman et al. 1994), leachates, residues, etc. This may be done in solution or substrate bioassay similar to what has been done in studies for induction of resistance to fungi, nodulation, carbon transport, respiration, water potential of root segments, net rhizodeposition of N, root response to localized K, and root competition to name a few examples (Whitcomb et al. 1969; Adeoye and Rawlins 1981; Schumacher and Smucker 1985; Merbach et al. 2000; van Brussel et al. 2002; Kaur and Singh 2007; Fernández et al. 2011). Lyu and Blum (1990), Klein and Blum (1990), and Lehman et al. (1994) working with nutrient bioassay systems found that percent inhibition increased as the proportion of root systems exposed to a given concentration of ferulic acid or *p*-coumaric acid was increased. (Note: Ferulic acid and *p*-coumaric acid are not systemic in their actions but local

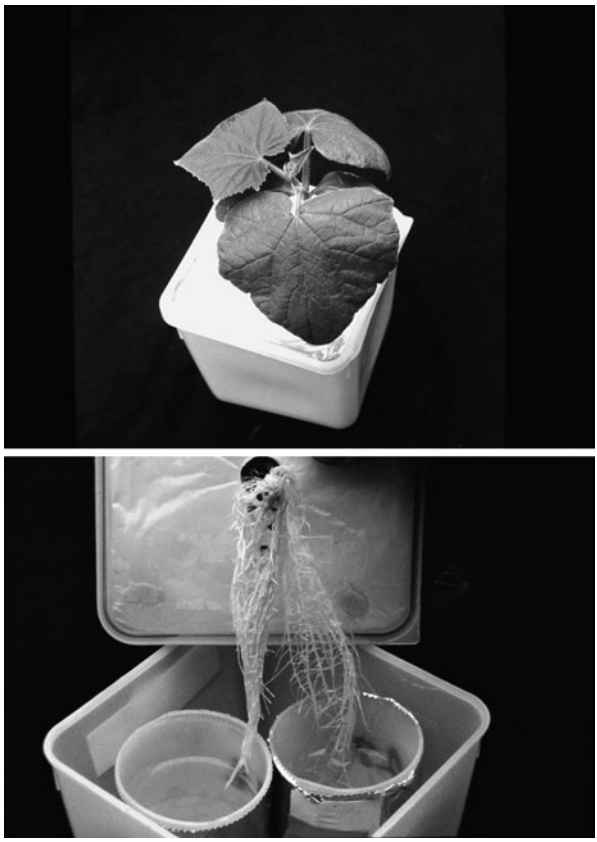

Fig. 6.3 An example of a split-root system containing a cucumber seedling . (Bottom figure taken from Blum (2011), figure used with permission of Springer Science and Business Media)

in action (Blum 2006, 2011).) The following are two examples (Options) of the way the potential role of treatment surface area may be determined.

Objective To determine how seedlings grown in nutrient, sand, or soil culture are stimulated or inhibited by a range of concentrations of IPA compounds, leachates, and residues and how effects of these treatments are modified as the proportions of the root and mycorrhizal systems in contact with these treatments are modified.

Materials and Methods *Option 1:* Morning glory (*Ipomoea hederacea* L.), pigweed (*Amaranthus retroflexus* L.), or prickly sida (*Sida spinosa* L.) seedlings were grown from seeds in flats containing sand. Flats were watered daily with Hoagland's nutrient solution. Once seedlings were sufficiently large enough, seedlings of equal size were gently removed from the sand. Seedling roots were then split between two containers (Fig. 6.3; 4.5 × 9 cm, 110 ml), located within a third container (10 × 10 cm). The lid of the third container was used to suspend seedlings by a foam collar into the two containers (A and B). Another function of the third container was to protect the solutions and the root systems from light. The pH, solute potential, and inorganic content of all treatment solutions were adjusted to

be identical (see Sects. 4.2.1, 4.3.3, and 5.2.3.1; Blum et al. 1992). That can be easily achieved for IPA compounds in nutrient solution but is much more difficult to achieve for leachates (e.g., leaf, residue, and "root exudates plus" leachates).

Treatment combinations were as follows:

a. A and B with reference solution (nutrient solution; pH 5.5),
b. A and B with treatment solution (phenolic acid in nutrient solution; pH 5.5), and
c. A with reference solution and B with treatment solution.

The concentration of the phenolic acid treatment solutions for b. and B of c. were varied (e.g., 0.125, 0.25, 0.5, and 0.75 mM phenolic acid [e.g., ferulic acid] or mixtures of phenolic acids or various concentrations of leachates). The portion of the root system in each container for a., b., and c. was varied between the A and B containers (e.g., values in percent; a. reference (A)/reference (B)—25/75, 50/50, 75/25; b. treatment (A)/treatment (B)—25/75, 50/50, 75/25; and c. reference (A)/treatment (B)—25/75, 50/50, 75/25). (Note: Instead of visual estimates, root displacement volume could also be used.) Distilled water was added to each container twice a day (morning and evening) to compensate for transpiration (i.e., containers were brought back to the original level). Solutions were completely replaced every other day so that the initial phenolic acid and nutrient concentrations, solute potentials, and pH values of the solutions could be maintained and microbial populations could be kept in check. Solutions were replaced 4 times. Bioassays were carried out in light banks (140–180 µmol/cm^2/sec) with a photoperiod identical to field conditions and a normal range of field temperatures. Minimum and maximum air temperature and water utilization were recorded daily. Root and shoot dry weights (100 °C) were determined at the end of the experiment. Root dry weight was determined separately for each container and used to calculate the proportion of roots treated with IPA compounds, leachates, etc. The seedlings were harvested after 5 treatments, i.e., 10 days. The basic experimental design was as follows:

a. References (reference solutions in A and B): 3 proportions × 4 replicates = 12,
b. Treatments (phenolic acid solutions in A and B); 3 proportions × 4 concentrations × 4 replicates = 48, and
c. Treatment (reference solution in A and phenolic acids in B): 3 proportions × 4 concentrations × 4 replicates = 48 (total = 108 experimental units per weed species).

The root systems were not inoculated with mycorrhizal fungi. However if the time period of the bioassay is sufficiently long, roots may be inoculated. Individual compounds (e.g., treatment solutions for phenolic acids) were dissolved in Hoagland's nutrient solution and, thus, Hoagland's nutrient solution was used as the reference solution (Hoagland and Arnon 1950). For methods to determine water utilization, see Blum and Gerig (2005). It might also be interesting to see how the metabolic profiles of seedling shoots differ for the seedlings with various percent treatment surface areas (see Sect. 6.6).

Option 2: Morning glory (*Ipomoea hederacea* L.), pigweed (*Amaranthus retroflexus* L.), or prickly sida (*Sida spinosa* L.) seedlings were grown from seeds in flats

containing sand. Flats were watered daily with Hoagland's nutrient solution. Once seedlings were sufficiently large, seedlings of equal size were gently removed from the sand. Seedling roots were then transplanted into pots (15 cm in diameter and 1767 cm^3 volume) with a sealed partition in the center of the pots and two holes in the base of each pot, one on each side of the partition. Weed root systems were inoculated with mycorrhizal fungi (Menge and Timmer 1982) and divided between the two compartments in a manner similar to that of the nutrient bioassay system of Option 1 and 75 g of a 2:1 soil-sand mixture containing one concentration of coarsely ground wheat residue with 4 different chemical compositions (Sect. 6.4.1) was added to both compartments of b. and one compartment of c. (see design below). The remaining compartments were filled with 75 g of a 2:1 soil-sand mixture containing the same concentrations of coarsely ground inactive or inert residues (see Putnam and DeFrank 1983; Putnam et al. 1983; For inactive or inert materials it may also be possible to use highly leached or aged inactive or inert wheat residues.). Soil was obtained from the wheat reference plots (Sect. 4.3.2). An effort was made to maximize the distribution of the root systems within each compartment. The top of the partition was located even with the soil level and seedlings were stabilized with rods attached to the sides of the pots. Equal amounts of water and nutrient solution were supplied on alternate days to each side of the partition. Since evapotranspiration from each compartment will vary depending on the proportion/size of the root system it contains, considerable care was taken to avoid different moisture levels within the compartments. Holes in the base of each compartment prevented the moisture content of the soil from going beyond field capacity and prevented the formation of an artificial water table. Leaching from compartments was controlled and minimized by adjusting the volumes of solution added. Adjustments, however, were such that all compartments contained roughly the same volume and had the same moisture levels for each solution addition. This was achieved by only saturating the soil each time compartments were supplied with solutions. Bioassays were carried out in light banks (140–180 µmol/cm^2/sec) with a photoperiod identical to field conditions and a normal range of laboratory temperatures. Both active and inactive coarsely-ground residues were analyzed for various chemical and physical characteristics (e.g., hydroxaminc acids, phenolic acids, various organic acids, FC reactive compounds, metabolic fingerprints or profiles (see Sect. 6.6), nutrients [e.g., NO_3^-, NH_4^+, K and P], C/N ratio, states of fragmentation, etc. (see Sect. 6.4.1)). Minimum and maximum air temperatures were recorded daily. The seedlings were harvested on day 14 and root and mycorrhizal dry weight in each compartment and shoot dry weight per pot (100 °C) were determined. Root and mycorrhizal dry weight was determined separately for each side of the partition and used to calculate the proportion of roots and mycorrhizae treated with active residues. Subsamples of the root/mycorrhizal systems from each compartment were analyzed for the presence and frequency of mycorrhizae before they were freeze-dried to determine effects of residues on mycorrhizal formation and development (see Kormanik and McGraw 1982). The basic experimental design was as follows:

a. References (compartments A and B with inert or inactive residues; see Option 1) 3 root and mycorrhizal proportions × 4 replicates = 12,
b. Treatments (compartment A and B with wheat residues); 3 root and mycorrhizal proportions ×4 wheat residues with different chemical compositions ×4 replicates =48, and
c. Treatments (compartment A with inert or inactive residues and B with wheat residues): 3 root and mycorrhizal proportions ×4 wheat residues with different chemical compositions ×4 replicates =48 (total =108 experimental units per weed species).

All system compartments had identical amounts of residue. Compartments contained the same amount of either inert or inactive residues or wheat residues with different chemical compositions. Different chemical compositions of residues in this instance were achieved by utilizing aging residues from the field. Different chemical compositions for residues could also be generated by growing plant materials under different physicochemical environments (Rice 1984; Hall et al. 1982, 1983) or possibly by using different crop varieties. Residues were coarsely ground to maximize contact between root and mycorrhizal surface areas and residues within a compartment were evenly distributed. Instead of analyzing residues, soil solutions (e.g., soil extracts or leachates) from each side of the pots could be analyzed by metabolic profiling (see Sect. 6.6).

Hypotheses If the actions of IPA compounds and compounds released from residues are systemic in action, then changing the proportion of the roots and mycorrhizae in contact with these compounds will probably not dramatically modify the observed stimulation or inhibition of the shoot biomass of seedlings.

If the actions of IPA compounds and compounds released from residues are not systemic in action, then changing the proportion of the roots and mycorrhizae in contact with these compounds will dramatically modify the observed stimulation or inhibition of the shoot biomass of seedlings.

Analyses Regression analysis can be used to determine how shoot biomass (dependent variable) is modified by treatment concentrations or residue chemistry and root (or soil solution chemistry) and mycorrhizal treatment surface area (independent variables).

Comments These approaches can provide theoretical insight regarding the way root and mycorrhizal surface treatment area can modify the potential effects of IPA compounds (or any other compounds of interest) in the absence of soil or complex mixtures of both organic and inorganic compounds in the presence of soils. However, due to the atypical nature of bioassay systems and the unknown distribution of roots, mycorrhizae, and active compounds (i.e., promoters, modifiers, and inhibitors) within field soils there presently appears to be no practical way to apply this insight to day to day plant-plant interactions occurring within field soils. It might, however, be very interesting to see how different or similar metabolic profiles of roots, mycorrhizae, and/or shoots from laboratory and field systems may be related to each other.

6.6 Using Omics Methods as Tools

Up to this point the primary research focus has been on secondary, tertiary, etc. effects (frequently just called secondary effects) of the action of allelopathic compounds such as phenolic acids on plants (e.g., nutrient and water uptake, cell expansion, germination, seedling emergence, and seedling growth and development) and how the effects of allelopathic compounds such as phenolic acids are modified by a range of physicochemical and biotic factors in model bioassay systems. In the process some insight has been gained regarding the potential primary sites and modes of action of some allelopathic compounds. However, due to the multiple sites of action for allelopathic compounds and the complex interactions between cells, tissues, and organs of seeds and plants, it has been difficult to determine with any certainty that the primary sites and modes of action identified were actually directly responsible for the ultimate observed effects on germination and seedling growth. Since initial actions of allelopathic compounds will occur at the molecular and cellular levels (e.g., gene expression, cell walls and membranes, and organelles), the location of their actions would suggest that the research focus for determining primary sites and modes of action should ultimately be at those level. A word of caution, however, effects at the molecular and cellular levels are not always expressed at the tissue, organ, seed or seedling levels because cells can frequently adjust or compensate (e.g., shift to alternative metabolic pathways) and at times eliminate (e.g., detoxifcation) the actions of external agents such as allelopathic compounds. Therefore, effects observed on the molecular and cellular levels have to be anchored to the effects observed at higher levels. In the past because of tools and methods available, researchers were largely limited to looking at individual or small subsets of cellular processes. With the development of omics methods in the last 10 years or so, the cellular processes and the metabolic products that can be looked at concurrently have increased enormously.

Omics methods such as genomics, proteomics, metabolomics, and physionomics have been used to study a broad range of topics related to plants and microorganisms (Bais et al. 2003; Summer et al. 2003; Dunn and Ellis 2005; Macías et al 2007; Duke et al. 2008, 2013; Prince and Pohnert 2010; Kim et al. 2011; Metz 2011; Sardans et al. 2011; see Sect. 6.1). These methods have allowed researchers to view at the molecular level the actions of a variety of external agents (e.g., herbicides, competitors, phenolic acids, microorganisms, herbivores, and salinity) on plants (Bais et al. 2003; Gidman et al. 2003; Johnson et al. 2003; Grossmann 2005; Grossmann et al. 2012; Badri et al. 2013; D'Abrosca et al. 2013; Marti et al. 2013) and the functions and actions (e.g., decomposition, detoxification, modification of herbivore feeding behavior, and synthesis of secondary metabolites) of microorganisms in the rhizosphere and/or in the soil (Narasimhan et al. 2003; Wallenstein and Weintraub 2008; Fan et al. 2009; Liebeke et al. 2009; Carlsen et al. 2012; Nuguyen et al. 2012; Badri et al. 2013). They have also been valuable in determining the composition of organic mixtures (predominately polar molecules, e.g., primary and secondary metabolites) in and released from plant tissues, litter and microorganisms and in soils (Fan et al. 2009; Liebeke et al. 2009; Carlsen et al. 2012; D'Abrosca et al. 2013;

Table 6.1 Some examples of the application of omics methods to plant-plant allelopathic interactions

Omics methods	Applications	References
Genomics	Inhibition of *Arabidopsis* by the phytotoxin (-)-catechin, Ca^{2+} signaling, genome-wide changes in gene expression	Bais et al. 2003
	Detoxification and transcription response to benzoxazolin-2(3H)-one (BOA) in *Arabidopsis*	Baerson et al. 2005
	Gene expression of *Arabidopsis* in response to allelochemicals in buckwheat	Golisz et al. 2008
	Effects of the phytotoxin on gene expression in *Arabidopsis*; induction of defense mechanisms against BOA	Duke et al. 2008
	Gene expression in juglone-treated rice roots	Chi et al. 2011
Proteomics	Comparative proteomics were used to unveil the molecular mechanism of allelopathic stimulation induced by the extracts of acyranthe	Li et al. 2011
Metabolomics	Effects of juglone, coumarin, catechol, *p*-hydroxybenzoic acid, ferulic acid and *p*-coumaric acid on the metabolome of wild wheat (*Aegilops geniculata* Roth)	D'Abrosca et al. 2013

Table 6.2 Some additional examples of how omics methods could be applied to plant-plant allelopathic interactions

Omics methods	Applications	References
Genomics	Secondary metabolism	Chen et al. 2009
Genomics and metabolomics	Combing genomic, metabolome analysis, and biochemical modeling	Fiehn 2001
Genomics and proteomics	Soil extracellular enzymes	Wallenstein and Weintraub 2008
Genomics, proteomics, metabolomics, and physionomics	Modes of action	Duke et al. 2013
Transcriptomics and proteomics	Complexity and coordination of root growth	Yamaguchi and Sharp 2010
Metabolomics	Fingerprinting	Johnson et al. 2003; Bertram et al. 2010
	Large-scale phytochemistry	Summer et al. 2003
	Identification of metabolites in complex mixtures	Xi et al. 2008
	Bacteria growing in soil extracts	Liebeke et al. 2009
	Fate of secondary compounds in soil	Carlsen et al. 2012
	Secondary metabolism of mircobes	Nguyen et al. 2012
	Signal molecules	Barofsky et al. 2009; Prince and Pohnert 2010
	Effects of soil microorganisms on leaf metabolites	Badri et al. 2013
	Rhizopshere and plant-microbes interaction	Narasimhan et al. 2003
Physionomics and metabolomics	Modes of action	Grossmann 2005; Grossmann et al. 2012

Marti et al. 2013). For maximum benefit the molecular results from different omics methods must also be anchored to each other as well as to the secondary, tertiary, etc. effects (see Fiehn 2001; Bais et al. 2003; Summer et al. 2003; Chen et al. 2009; Yamaguchi and Sharp 2010; Reaves and Rabinowitz 2011; Sardans et al. 2011; Prince and Pohnert 2010).

Unfortunately with minor exceptions these methods have not been directly applied to the study of plant-plant and plant-microbe allelopathic interactions. Examples that the author could find for potential allelopathic interactions are provided in Table 6.1 and some additional examples that could be readily applied to potential allelopathic interactions are provided in Table 6.2. Given the enormous potential of these methods as tools in resolving basic questions regarding primary sites and modes of action of physiochemical and biotic factors including allelopathic compounds, identifying and quantifying the compositions of leachates, exudates, soil extracts, plant tissues and litter, and relating or anchoring molecular processes to each other and to secondary, tertiary, etc. effects, researchers should now take advantage of these methods for the study of plant-plant and plant- microbial allelopathic interactions in relevant laboratory bioassay systems and field systems.

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Chapter 7
Laboratory Model Systems and Field Systems: Some Final Thoughts

Abstract This Chapter compares field systems with past and present laboratory bioassay systems, provides some thoughts on how to minimize the impacts of atypical factors in present seed and seedling laboratory bioassays, points out which factors limit our ability to design field-relevant model systems, suggests future directions for laboratory and field research on plant-plant allelopathic interactions in a question format, and outlines the central tenets (i.e., opinions, doctrines, or principles) articulated in this volume.

7.1 Introduction

Because of the reductionist nature of past and present laboratory bioassay systems (Sects. 3.2 and 7.2) and the perceived unnatural treatment concentrations required for stimulation or inhibition within laboratory bioassay systems, the observed allelopathic effects in the laboratory have been categorized by critics as atypical, misleading, and false for field systems (e.g., Harper 1975, 1977; Stowe 1979). In other words according to the critics, the stimulation or inhibition observed for putative allelopathic compounds tested in laboratory bioassay systems do not reflect adequately or do not reflect at all the effects of these compounds in the field. In addition critics have pointed to a past history of poorly designed laboratory bioassays, false assumptions and misconceptions about laboratory bioassays, and erroneous conclusions and generalizations derived from laboratory bioassays about field systems (Sects. 1.7). The latter are unfortunate but not uncommon in the early stages of most, if not all, evolving scientific disciplines. Most critics, however, will agree that laboratory bioassay systems can and do provide valuable theoretical insights regarding potential primary sites and modes of action of putative allelopathic compounds.

The author would agree that the past and present reductionist nature of laboratory bioassays is a problem when attempting to use laboratory bioassay systems as surrogates for field systems. After all they only model a small part of any given field system and, depending on the system used, have missing or atypical factors (see Chaps. 2 and 3). However, it is the assertion of the author that by designing laboratory bioassay systems that are more holistic or more field-relevant and by reducing or eliminating atypical factors, laboratory bioassay systems can be linked more closely to field systems and, thus, make laboratory bioassay systems more

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useful and insightful when it comes to identifying primary sites and characterizing modes of action, processes, and effects of plant-plant allelopathic interactions.

7.2 Comparison of Field and Present Laboratory Model Systems

A first step in designing laboratory bioassay systems which are more field-relevant is to understand the differences and similarities of field systems and laboratory systems. Many of the essential similarities and differences have already been described in previous chapters, in particular Chaps. 2 and 3. Only a summary of their major similarities and differences are provided here. The reader, however, should recall that the focus in this volume has been on the actions of hydrophilic compounds in bioassay systems at the exclusion of the actions of hydrophobic and volatile compounds. That is with the exception of field and residue bioassays where the actions of all three types of compounds can occur. A hydrophilic compound (molecule) typically has an affinity for water, is usually charged or has polar side groups, and is capable of hydrogen bonding enabling it to more readily adsorb/absorb water and to be dissolved in water or other polar solvents. Some hydrophilic compounds such as calloids do not dissolve. A hydrophobic or lipophillic compound (molecule) typically is repelled by water (insoluble), tends to be electrically neutral [no charge], and non-polar enabling it to more readily dissolve in neutral or non-polar solvents or molecular environments. Molecules can have both hydrophilic and hydrophobic sites. Fatty acids for example which are amphipathic have a hydrophilic head and a hydrophobic tail allowing them to dissolve in both polar (e.g., water) and non-polar solvents (e.g., oils). A volatile organic compound has a high vapor pressure, a low boiling point, tends to be hydrophobic, and evaporates or sublimates from liquids or solids at room temperature. The level of volatility for organic compounds varies from low to high. See the following references for additional details (Gauthier 1997; McNaught and Wilkinson 1997).

7.2.1 Inputs of Water-Soluble Compounds

Field soil systems have a number of potential input sources of water-soluble compounds (e.g., leaf leachates, “root exudates plus”, leachates of litter, residues, and organic matter). The input concentrations, volumes, and frequencies of these sources are largely regulated by environmental events (e.g., timing, volume and frequency of rain events, growth rates of roots and mycorrhizae, substrate mechanical resistance to root and mycorrhizal growth, timing of senescence and mortality, and activity of soil organisms to name a few). The distributions of inputs (i.e., locations on and in the soil) and concentrations, volumes, and frequencies of inputs are, thus, highly variable and largely asymmetrical over time and location in field

soils. The inputs are composed of complex mixtures of water-soluble organic and inorganic compounds (i.e., promoter/modifier/inhibitor complexes) that, depending on their compositions, concentrations, environments, and locations, may be neutral (inactive) or function as promoters or inhibitors for seed, seedling, and older plant processes or end products (e.g., biomass of tissues, organs, seedlings, and older plants) being monitored. Modification of the water-soluble organic compounds by soil organisms and the synthesis of new organic compounds by them is another type of input to field soil systems which can also determine the outcome. In field systems transfer between plants by mycorrhizae and grafts may also be considered as inputs or losses depending on the direction of transfer. See Sects. 3.6 and Chap. 1 of Blum (2011) for additional details.

The inputs of water-soluble compounds to laboratory bioassay systems are determined by the composition, concentration, volume, and frequency of treatment solutions (e.g., identified putative allelopathic (IPA) compounds, leachates, exudates, etc.) and/or the living or dead plant material (e.g., actually leachates of materials such as tissues, litter, or residues) added to a bioassay system. The composition, concentrations, volumes, and frequencies of these inputs are, thus, determined by the researcher (e.g., where and how inputs were collected, processed, formulated, and applied). Distributions of inputs within bioassay systems can be variable (e.g., horizontal or vertical gradients in solid media) but may also be fairly homogeneous (e.g., solution cultures). The frequency of input may consist of a single event, multiple events at regular intervals, or continuous additions. The inputs may be composed of a single organic compound or a range of mixtures of organic and/or inorganic compounds which, depending on the bioassay system and environment, will have no effect (be neutral or inactive) or function as promoters or inhibitors for seed, seedling, and older plant processes or end products being monitored. Modification of water-soluble organic compounds by microorganisms and synthesis of new compounds by them is another type of input for laboratory systems which have not been sterilized or sterilized and inoculated with microorganisms. These modified or synthesized compounds can also influence the outcome. Media for laboratory bioassay systems range from liquids to solids such as nutrient solutions and soil, respectively. Laboratory bioassays generally tend to focus on only one type of input (e.g., IPA compounds, leachates, exudates, litter, or residues) and a single input event in the field (e.g., a rain event, litter event, etc.).

7.2.2 Losses of Water-Soluble Compounds

For field systems the term loss refers to the removal or transfer of water-soluble compounds from soil solution at a particular location. The primary locations of interest for plant-plant allelopathic interactions are the spermosphere of seeds, the rhizosphere of roots, and the mycorrhizosphere of mycorrhizae for the species of interest. There are a number of soil sinks or locations to which water-soluble compounds are partitioned or transferred, respectively, in field soil systems. Among them are:

a. sorption to soil organic and inorganic particles,
b. conversion, utilization, and polymerization by soil organisms,
c. uptake by seeds, roots and mycorrhizae by the species of interest,
d. uptake by associated seeds, roots, and mycorrhizae other than the species of interest,
e. oxidation/reduction and polymerization not associated with soil organisms, and
f. horizontal and vertical movements primarily due to capillary action, transpirational pull, and gravitational flow.

The transfer between plants by mycorrhizae and grafts in field systems could also be considered as losses or inputs depending on the direction of transfer. See Sect. 3.6 and Chap. 1 of Blum (2011) for additional details. Losses from laboratory bioassays depend on the type of system being utilized. For example:

a. Solution culture
    1. utilization and conversion by microorganisms (primarily bacteria and fungi),
    2. uptake by seeds, roots and mycorrhizae by the species of interest (Note: This frequently occurs in the absence of other plant species.), and
    3. oxidation/reduction not associated with microorganisms. (Note: Oxidation can be an important process, particularly for aerated solutions.)
b. Solution additions to soil culture
    1. sorption to organic and inorganic soil particles,
    2. utilization, conversion, and polymerization by soil microorganisms (primarily bacteria and fungi),
    3. uptake by seeds, roots and mycorrhizae by the species of interest (Note: This usually occurs in the absence of other plant species.),
    4. oxidation/reduction and polymerization not associated with soil microorganisms,
    5. vertical movement primarily due to gravitational flow (i.e., leaching below seeds, roots, and mycorrhizae,
    6. horizontal and vertical movement by capillary action and transpirational pull, and
    7. losses by leachates from containers with holes in the bottom.

7.2.3 Timing and Frequency of Inputs and Losses

Timing and frequency of inputs of water-soluble compounds to field soils are highly variable and largely asymmetrical over time and location. The variability and asymmetry of inputs stems from the fact that:

a. the chemical composition of living tissues changes over time,
b. the timing, frequency and volume of rain events in temperate regions of the world are unpredictable and, therefore, inputs by leaf leachates are irregular and unpredictable,

c. production and release of "root exudates plus" by roots and mycorrhizae will vary with the age and distribution of roots, growth environment, and the action of soil organisms,
d. the input by aboveground and belowground residues in the temperate regions of the world occur primarily in the fall but also can occur anytime during the growing season particularly after stressful events such as drought (Note: In managed systems the timing, mode, frequency, and distribution of inputs can be manipulated by management practices.), and
e. the release of water-soluble compounds from residues varies with tissue type, time of senescence and mortality, rain events, soil moisture, weathering, and action of soil organisms (e.g., fragmentation, decomposition, and conversion and synthesis of new compounds).

The variability and asymmetry of input events creates fluctuations (i.e., peaks and valleys) in inputs of water-soluble compounds throughout the growing season. However, there is sufficient overlapping of different input events to produce essentially some continuous input of water-soluble compounds, not only during the growing season but also at other times of the year. The rates and amounts of input vary dramatically with environmental events and management practices (e.g., irrigation, plowing, etc.) during any given season of a year or year.

The rates of soil losses (e.g., sorption, utilization and conversions by soil organisms, uptake by seeds, roots and mycorrhizae, etc.) tend to follow the rates of inputs as long as environmental conditions are not limiting. It is, however, not uncommon particularly for mature field systems to find that inputs and losses reach a quasi steady state where available compounds such as phenolic acids for example are detectable in the soil and soil solution throughout the year at fairly constant concentrations (Whitehead 1964; Whitehead et al. 1981, 1982; Blum et al. 1991; Muscolo and Sidari 2006). However, when it comes to specific individual water-soluble compounds, they may only be detectable in the soil for specific time periods.

In laboratory systems, the timing, frequency, inputs, and losses are associated with a single event or tend to be regular and very predictable (i.e., very symmetrical) for multiple events and continuous inputs. Environmental conditions as a rule are fairly constant over time. In general after a treatment event, the concentrations of water-soluble compounds tend to decline fairly rapidly, and, given sufficient time, will go below detection limits. The rates of decline, however, are compound specific.

7.2.4 Treatment Surface Areas

In field systems the portion of seed, root, and mycorrhizal surface area contacted by available and active water-soluble organic and inorganic compounds is highly variable in time, frequency, and location and to a large extent is unpredictable. At any given point in time the likelihood that the entire root and mycorrhizal surface area of a developing seedling or older plant of interest are impacted is small and the larger and more extensive the root and mycorrhizal system, the smaller the

likelihood. For seeds, because of their small surface area, full surface area contact is common when it occurs. In most laboratory bioassays the entire seed, root, and mycorrhizal surface area are usually treated. For attempts to correct this disparity between laboratory and field systems see Sect. 6.5.

7.2.5 Microorganisms (Microflora and Fauna)

Field systems contain very diverse numbers and types of soil organisms (micro-, mesa-, and mega-organisms) which are highly variable in their actions depending on their location (e.g., bulk soil, spermosphere, rhizosphere, mycorrhizosphere, rhizoplane, and endorhizosphere) and environment (see Sects. 3.6 and 3.7). Mycorrhizae are frequently present. Laboratory bioassay systems generally contain only microorganisms, primarily bacteria and fungi (in soils actinomycetes are also common) and, due to the small size of most bioassay systems, consist primarily of organisms that function in the spermosphere, rhizosphere and mycorrhizosphere, on the rhizoplane, and in the endorhizosphere. Mycorrhizae are frequently absent. This is true for all solution cultures and most solid-media cultures. For solid media such as soil, the bulk soil is frequently absent in small systems. However, the larger the soil system, the more likely that bulk soil and bulk-soil microorganisms will be present. In addition present data indicate that selection and induction of specific active soil organisms in laboratory bioassay systems are considerably greater than in field systems. Thus, diversity, function, and action of microorganisms in laboratory bioassays are atypical when compared to field systems.

7.2.6 Media

Composition of media in terrestrial field systems usually consists of combinations of sand, silt, clay, and organic matter with a broad range of chemical compositions and physical structures. Soil moisture and nutrition vary over time and with location. Media of field systems are, thus, highly variable over time and with soil type and location. Media in laboratory bioassays range from solutions to solids and tend to be very homogeneous in character. Solid media such as soil are also frequently air-dried and sieved, adulterated with substances such as sand, vermiculite, perlite, peat, etc., and supplemented with nutrients (e.g., NO_3^-, NH_4^+, P and K) and water.

7.2.7 Plant Densities

Field systems contain mixtures of plant species (producers and receivers) with a broad range of individual densities and seed, root, and mycorrhizal surface areas.

Laboratory systems generally use single individuals or an arbitrary density of a single species. The putative allelopathic species is absent and represented only by IPA compounds, leachates, exudates, extracts, etc. with the exception of the stair-step system for "root exudates plus" where the putative allelopathic species and the bioassay species are present (see Sect. 4.2.3.1). For additional details regarding density see Sect. 3.5.

7.2.8 Physicochemical Environments

The physicochemical environments (e.g., light intensity, day/night temperatures, soil composition, soil moisture, soil pH, rain events, organic and inorganic compounds, etc.,) of field systems are highly variable over time (e.g., day/night, season, etc.) and location. The physicochemical environments of laboratory bioassays as a general rule are fairly constant and frequently arbitrary in the way they were chosen.

7.2.9 Available Water-Soluble Compounds

The end product of inputs and losses from field systems leaves behind net or residual amounts (mM or mol/g soil) of water-soluble compounds. (Note: The losses include what has interacted with and been taken up by seeds, roots, and mycorrhizae of all the species present.) For most systems, unless there has been a recent disturbance (e.g., rain event or mortality event), net or residual concentrations of water-soluble compounds in the soil tend to move towards some quasi steady state. Unfortunately residual amounts in the soil (e.g., soil solutions and soil extracts) provide little, if any, insight regarding the way organic compounds (also inorganic compounds such as nitrogen) are partitioned in the soil. A more complete insight regarding partitioning of organic compounds in soil systems may be gained by using radioactive isotopes of the organic compounds of interest (Haider and Martin 1975; Martin and Haider 1976, 1979; Haider et al. 1977; Shann and Blum 1987), by monitoring various soil processes (see Blum and Shafer 1988; Blum et al. 1999a; Blum 2004, 2006, 2011; Muscolo and Sidari 2006), and/or by metabolic profiling of soils and soil solutions (Summer et al. 2003; Dunn and Ellis 2005; Xi et al. 2008; Prince and Pohnert 2010; and Sardans et al. 2011; Sect. 6.6). To a limited extent, inputs may be modified by researchers in the field by various management practices. However, modifying or regulating how compounds are partitioned to soil sinks is extremely difficult even under the best of circumstances.

So why even test soil solutions (e.g., soil extracts) from field systems in laboratory bioassays? Water-soluble organic and inorganic compounds in soil solutions (i.e., residual amounts) constitute the background amounts (frequently a steady state amount) in which seeds germinate and roots and mycorrhizae grow. That background at times can be sufficient to stimulate or inhibit plant processes and provide

indirect evidence for potential plant-plant allelopathic interactions. The absence of an effect, however, provides no evidence for or against potential plant-plant allelopathic interactions since the residual amounts do not include potential amounts that may have already, in a theoretical sense, interacted with seeds, roots, and mycorrhizae.

For laboratory bioassay systems the actual amounts of input of available water-soluble compounds (e.g., IPA compounds or simple mixtures) is known or there is at least some general idea of the input of available water-soluble compounds (e.g., amount and frequency of addition of leachates, tissues, litter, or residues). However, available concentrations of organic compounds including allelopathic compounds are frequently modified and at times manipulated by eliminating soil sinks (e.g., use of solution culture, sand culture, or sterilized systems) or changing the nature of soil sinks by adjusting pH, by adding inert materials such as sand to soil, and by adding organic matter and inorganic compounds including nutrients, etc. In the process researchers increase or decrease the potential amounts of promoters, modifiers, and inhibitors available to interact with seeds, roots, and mycorrhizae.

7.2.10 Doses (Active Water-Soluble Compounds)

Just because water-soluble compounds are present in field soils does not mean that they will interact with seeds, roots, and mycorrhizae. The available compounds must first reach the surfaces of seeds, roots and mycorrhizae and then be in the appropriate chemical and physical states before they can interact with seeds, roots, and mycorrhizae. Thus, the relationship between available concentrations in field soils and dose are in most instances not directly related and, therefore, not predictable. Dose is defined here as the actual concentration of active organic and inorganic compounds which interacts with seeds, roots and mycorrhizae. The relationship becomes even more unpredictable when one attempts to single out this relationship for individuals growing in mixtures of plant species.

Unpredictable relationships for concentration and dose are also undeniably apparent in most laboratory bioassays. The exception would be solution culture bioassays in which the proximity of compounds to seeds, roots, and mycorrhizae is a given. Even for solution-culture bioassays, however, the chemical and physical states must still be appropriate for contact and uptake. For example simple phenolic acids do not readily appear to interact with roots if the solutions are neutral or above in pH (Harper and Balke 1981; Blum et al. 1985b). This same pH mediated response also appears to occur in soil (Blum et al. 1989). Due to the small size of most laboratory bioassay systems and the nature of inputs to such systems, contact and, thus, potential dose should logically be substantially higher in laboratory systems than in field systems. However, the rapid decline of stimulatory and inhibitory water-soluble organic compounds and the associated rapid rate of recovery of plant processes observed in the laboratory for some bioassay systems suggest that this may not be entirely true for all systems.

7.2.11 Response Times

Response times of seeds, seedlings, and plants to active organic and inorganic compounds in field systems as a general rule are considerably slower for field systems than most laboratory systems because processes such as germination of seeds and growth of seedlings and older plants are frequently limited by resources and/or periods of harsh environments in the field. Laboratory bioassay systems are frequently designed to maximize germination, growth, etc. by providing adequate water, nutrients, an appropriate range of temperatures, etc., but even for laboratory bioassays, response times can vary substantially. For example, the response times of seeds or seedlings in solution cultures are much shorter than the response times in substrate culture (see Sect. 5.2.3; Blum and Gerig 2006; Blum 2011).

7.2.12 Final Comment

What should be obvious to the reader at this point is that field systems and our past and present laboratory systems are very different systems when it comes to inputs, source-sink relationships, timing and frequency of inputs and losses, contact surface areas, populations and actions of microorganisms, species densities, response times, etc.

7.3 Is the Present Criticism by Critics Regarding Plant-Plant Allelopathic Interactions in the Field Credible?

The concentrations (inputs) required for an observable effect (stimulation or inhibition) in laboratory bioassays by putative allelopathic compounds or complex mixtures containing promoters, modifiers, and inhibitors are determined by the bioassay species chosen, the bioassay system chosen, the process measured, and the management of that system by the researcher. For example inhibitory treatment concentrations required for solution bioassay systems will be much lower than for soil bioassay systems (see Sect. 3.2.2), and much higher for individual compounds than for individual compounds in complex mixtures (see Blum et al. 1985a, 1989; Blum 1996). Minimum concentrations required for an effect in laboratory bioassays are, thus, a moving target. When we add that the inputs and actual available concentrations of allelopathic compounds for field soils are unknown (Note: All we presently have are residual concentrations in soils) and that dose (active compounds that actually interact with seeds, roots and mycorrhizae) in both laboratory and field systems are also unknown, then the criticism based on minimum concentrations required for an effect in laboratory systems and the static and residual concentrations determined in field systems are not appropriate. To make a fair comparison requires reliable data for the doses experienced by seeds, roots and mycorrhizae in laboratory and field systems and laboratory systems that are roughly equivalent to field systems.

Harper, Stowe, and Connell (Harper 1975, 1977; Stowe 1979; Connell 1990), however, have raised legitimate questions regarding the atypical behavior of laboratory bioassays and the overall difficulty of identifying cause and effect for plant-plant interactions in field systems (e.g., identifying resource competition, allelopathic interactions, and indirect interactions). Throughout this volume an attempt has been made to address the questions regarding the atypical behavior of laboratory bioassays by identifying atypical factors and behaviors of laboratory bioassay systems and suggesting ways to reduce or eliminate such atypical aspects to make laboratory bioassays more relevant to field systems. How to separate different types of plant-plant interactions have been addressed elsewhere (for example see Weidenhamer et al. 1989; Connell 1990; Thijs et al. 1994; Weidenhamer 1996; Inderjit and del Moral 1997).

To answer the original question, the criticisms based on comparisons of required effective concentrations for laboratory bioassays and the residual concentrations found in field systems are entirely inappropriate. The questions regarding the atypical behavior of laboratory systems and the concerns regarding the difficulty of identifying actual cause and effect under field conditions are appropriate. Therefore given the nature of past and present laboratory model systems and field systems, the evidence cited by both detractors and advocates against or for plant-plant allelopathic interactions in the field are still an open question except for the most extreme cases.

7.4 Improving the Value of Laboratory Bioassay Systems

Now that it has been established in general how inputs and losses, etc., of water-soluble compounds differ for field systems and past and present laboratory bioassay systems, what can be done to make laboratory bioassay systems more relevant to field systems and more useful and insightful when determining the real time modes of action and processes involved in plant-plant allelopathic interactions in the field? There are, of course, limits to what can be done, but there are still things that can and should be done.

7.4.1 Bioassay Species

Using the appropriate species, appropriate stage of its life cycle, and appropriate density are obvious. However, how to include the influences (or modifications) of other plant species found in field systems to laboratory bioassay systems is not all that obvious. In fact that turns out to be an extremely complex factor to include. The presence of other species would not only modify available organic compounds including allelopathic compounds by removing and adding organic and inorganic compounds to media but would also modify the physiochemical and biotic environments of media.

7.4.2 Physicochemical Environments

Depending on the objective, a whole range of different system environments can be created and used when addressing hypothetical or theoretical questions. However, when modeling field-relevant systems, prescribed limits for laboratory bioassay systems are set by the field system under study. In other words the field system under study determines the types and ranges of factors (e.g., type of medium, pH, nutrition, moisture of the medium, photoperiod, day/night temperatures, types and sources of input and losses, light quality and quantity, space, etc.) that should define a model system. Every effort should, therefore, be made to create model systems that are more field-relevant (i.e., as close as possible to the field system).

7.4.3 Biotic Environments

Unfortunately our ability to reproduce biotic environments of field systems (e.g., presence of roots of species other than the bioassay species, root densities and distributions within the containers, symbiotic relationships, and bulk soil, root, and mycorrhizal associated flora and fauna) is extremely limited. Throughout the chapters of this volume the author has described the problems and difficulties associated with creating and maintaining realistic biotic environments within laboratory model systems and they will not be repeated here. Let it be said, however, that this has been and continues to be an Achilles' heel for the development of field-relevant laboratory bioassays.

7.4.4 Treatments

Once the biotic and physicochemical factors of a model system have been chosen, what remains to be determined are the treatments that will be applied to the model system. Unfortunately most published research suggests that researchers pay little attention to the relevance of their treatment protocols to field systems in spite of harsh criticism from critics of laboratory plant-plant allelopathic interaction studies. Ultimately the relevance of treatment protocols to field systems and the use of treatments as tools to identify and characterize system behavior should not be underestimated.

7.4.4.1 Effective Treatment Concentrations in Field-Relevant Laboratory Bioassays

In a laboratory bioassay the treatment concentration (see Sect. 3.2.2) required to bring about a stimulation or an inhibition of plant processes depends upon the

relationship between inputs and losses to sinks other than the bioassays species and the sensitivity of the bioassay species. The nature of this relationship is determined by the following:

a. the organic and inorganic composition and concentrations of treatment solutions and the available (water-soluble) organic and inorganic compounds released from materials (e.g., litter or residues) added and the mode of their application,
b. the frequency, timing, and volume of treatment solutions or mass of materials added to a system,
c. the media characteristics (e.g., type [e.g., solution or solid], solute potential, pH, organic and inorganic compounds present, and in addition for solid media, mineralogy, texture, structure, and content and nature of organic matter),
d. the types, populations, and activities of microorganisms and presence or absence of symbiotic relationships,
e. the growth environment other than that directly related to media (e.g., quality and quantity of light, duration of light and dark periods, temperature, relative humidity, pH, etc.),
f. the size of potential treatment surface area in solution culture or the size and distribution in solid-media culture and competition between seeds, roots and mycorrhizae, and
g. the sensitivity of the bioassay species as determined by its genetic composition, stage of development, state of health, acclimation to previous and present environments, presence or absence of symbiotic relationships and a species ability to sequester and/or deactivate promoters or inhibitors,

There are others, of course, but these represent the most common factors that ultimately determine an effective treatment concentration for a laboratory bioassay system.

The types, composition, and quantities of inputs in most cases are determined by the researcher. Losses are determined by the system chosen in the laboratory by the researcher. The absolute range of sensitivity of a bioassay species is genetically fixed but the functional range is shaped by the environment. The bottom line is that effective treatment concentrations vary substantially from laboratory system to laboratory system and researchers may manipulate a system or choose a system that will give them a desired result. Such manipulations, however, distort one of the primary goals of many bioassays, which is to run bioassays with inputs and losses that are relevant to the field systems under study.

At this point the reader might ask: What are realistic or, better yet, field-relevant treatment concentrations that should be used in laboratory bioassays? To be honest we really do not know. Why not? There are several reasons that come immediately to mind.

a. The author is not aware of any attempts to completely characterize the composition of promoters, modifiers, and inhibitors within the bulk soil, the rhizosphere, or the mycorrhizosphere for field systems when putative allelopathic interactions are observed. What we presently have are estimates of residual concentrations for individual IPA compounds or small groups of similar IPA compounds at specific points in time for bulk soils and in rare instances in the rhizosphere.

b. Presently it is not know how to create model treatment solutions which will mimic the promoter/modifier/inhibitor complexes in field systems. The actions of individual IPA compounds or the actions and interactions of simple mixtures of similar IPA compounds are not necessarily representative of the actions and interactions of promoter/modifier/inhibitor complexes. Leaf and residue leachates, litter, and residues constitute promoter/modifier/inhibitor complexes but the compositions of the active components for these treatment materials have not been adequately characterized.
c. Presently it is not know how to compensate or account for the atypical nature and behavior of laboratory-bioassay systems. By atypical nature and behavior (i.e., atypical compared to field systems), the author is referring, for example, to the composition and frequency of treatment solutions, the physicochemical and biotic changes associated with treatments, the behavior of microorganisms within laboratory bioassay systems, the frequent absence of symbiotic relationships, the distribution of organic compounds including allelopathic compounds throughout the media of bioassay systems, and the differences in source-sink relationships.
d. There are other reasons as well; see Chap. 3.

Resolving these issues could go a long way toward developing realistic and field-relevant bioassay systems in the laboratory.

7.4.4.2 Treatment Inputs as a Tool

In the meantime until these issues are resolved, applications of effective treatments of laboratory bioassay systems with organic compounds (IPA or others of interest) and mixtures of organic and inorganic compounds can be used as a tool to probe laboratory model systems for the behaviors of key modifying factors and identification of the specific roles of individual organic compounds within complex mixtures of organic and inorganic compounds. In the process in combination with field observations, the behavior of typical and atypical factors of laboratory bioassay systems can be identified and quantified. Such knowledge could then be used to adjust atypical factors to make laboratory bioassay systems more relevant to field systems. Ultimately that would lead to a better understanding of the primary sites, modes of action, and system processes associated with plant-plant allelopathic interactions and to eventually our ability to relate the behavior of laboratory bioassay systems to field systems.

7.5 Future Directions: Questions that Need Answers

It should be clearly evident at this point that there are still considerable uncertainties regarding the modes of action involved in plant-plant allelopathic interactions. These uncertainties revolve around a series of central issues such as inputs, turnover rates, roles of modifiers, available and active concentrations, etc. Laboratory

model bioassay systems have been, are, and will continue to be an important tool for resolving such uncertainties from a theoretical standpoint. However, developing and designing field-relevant laboratory model bioassay systems which can function as surrogates for field systems is not a simple matter. A number of potential adjustments to and additions of factors for laboratory bioassays have already been proposed in Chaps. 2 and 3. These include adjustments in the way treatment materials should be collected, stored, and applied to bioassay systems and how various factors of bioassay systems can/should be manipulated and managed. A number of these proposed adjustments and additions have been included in the hypothetical bioassays described in Chaps. 4 and 6. Also there is already considerable information in the literature on how specific adjustments to and additions of factors may potentially modify source-sink relationships and effective treatment concentrations (e.g., the concentration required for a given percent stimulation or inhibition; see references in Sects. 1.4, 3.2.1, 3.7, and Chaps. 4 and 6). However, much of this information cannot be applied directly to field systems because factors were studied in isolation, were missing, and/or, if present, were atypical in their behavior. What is needed is an amalgamation or unification of these various factors within the confines of field-relevant laboratory bioassay systems. Unfortunately real time comparisons of systems will have to wait until a means of determining real time inputs, sink-source relationships and turn-over rates for field systems can be developed and until it can be determined how to apply this insight to laboratory model bioassay systems. In the meantime there are numerous questions that can be addressed. What follows are some of the questions that need answers.

a. What are the real-time organic and inorganic compositions of leaf and "root exudates plus" leachates of living plants and the leachates of litter and residues?
b. What are the real time rates of input of available organic and inorganic compounds to soil by living plants, plant litter and residues, flora, fauna, and residual organic matter?
c. What roles do organic, inorganic, physical, and biotic modifiers play in the expression of plant-plant allelopathic interactions? How and when do they modify the expression of these interactions?
d. What are the turnover rates (i.e., input and losses) of potential allelopathic compounds and their modifiers? Can such turnover rates be used to obtain a better estimate of actual available allelopathic compounds in soils?
e. What are the source-sink relationships within soil systems? How can these relationships be quantified or manipulated?
f. How can we differentiate between total, available, and active-functional allelopathic compounds in the soil?
g. By what mechanism and at what rate are potential allelopathic compounds and their organic and inorganic modifiers transported in the soil?
h. How do distribution and density of sensitive and non-sensitive roots in the soil influence plant-plant allelopathic interactions?
i. What happens to root growth when roots reach or come close to a source of allelopathic compounds?

j. What factors or processes regulate the sensitivity or resistance of seeds, seedlings, and older plants to allelopathic compounds?
k. What roles do soil organisms play in the expression of allelopathic interactions? How do we quantify those roles?
l. How do soil organisms on the rhizoplane and in the rhizosphere and mycorrhizosphere impact plant-plant allelopathic interactions? How are they different and how are they similar? If they are similar, do they simply produce a declining concentration gradient of allelopathic compounds from the bulk soil to the root surface, or if they are different, how do the zones function individually as utilizers, modifiers, sources, and sinks?
m. How does the transport of organic compounds by the hyphae of mycorrhizae to and away from roots impact plant-plant allelopathic interactions?
n. How do root grafts impact the identification and characterization of plant-plant allelopathic interactions?
o. How do physicochemical and biotic environments influence the expression of allelopathic effects?
p. How can environmental factors be manipulated to enhance or reduce plant-plant allelopathic interactions?
q. What determines when, where, and how a complex mixture of available compounds within soils is active or inactive?
r. How can we create model bioassay systems that more closely mimic field systems?

Answering these questions, among others, should be our goal. Properly designed laboratory bioassays can provide considerable insight into these questions. However, it will take considerable ingenuity and perseverance to find answers to these and other pertinent questions.

7.6 Central Tenets (i.e., Opinions, Doctrines, or Principles) Articulated in this Volume

7.6.1 Plant-Plant Allelopathic Interactions

As presently used in the literature, plant-plant allelopathic interactions are due to the actions of active organic compounds (i.e., allelopathic compounds) released into the atmosphere and/or the soil by living plants or decomposing recognizable plant litter and residues where these organic compounds may influence associated plants in a positive or negative manner. The focus in this volume has been on the water-soluble compounds in soil, where these released organic compounds can be oxidized or reduced by chemical, microbial, or faunal processes before influencing associated plants in a positive or negative manner. The positive or negative influences on plants growing in soil can either be due to direct (e.g., impact on root membranes or cell processes) or indirect (e.g., impact on symbiotic relationships,

i.e., formation, development, and function of nodules or mycorrhizae) actions of allelopathic compounds (see Molisch 1937, 2001; Willis 1985, 1994, 2007; Blum et al. 1999b; Blum 2011; also see Chap. 1). (Note: The term allelopathic compound as presently used in the literature is frequently used as a substitute for identified putative allelopathic (IPA) compounds.) However:

a. since the primary source of all organic compounds in terrestrial systems are photosynthetic organisms (e.g. higher plants, algae, and photosynthetic bacteria),
b. since the qualitative and quantitative release of organic and inorganic compounds from primary sources to the soil or atmosphere vary dramatically depending on the source, timing and processes by which they are released (see Sects. 1.3 and 1.4),
c. since the secondary, tertiary, etc. sources of organic compounds within the soil stem from the actions of physicochemical processes (e.g., oxidation, reductions, and polymerization) or biotic processes (e.g., faunal and microbial processes [i.e., fragmentation, decomposition, metabolic and catabolic processes, and polymerization], also see Sects. 3.2.2.8 and 3.2.2.9),
d. since at least theoretically any organic and inorganic compounds contacting a sensitive species can be stimulatory, neutral, or inhibitory depending on its concentration and physiochemical state, the sensitivity of the species being contacted, and the processes being impacted (or monitored),
e. since soils contain three recognizable fractions of organic and inorganic compounds: total (free [in solution], reversibly sorbed, and irreversibly sorbed or fixed), available (free and reversibly sorbed), and active (the free and reversibly sorbed compounds that actually contact and interact with seeds, roots, and mycorrhizae),
f. since only active organic compounds which interact with seeds, roots, and mycorrhizae appear to be involved directly in plant-plant allelopathic interactions,
g. since we presently lack the necessary tools to distinguish the active fraction from the available fraction in the soil,
h. since available and active compounds in soils are unlikely to be linearly related (i.e., their relationships are unpredictable),
i. since available and active organic compounds in the soil at any given point in time are determined by inputs (e.g., leachates, "root exudates plus", and microbial conversions and synthesis) and losses (e.g., soil particle sorption, root uptake, microbial utilization, and gravitational flow; also see Sects. 7.2.1–7.2.3),
j. since organic and inorganic compounds extracted or leached from leaves, roots, litter, and residues do not necessarily correspond or are not directly related to the qualitative and quantitative compositions of available and active organic and inorganic compounds in the soil at a given point in time or over time,
k. since concentrations of available or free compounds extracted from soils represent only the residual concentrations in the soil and not the actual available or active concentrations,

l. since turnover rates of available or free compounds in soils are extremely difficult, if not impossible, to determine by present methods available (e.g., using soil extracts at various time intervals)
m. since for plant-plant allelopathic interactions the actions of active individual organic compounds within complex mixtures of organic compounds can be independent, joint action (i.e., synergistic, additive, or antagonistic, see Sect. 5.3), or modifying in action,
n. since available neutral and active organic and inorganic compounds in complex mixtures of organic and inorganic compounds can act as modifiers of available active organic compounds present (see Sect. 5.3),
o. since a complex mixture composed of individual organic compounds at ineffective (or neutral) concentrations (e.g., too low and neutral for stimulation or inhibition) can still have an effect as long as the total concentration of all these neutral organic compounds making up the mixture functions as a promoter or inhibitor,
p. since minimum concentrations required for stimulation or inhibition of individual identified putative allelopathic (IPA) compounds are generally not consistent with minimum concentrations required when these compounds are embedded within complex mixtures of compounds (Note: Possible exceptions are IPA compounds that are very toxic at very low concentrations.),
q. since effects of IPA compounds are moderated by pre- and post-environmental stresses (Gawronska and Golisz 2006; Pedrol et al. 2006, see Sect. 5.2.3.1.5), and
r. since the magnitude of effects observed for plant-plant allelopathic interactions are not only determined by the sensitivity of the species and the types of active organic compounds present and their concentrations but also by the physiochemical (e.g., pH, neutral [and active] organic compounds, inorganic compounds, temperature, soil moisture, leaching, aeration, soil compaction, etc.) and biotic environments (e.g., microbial utilization and root and mycorrhizal uptake [see Sect. 5.2.3]), the past focus on available (free) individual or even simple mixtures of identified putative allelopathic (IPA) organic compounds as the primary driving forces of all positive or negative plant-plant allelopathic interactions (i.e., representing a "Growth Promoter" or "Herbicide" paradigm) is questionable and at best uncertain. Given this uncertainty, the role or roles of IPA organic compounds as the primary driving force of plant-plant allelopathic interactions must be carefully re-evaluated by researchers on a case by case basis.

When we add that:

a. with the exception of some unique compounds produced by microorganisms (e.g., see Duke 1986; Demain and Fang 2000; Owens et al. 2013) and a few released by plants, individual IPA compounds do not have high levels of biological activity (Boyette and Abbas 1995; Macías 1995; Rice 1995; Duke and Dayan 2006; Dayan 2007),

b. when it comes to stimulatory or inhibitory effects of IPA compounds, the actions of ethylene, a volatile hormone, and abscisic acid, a slightly water-soluble hormone, have been documented for higher plants (e.g., Molisch 1937, 2001; Holappa and Blum 1991; Li et al. 1993; Berner et al. 1999; Inderjit et al. 2009; Zhao et al. 2011; Soltys et al. 2012) but there appear to be no other data at this point that would suggest such hormone-like actions (or systemic action) for other water-soluble IPA compounds, and
c. the data that are available for water-soluble or hydrophilic and some hydrophobic IPA compounds indicate that their actions are local (see Macías et al. 2004; Blum 2011; Sect. 1.2; Chap. 5), then the uncertainty of the role or roles of most IPA compounds as the primary driving forces of plant-plant allelopathic interactions in natural and managed systems become even more clouded.

These observations (a. to c.) are based on a limited data base and have as their primary focus water-soluble compounds The current state of cloudiness alluded to in c. is a consequence of the present lack of knowledge regarding the actual functional roles of most IPA compounds in natural and managed systems.

In summary:

a. The inputs of organic and inorganic compounds into the soil by plant tissues, litter and residues modifies the nature and function of promoter/modifier/inhibitor complexes in the soil in such a way that the processes of associated plants are stimulated or inhibited directly or indirectly. The promoter/modifier/inhibitor complexes in the soil are composed of the baseline or the residual compounds in the soil and the inputs by plant tissue, litter and residues. Modifications of the promoter/modifier/inhibitor complexes (e.g., shifting the balance from promoters to inhibitors) in the soil may occur due to the direct input of compounds by plant tissue, litter, and residues with high activity (e.g., individual or mixtures of highly active organic compounds), due to the direct inputs of a complex series of compounds with low activity (e.g., their activity is determined by the sum of their actions), and/or due to indirect inputs resulting from modifications of direct inputs by chemical or biotic processes.
b. The stimulation or inhibition by allelopathic compounds in soils will most likely be a product of the actions of all free and active organic and inorganic compounds in the soil which act individually or in combination as promoters, modifiers, or inhibitors depending on their individual concentrations and environments and the sensitivity of the receiving seed, seedling, or older plant process impacted or monitored.
c. That in an absolute sense, there is no such thing as a compound which is always an allelopathic compound. There are only organic compounds which function as promoters or inhibitors under the appropriate conditions. In other words under appropriate conditions and appropriate concentrations, organic compounds function as allelopathic compounds. Theoretically this is true but from a practical perspective, there clearly are organic compounds (e.g., compounds with hormone-like or systemic actions) in nature which at very low concentrations readily

affect (stimulate or inhibit) plant processes and other organic compounds which require very high or unnatural concentrations to affect plant processes unless they act in concert with other compounds. The majority of individual organic compounds will likely affect plant processes somewhere between these two extremes.

d. That organic compounds can function not only as promoters and inhibitors but also as modifiers of promoters and inhibitors.
e. That physicochemical and biotic factors can also modify the actions of promoters and inhibitors.
f. That the present description for plant-plant allelopathic interactions must be broadened to include physicochemical (e.g., pH, solute potential, inorganic compounds, and soil moisture) and biotic (e.g., roots, mycorrhizae, microorganisms, and fauna) modifiers and promoter/modifier/inhibitor complexes (Note: These are in addition to highly active individual compounds including IPA compounds.).

If we accept the above, then a modified version of the description for plant-plant allelopathic interactions for water-soluble (also hydrophobic and volatile) compounds would become:

Plant-plant allelopathic interactions are due to the combined actions of organic compounds already in the soil and those released from living plants or decomposing recognizable plant litter and residues where these may be oxidized or reduced by chemical, microbial, or faunal processes before influencing in a positive or negative manner associated plants. The positive or negative influences on plants can either be due to direct (e.g., impact on root membranes or cell processes) or indirect (e.g., impact on symbiotic relationships, i.e., formation, development, and function of nodules or mycorrhizae) actions of allelopathic compounds (individual or mixtures of highly active organic compounds) and/or active promoter/modifier/inhibitor complexes composed of individual compounds with individual low or no activities (i.e., the summed action is active in this instance). For promoter/modifier/inhibitor complexes stimulation will occur whenever promoters dominate and inhibition when inhibitors dominate. The magnitude of the positive or negative influence will be determined not only by the individual or summed actions of promoters and inhibitors on sensitive (or susceptible) species but also by the organic, physicochemical, and biotic modifiers present. This assumes that other factors (e.g., competition, disease, pH, solute potential, nutrition, physical state of the soil, direct transfer between plants, etc. (see Blum 2011)) are not primarily responsible for the effects observed.

The author should hasten at this point to state that he is not seeking to diminish or negate the potential role of water-soluble IPA compounds in plant-plant allelopathic interactions of natural and managed systems but simply to point out that in most cases their actual role is not clearly understood. What the present data suggest is that with a possible few exceptions (i.e., very toxic IPA compounds) most allelopathic effects in natural and managed systems appear to be due to complex mixtures of available organic and inorganic compounds (e.g., in leachates, exudates, and soil solutions) not due to individual compounds with the proviso or stipulation that effects (stimulation or inhibition) of complex mixtures on sensitive species require mixtures composed of the appropriate combinations of compounds with

the appropriate total active concentrations and suitable physicochemical and biotic environments (Blum 2006, 2011; Chaps. 4 and 6).

Given this complexity, why the emphasis and reliance on a single group of compounds, i.e., simple phenolic acids in both this and the previous volume (Blum 2011)?

a. Phenolic acids are commonly found in all higher plants and most soils (Bates-Smith 1956; Haider and Martin 1975; Haider et al. 1977; Harborne 1990; Blum et al. 1991; Dalton 1999; Blum 2004).
b. Simple phenolic acids serve a variety of plant and ecosystem functions (Harborne 1982, 1990; Goodwin and Mercer 1983; Siqueira et al. 1991; Appel 1993).
c. Historically simple phenolic acids have been the most frequently cited IPA compounds in the literature (for example see Rice 1984; Blum 2011).
d. The behavior of phenolic acids in soil systems is somewhat similar to the behavior of other organic acids (e.g., acetic acid, butyric acid, citric acid, formic acid, hydroxamic acids, lactic acid, tannic acids, and tartaric acid) which have also been classified as IPA compounds (see Patrick 1971; Rice 1984).
e. Simple phenolic acids such as cinnamic and benzoic acid derivatives with their various side groups or chains are readily available in pure form from commercial sources.
f. Simple phenolic acids such as cinnamic and benzoic acid derivatives are water soluble and released from living plants and plant litter and residues (see Rice 1984, Blum 2011; Sects. 1.3 and 1.4).
g. The functions of their various side groups or chains can be tested in laboratory bioassay systems (see Chap. 5).
h. Simple phenolic acids provide an excellent opportunity to identify primary sites and characterize modes of actions for simple organic acids (see Chap. 5).
i. Simple phenolic acids have been found to be stimulatory or inhibitory to seed, seedling and plant processes (see Chap. 5).
j. The actions of simple phenolic acids in mixtures of phenolic acids or other organic compounds have been found to be additive, partially antagonistic, or totally antagonistic (see Chap. 5; Sect. 6.2).
k. The actions of phenolic acids are modified by a range of environmental factors (e.g., pH, solute potential, soil type, soil moisture, and soil microbes; see Chap. 5).
l. Effects of simple phenolic acids on plant processes are partially or wholly reversible whenever the active concentrations contacting seed, root, and mycorrhizal surface areas are reduced or eliminated (see Chap. 5, Blum 2011).
m. Simple phenolic acids are readily utilized, modified, detoxified, taken up by plant roots and microorganisms, sorbed by soil particles, and leached within soils (see Blum 2011).
n. Reliable extraction procedures for tissues, litter, residues, and soils have been developed (see Blum 2011; Dalton 1999).
o. Simple phenolic acids, once extracted, are readily isolated, identified, and quantified by chromatographic techniques (see Harborne 1984; Blum 2011).

p. Their ubiquitous distribution in nature and apparent rapid turnover rates in soils have generated considerable controversy regarding their importance in plant-plant allelopathic interactions under field conditions (see Schmidt and Ley 1999; Blum 2004, 2006, 2011).

All of these aspects, as well as others, make simple phenolic acids individually or imbedded in simple or complex mixtures of compounds excellent model compounds for the exploration, probing, and testing of hypotheses related to plant-plant allelopathic interactions in laboratory bioassay systems. The bottom line is that using phenolic acids as well as other IPA compounds as model compounds in laboratory systems has and will continue to provide researchers with additional insight regarding the primary sites, modes of action, and the roles of organic compounds (i.e., natural products) in plant-plant allelopathic interactions of natural and managed ecosystems (see Blum 2011; Chap. 5).

7.6.2 Laboratory Bioassay Systems

The author feels that there is nothing inherently wrong with dose response bioassays of identified individual putative allelopathic compounds as long as they are properly designed and characterized and their limitations are properly acknowledged (see Sect. 1.5). They can play a pivotal role in:

a. identifying potential primary sites, modes of action, and effects of compounds individually or in combination with other compounds,
b. determining potential types of actions and interactions of individual compounds in the presence of other compounds,
c. determining the way various factors associated with natural and managed systems can modify potential modes of action and effects, and
d. providing a means by which atypical behaviors of factors of laboratory bioassay systems can be characterized, quantified, and hopefully eliminated.

A basic knowledge of the way actions and interactions of IPA compounds and other compounds influence the effects of sensitive seeds, seedlings, or older plants under a variety of environmental conditions is essential if we are to eventually identify and characterize plant-plant allelopathic interactions within natural and managed systems. Present data at hand clearly suggest that the answers to our questions regarding plant-plant allelopathic interactions (i.e., when, where, and how?) lie somewhere among the complex actions and interactions of organic and inorganic compounds of a given plant-microbial-faunal-soil system and its environment. For example by using dose-response bioassays, multifactor bioassays, regression analysis, sorbents, and omics methods (see Chap. 6) as tools researchers can begin to identify and characterize the roles of these complex actions and interactions for plant-plant allelopathic interactions. How the answers from laboratory bioassays may relate specifically to the behavior of promoter/modifier/ inhibitor complexes

acting in natural and managed systems is, of course, another question. To answer that question will require specific detailed knowledge about:

a. the combinations, concentrations, and environments required for positive or negative allelopathic effects in natural and managed systems,
b. when, where, and length of time such combinations, concentrations, and environments occur or exist in natural and managed systems, and
c. what determines when seeds, seedlings, or older plants within natural and managed systems are susceptible to the summed action (i.e., stimulation or inhibition) of complex mixtures composed of individual organic and inorganic compounds (promoter/modifier/ inhibitor complexes) with low activities (a more holistic paradigm; also see Sect. 7.5).

This is not to imply that the promoting or inhibiting actions of individual promoters or inhibitors with high activity do not occur in natural and managed ecosystems. They, no doubt, do but just like promoter/modifier/ inhibitor complexes their actions will also be modified by the presence of a range of modifying factors associated with each individual type of ecosystem.

The following are several approaches that can be taken when studying plant-plant allelopathic interactions within a given laboratory system:

a. Determining how individual factors may influence or modify the stimulation or inhibition observed.
b. Determining how the addition of factors may influence or modify the stimulation or inhibition observed.
c. Determining how the modification or removal of factors may influence or modify the stimulation or inhibition observed.
d. Determining potential relationships between factors and effects by correlation, regression, and/or principle component analyses.

Chapters 5 and 6 provide some examples for such approaches. What is required is the design of more field-relevant bioassay systems which will allow researchers to ask and answer probing questions or test specific hypotheses that will lead to a better understanding of primary sites and modes of action for allelopathic compounds and system processes in field systems (see Chaps. 2, 3, and 6). For example the focus should shift from simple dose response studies that ask what is the minimum concentration at which a given IPA compound stimulates or inhibits a particular plant process under a given set of environmental conditions (representing the “Growth Promoter” or “Herbicide” Paradigm) to asking how is the minimum concentration for stimulation or inhibition of an IPA compound modified by the presence of other IPA and organic compounds under a range of environmental conditions? Additional questions that could be asked for sensitive species are:

a. How is the activity of a given IPA compound modified by soil pH, moisture, solute potential, inorganic ion content, aeration, organic matter content, particle size, clay type and content, etc.?

b. How is the activity of a given IPA compound modified by its distribution and mobility within the soil and the distribution and growth of root and mycorrhizal systems in the soil?
c. How do the activities of microorganisms on the rhizoplane and in the rhizosphere, in the mycorrhizosphere, and the bulk soil directly or indirectly influence the effects of individual IPA compounds and IPA compounds in the presence of other organic and inorganic compounds?
d. How do active and neutral individual compounds within promoter/modifier/inhibitor complexes influence or determine the ultimate observed effects?
e. What are the primary sites and modes of action of IPA compounds or complex mixtures composed of promoters, modifiers, and inhibitors? How are these sites and modes of action similar or how are they different?
f. How do promoters, modifiers, and inhibitors interact to bring about a stimulation or inhibition?
g. Under what circumstances do promoters, modifiers, and inhibitors act independently?
h. What are the essential factors of a system that determine the ultimate outcome?

Throughout this volume the author has made an attempt to provide the reader with guidelines for the design and construction of relevant laboratory bioassays with the hope that researchers investigating plant-plant allelopathic interactions will be motivated to improve their designs by reducing or eliminating atypical factors (see Chap. 3; Sect. 7.4) and to include as many relevant factors as possible in their laboratory bioassays. Considerable ingenuity and perseverance will be required to achieve these goals but ultimately it is essential to our understanding of the roles of plant-plant allelopathic interactions in natural and managed ecosystems.

Laboratory bioassays, of course, do have their limits. They can provide considerable insight regarding the potential primary sites, modes of action, system processes, and the conditions that will lead to a stimulation or inhibition of sensitive species in natural and managed systems. They cannot provide data for day to day activities within such systems but, if done correctly, laboratory bioassays can provide circumstantial evidence for plant-plant allelopathic interactions within natural and managed ecosystems. In the final analysis, a level of uncertainty regarding the day to day presence or absence of plant-plant allelopathic interactions in natural or managed systems will always exist. For what it is worth, such levels of uncertainty will also exist for other plant processes including plant-plant resource competition. For reasons why such uncertainties exist, see Harper (1975, 1977), Willis 1985, 2007; Weidenhamer et al. (1989), An et al. (1993), Connell (1990), Thijs et al. (1994), Weidenhamer (1996), Inderjit and del Moral (1997), Sinkkonen (2001, 2003, 2007), Belz et al. (2005), and Blum (2011).

7.6.3 Personal Note

When the author first started working with individual phenolic acids (i.e., gallic and tannic acids), he was looking for their acute effects on nodulation and nitrogen

fixation of legumes located in old-fields of central Oklahoma (representing a herbicide paradigm; Blum and Rice 1969). That was still the focus of the research in the author's laboratory in the early eighties when the author and his students began detailed studies of the effects of phenolic acids on seeds and seedlings using laboratory bioassays. However, once we began to look at the effects of simple mixtures of phenolic acids and their actions and interactions on germination and seedling processes and on how these effects in turn were modified by other environmental factors including other organic compounds, a shift in paradigm was necessary (e.g., Blum et al. 1984, 1985a, 1987, 1989). The data generated during this period by laboratory bioassays clearly indicated that the actions of simple phenolic acids in field systems must be much more complex, convoluted, and ancillary than was originally anticipated (e.g., their actions are more chronic than acute (requiring a more holistic paradigm; see Blum 2011; Chap. 5)). Now this also appears to be true for a whole range of other IPA compounds.

The author was, of course, not the first to notice the complex, convoluted, and ancillary nature of IPA compounds for plant-plant allelopathic interactions. Muller (1953) and Harper (1975, 1977), for example, suggested that the likelihood of a simple explanation for the modes of action of allelopathic compounds and functions of plant-plant allelopathic interactions was unlikely. To quote Cornelius H. Muller (1953):

> The natural habitat, even in a relatively simple community of the desert, is far too intricate a system of influences and factors, physical and biological, to hope that there may be found a single factor controlling the complicated life of a perennial species. An explanation when it is arrived at, will be at least as intricate as the situation it seeks to describe. (Quotation used with permission of Botanical Society of America)

7.7 Final Comments

Although the focus of this volume has been on hydrophilic (water-soluble) compounds such as simple phenolic acids, the presence and actions of other compounds such as hydrophobic and volatile compounds cannot be ignored. They add additional dimensions to understanding the roles of plant-plant allelopathic interactions within natural and managed systems. Compared to hydrophilic compounds such as simple phenolic acids, there are differences in pathways of inputs and losses, solubility, mechanisms of transport, potential modes of action, the nature of their response to environmental factors, and the concentrations required for an effect. In regard to solubility, however, the distinction between hydrophilic, hydrophobic, and volatile compounds may become somewhat less distinct when and if researchers begin to consider solubility, not in terms of absolute solubility but in terms of relative concentrations required for biological activity (Weidenhamer et al. 1993). That may also provide a useful context for the study of independent actions, joint actions (synergistic, additive, and antagonistic), and modifying actions of all these different types of compounds.

Finally, in recent years new tools and techniques have been introduced to identify primary sites and study the modes of action for allelopathic compound. Example for these are omics methods (genomics, proteomics, metabolomics, and physionomics; see Macías et al. 2007; Chen et al. 2009; Leão et al. 2009; Duke et al. 2013; also see Sect. 6.6), the use of silenced transgenic plants (Interjit et al. 2009) and mutant lines (Xu et al. 2012), and quantitative evaluations of total activity in soils (Hirdate et al. 2010). It is hoped that once such tools and techniques, as well as others, are fully implemented that considerable progress will be made in identifying and characterizing the range of organic compounds produced/released from plants and microorganisms which can potentially function as allelopathic agents and the environmental conditions (e.g., presence of other organic compounds, pH, nutrition, water potential, microbial utilizers, etc.) required for their actions on sensitive species.

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