Ajit Varma · Ram Prasad
Narendra Tuteja *Editors*

# Mycorrhiza - Eco-Physiology, Secondary Metabolites, Nanomaterials

*Fourth Edition*

Springer

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# Foreword

The pressure on plant production systems is steadily increasing. At first, areas which could be used for the cultivation of plants are getting smaller because more and more space is used for other anthropogenic activities. Secondly, environmental constraints like soil erosion, salinization, or flooding lead to periodical yield losses and finally to the decision to give up a particular region for plant production. Thirdly, the use of pesticides becomes difficult, because the application of more and more compounds is not permitted anymore or they have lost their effectiveness. The development of new agents is time and cost intensive, and it is questionable if there will be enough of such new agents to substitute the compounds which are disappearing from the market. Under these circumstances, the application of plant-interacting microorganisms in plant production systems becomes more and more a realistic alternative and might be the only chance in the future to produce enough food for a growing world population. Among such microorganisms, mycorrhizal fungi fill a particular position. With their hyphae colonizing at the same time the root and the surrounding soil, they connect the inside and the outside of the plant. In this so-called mycorrhizosphere, they bring together all physical, chemical, and biological factors of the terrestrial environment with the physiology of the plant.

The book "Mycorrhiza: Eco-Physiology, Secondary Metabolites, Nanomaterials" gives an excellent overview of the current state of the art from basic to applied mycorrhizal research. It covers different types of interactions including those between the orchid mycorrhizal fungus *Piriformospora indica* and non-orchid plants. Several chapters describe more basic aspects but nevertheless important for application. Carbon flux in mycorrhizal plants has more and more to be the basis for predicting the outcome of mycorrhizal interactions. Functional diversity must be managed for an adapted application in the field. Also, plant–fungus signaling needs a better understanding. Most chapters, however, describe where and how mycorrhizal fungi can be used in plant production under difficult conditions and show in this way how broad the possibilities for application can be. I therefore congratulate the editors that they brought together so many different facets of basic and applied mycorrhizal

research. I also congratulate you on holding this book in your hand and ask you to read at least some of the highly interesting chapters.

Erfurt, Germany
20 March 2017

Philipp Franken

# Preface

German pathologist A.B. Frank (1885) coined the term Mycorrhiza which literally means fungus roots. These fungi aid in the productivity of plants *via* the formation of dynamic associations with plant roots. Mycorrhiza is considered a fundamental part of the root colonization and stabilization of plants on terrestrial habitats. The symbiotic associations formed are an important subject to evaluate various opportunities using modern tools of biotechnology. The possibilities of genetically manipulating these associations have led to the optimization of plant productivity in ecosystems with minimal risk of environmental damage.

This volume of the mycorrhiza book gives exemplary insight into the advancements in mycorrhizal studies. This edition extensively illuminates the ecophysiological aspects, secondary metabolite production, and interaction of mycorrhiza with nanomaterials. The ability of mycorrhiza to provide resistance against various abiotic and biotic stresses has been explored in various parts of this edition. In addition to providing resistance, mycorrhizas are known to increase secondary metabolite production of plants. Therefore, various studies have been conducted to elucidate the mycorrhiza-induced increase of secondary metabolites in various economically important and medicinal plants. Interaction studies of nanomaterials with mycorrhiza have also been a subject of recent interest.

It is hoped that this new edition will interest readers in the latest outcomes of mycorrhiza research and also encourage young researchers to prove the challenging field of these studies.

This volume consists of 18 chapters covering the diverse mycorrhizal associations by 57 eminent academicians and subject specialists.

We are grateful to the many people who helped to bring this volume to light. We wish to thank Hanna Hensler-Fritton, Isabel Ullmann, and Man-Thi Tran Springer Heidelberg, for generous assistance and patience in finalizing the volume. Finally, special thanks go to our families, immediate, and extended, not forgetting those who have passed away, for their support or their incentives in putting everything together. Editors in particular are very thankful to Dr. Ashok K. Chauhan, Founder President of the Ritnand Balved Education Foundation (an umbrella organization of

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# Chapter 1
# Carbon Fluxes in Mycorrhizal Plants

**Veronika Řezáčová, Tereza Konvalinková, and Jan Jansa**

**Abstract** Although declared as a research priority more than 40 years ago, the knowledge about the magnitude and mechanisms of carbon (C) fluxes between plants and their mycorrhizal fungal symbionts remains fragmentary. In spite of a number of experiments with isotopically labeled C documented rapid and directed C transfer from the host plant to its mycobionts, the molecular mechanisms and their regulation involved in such a transport remain largely unknown. It seems that in many arbuscular mycorrhizal (AM) symbioses, the C costs remains well below 10% of the C fixed photosynthetically by the host plants. Higher values were detected in the past only under specific situations such as in young plants, under low light intensities, and/or for particular partner combinations, involving very costly (in terms of C demand) and little nutritionally beneficial AM fungi such as *Gigaspora* sp. Ecological context of the common mycorrhizal networks in terms of redistribution of symbiotic C costs and nutritional benefits on one hand and C movement through soil food webs beyond mycorrhizal hyphae on the other are briefly discussed in this chapter, and further research challenges and open knowledge gaps with respect to C fluxes in mycorrhizal plants are outlined.

## 1.1 Introduction

Mycorrhiza is one of the most common inter-species interactions on Earth, involving great majority (>90%) of plant species (Smith and Read 2008) and several groups (and functional guilds) of soil fungi (Nguyen et al. 2016; Prasad et al. 2017). This interaction involves bidirectional flows of matter between the symbiotic partners, exchanging mineral nutrients such as nitrogen (N) and phosphorus (P) for the reduced carbon (C) originating from plant photosynthesis (Ferrol et al. 2002). Several different types of the mycorrhizal symbiosis evolved during the history, involving different (often disjunctive) groups of symbiotic partners at both plant and fungal sides (Cairney 2000). Yet, the main function (nutrient for C

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trading) is stunningly uniform across the different mycorrhizal types, with some remarkable deviations from this general pattern such as plant-bound C fluxing in orchid protocorms or mycoheterotrophic plants (Leake and Cameron 2010; Bever 2015).

Most efforts in mycorrhizal research have so far been dedicated to uncovering principles and diversity in nutritional and/or growth benefits the symbiosis confers to the plants or how the diversity of taxa and functions in the fungal communities affects the productivity/stability/diversity of the plant communities and vice versa (van der Heijden et al. 1998; Johnson et al. 2004; Munkvold et al. 2004; Cavagnaro et al. 2005). Less efforts have been dedicated to the role of mycorrhizas in sustainable soil use and in establishing and maintaining soil physical properties (e.g., aggregate stability, water conductivity, etc.) and to non-nutritional benefits such as improved biotic resistance of the plant (Newsham et al. 1995; Rillig 2005; Rillig et al. 2015). Comparatively, very little efforts have so far been invested into quantification of C fluxes in the mycorrhizal symbiosis, and to the underlying molecular mechanisms (Slavíková et al. 2017). The purpose of this chapter is to synthesize current knowledge on the influence of mycorrhiza on the C fluxes between atmosphere, plant, mycorrhizal fungi, and the soil. In this chapter, we focus mainly on the arbuscular mycorrhizal (AM) symbiosis, which is pertinent to most (>60%) plant species on Earth and also for most agricultural systems (Jemo et al. 2014; Sochorová et al. 2016), acknowledging similarities and differences between the different mycorrhizal types.

## 1.2 Magnitude of C Flow from Plants to the Mycorrhizal Fungi

Mycorrhizal fungi derive most of their C from their plant hosts, with only a little fraction (if any) of the C originating from the dead organic matter (Olsson and Johnson 2005; Hobbie et al. 2014; Lindahl and Tunlid 2015). Establishment of mycorrhizal symbiosis often increases allocation of C to the roots and further to the mycorrhizal fungi (Slavíková et al. 2017, and references therein), affecting whole plant C balance (Wright et al. 1998) and also the rate of plant photosynthesis, either directly through improved mineral nutrition or indirectly through increased below-ground C sink strength (Fig. 1.1, Douds et al. 2000; Kaschuk et al. 2009; Valentine et al. 2013). Due to the complexity of the interactions between the C and P economies (e.g., nutritional benefits conferred by the mycorrhizal association to the plant may stimulate host plant growth and thus C accumulation under nutrient limiting conditions to a great extent or completely compensate theoretical C allocation to the mycorrhizal fungus in a mycorrhizal plant of the same size as the nonmycorrhizal plant), there are different, partly contradicting concepts for calculation of mycorrhizal costs and benefits, sometimes resulting in conflicting

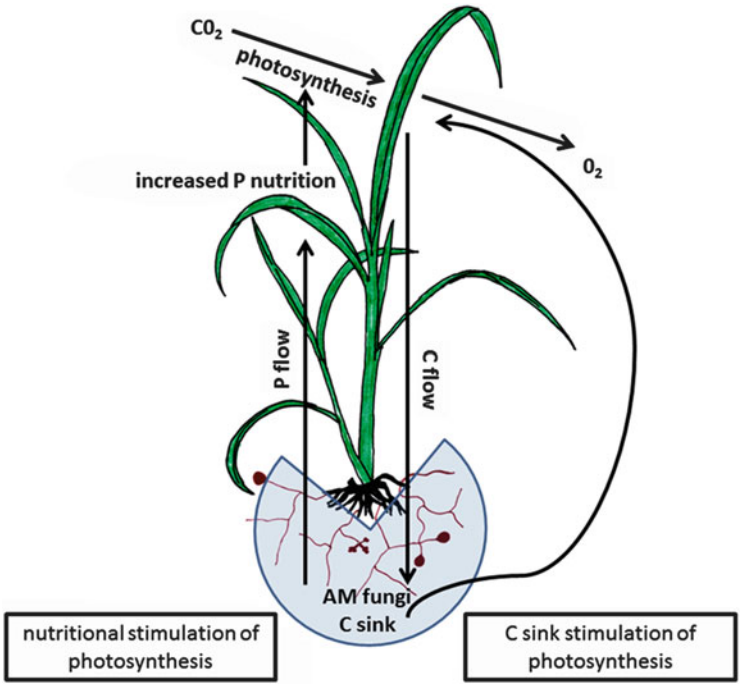


**Fig. 1.1** Two possible pathways how establishment of arbuscular mycorrhiza could feed back on the rates/efficiency of photosynthesis of its plant host

predictions (Fitter 1991; Tinker et al. 1994; Landis and Fraser 2008; Correa et al. 2011).

In spite of the wealth of theories and predictions, the flux of C from the plant to the fungus could be quantified, particularly by employing isotopic C labeling, and relative C expenditure to mycorrhizas (e.g., the fraction of plant C budget allocated to the fungus) could be calculated from such data. Previously, mycorrhizal C cost of AM symbiosis was reported to reach between 4 and 20% of the photosynthetically fixed C by the plant (Smith and Read 2008). Yet, the value of 20% has only been recorded once for young cucumber plants under artificial environmental conditions (Jakobsen and Rosendahl 1990), but it has been frequently cited and also widely generalized up to a global ecosystem level (e.g., Brzostek et al. 2014). More recent research by Tomé et al. (2015) and by Slavíková et al. (2017) reported mycorrhizal C expenditure to reach only a few percent of the plant C budget (see Table 1.1 for more details), which is even below the previously reported low end (4%) of the C allocation to AM fungi. Yet, not all studies reported/measured C allocation to all relevant system compartments such as plant, soil, and the respiration losses above- and belowground. From the handful of studies including all relevant system compartments (coincidentally, all employing short-term pulse $^{14}CO_2$ labeling, Table 1.2), we learn that the shoot respiration could reach between 1 and 6%

**Table 1.1** Mycorrhizal carbon (C) costs as a fraction of the total C budget of a host plant reported for various combinations of fungal and plant partners at different environmental contexts and assessed by different approaches

| Reference | Plant-fungal partner combination | | | Isotope | Length of labeling period | Length of chase period | Above-ground respiration assessed | Below-ground respiration assessed | Mycorrhizal cost (% of recorded C budget) | Note |
|---|---|---|---|---|---|---|---|---|---|---|
| | Host plant species | AM fungal species as reported | Current AM fungal name | | | | | | | |
| Pang and Paul (1980) | *Vicia faba* | *Glomus mosseae* | *Funneliformis mosseae* | $^{14}C$ | 48 h | 4.5 days | – | + | 11[a] | C in all measured compartments allocated to AM minus NM roots and belowground respiration |
| Paul and Kucey (1981) | *Vicia faba* | *Glomus mosseae* | *Funneliformis mosseae* | $^{14}C$ | 48 h | 96 h | + | + | 4 | Fraction of the whole assimilated C in mycorrhizal hyphae and fungal respiration |
| Kucey and Paul (1982) | *Vicia faba* | *Glomus mosseae* | *Funneliformis mosseae* | $^{14}C$ | 48 or 8 h | 96 or 116 h | + | + | 3.5–4.2 | C in all measured compartments allocated into mycorrhizal respiration and biomass |
| Snellgrove et al. (1982) | *Allium porrum* | *Glomus mosseae* | *Funneliformis mosseae* | $^{14}C$ | 30 min | 48 h | + | + | 7 | Total fixed C in roots of AM minus NM plants |
| Koch and Johnson (1984) | *Citrus aurantium*, *Poncirus trifoliata* × *Citrus sinensis* | *Glomus intraradices* | *Rhizophagus intraradices* | $^{14}C$ | 8.5 min | 2 h | – | – | 6–10 | Difference of the total assimilated C to the half-roots between AM and NM parts in split-root system × 2 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Harris et al. (1985) | *Glycine max* | *Glomus fasciculatum* | *Rhizophagus fasciculatus* | $^{14}C$ | 16 h | 68 h | + | + | 8–17 | Total photosynthate allocated into AM biomass, AM respiration, root exudates + soil of AM plants (deduced from comparison of dually colonized (mycorrhizal + *Rhizobium*) vs. NM and NM + *Rhizobium* plants) |
| Douds et al. (1988) | *Poncirus trifoliata* × *Citrus sinensis* | *Glomus intraradices* | *Rhizophagus intraradices* | $^{14}C$ | 10 min | 2 h | – | – | 5.6–7.8 | % assimilated C allocated to roots of AM minus NM plants |
| Wang et al. (1989) | *Panicum coloratum* | *Gigaspora margarita* | | $^{11}C$ | 100–120 min | 200 min | – | – | >3.9 | In the short-term study focused on $^{11}C$ fluxes was not possible to calculate %C in all measured compartments. The authors quote that allocation to mycorrhizal part of the roots was probably more than 3.9% higher than to the nonmycorrhizal roots |

(continued)

**Table 1.1** (continued)

| Reference | Plant-fungal partner combination | | | Isotope | Length of labeling period | Length of chase period | Above-ground respiration assessed | Below-ground respiration assessed | Mycorrhizal cost (% of recorded C budget) | Note |
|---|---|---|---|---|---|---|---|---|---|---|
| | Host plant species | AM fungal species as reported | Current AM fungal name | | | | | | | |
| Jakobsen and Rosendahl (1990) | *Cucumis sativus* | *Glomus fasciculatum* | *Endogone arenacea* | $^{14}C$ | 16 h | 80 h | + | + | 20 | % of assimilated C consumed by fungal biomass and its respiration |
| Peng et al. (1993) | *Citrus volkameriana* | *Glomus intraradices* | *Rhizophagus intraradices* | None | | | +[b] | + | 7[a] | % C of the net C assimilation flow into root and soil respiration (AM minus NM) |
| Pearson and Jakobsen (1993) | *Cucumis sativus* | *Scutellospora calospora, Glomus caledonium, Glomus* sp. | *Scutellospora calospora, Funneliformis caledonium, Glomus* sp. | $^{14}C$ | 16 h | 70 h | – | + | 8.5–18.6[a] | % of assimilated C allocated by AM minus NM plants to belowground (roots, ERM, belowground respiration) |
| Wright et al. (1998) | *Trifolium repens* | Field AM fungal community | | None | | | +[b] | + | 15 | % of the net amount of $CO_2$ assimilated by AM plants respired by AM minus NM roots |
| Johnson et al. (2002a) | Grassland—24 plant species | Field AM fungal community | | $^{13}C$ | 3.5 h | 24 h | +[d] | + | 3.9–6.2 | % of the fixed C passed through the ERM—no accumulation of $^{13}C$ observed in the substrate |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Johnson et al. (2002b) | Grassland—24 plant species | Field AM fungal community | | $^{14}C$ | 3 h | 70 h | +[d] | + | 3.4 | % C allocation of the photosynthetically fixed C by the plant into AM mycelium (incorporation into + release from AM fungi) |
| Grimoldi et al. (2006) | *Lolium perenne* | *Glomus hoi* | | $^{13}C$ | 16 h | 6–7 h | + | + | 4.8–6 | % C of daily gross photosynthesis allocated to the AM fungi |
| Heinemeyer et al. (2006) | *Plantago lanceolata* | *Glomus mosseae* | *Funneliformis mosseae* | $^{13}C$ | 3.5 h | 2 h | – | + | <1 | % C of net photosynthesis allocated to ERM |
| Drigo et al. (2010) | *Festuca rubra* | Field AM fungal community | | $^{13}C$ | 16 h | 6 days | – | – | 8.8–9[a,c] | % of total fixed C in the assessed compartments incorporated into the AM fungi (NLFA) |
| Lendenmann et al. (2011) | *Medicago truncatula* | *Glomus intraradices, Glomus claroideum, Gigaspora margarita* | *Rhizophagus intraradices, Claroideoglomus claroideum, Gigaspora margarita* | $^{13}C$ | 1 h | 5 days | – | + | 1.7–12.9[a] | % C in all measured compartments allocated belowground (roots, substrate and belowground respiration), difference between AM a NM plants |
| Calderón et al. (2012) | *Sorghum bicolor* | *Glomus clarum* | *Rhizophagus clarus* | $^{14}C$ | 3 h | 24 days | + | + | 4 (6.8[a]) | % photoassimilated C allocated belowground, difference between AM and NM plants |

(continued)

**Table 1.1** (continued)

| Reference | Plant-fungal partner combination | | | Isotope | Length of labeling period | Length of chase period | Above-ground respiration assessed | Below-ground respiration assessed | Mycorrhizal cost (% of recorded C budget) | Note |
|---|---|---|---|---|---|---|---|---|---|---|
| | Host plant species | AM fungal species as reported | Current AM fungal name | | | | | | | |
| Tomé et al. (2015) | *Fragaria ananassa* var. *elsanta* | Mix *Funneliformis mosseae* and *Rhizophagus intraradices* | | $^{13}C$ | 6 h | 1 and 7 days | – | – | 1.8–4.3 | % of total fixed C allocated to AM fungal mycelium |
| Slavíková et al. (2017) | *Medicago truncatula* | *Rhizophagus irregularis* | | $^{13}C$ | 2 h | 6 days | +[b] | + | 2.3 (2.9) | % of the plant C budget allocated to the AM fungi—comparison between AM and NM plants of C allocation to substrate (or belowground) |

Values were estimated with or without including above- and/or below-ground respiration
*AM* arbuscular mycorrhizal, *NM* non-mycorrhizal, *ERM* extraradical mycelium, *NLFA* neutral lipid fatty acid
[a]Our calculation from the numbers provided in the publication
[b]Dark shoot respiration
[c]Approximate values deduced from graphic presentation of results
[d]Approximate figures of shoot respiration deduced from sequentially harvested pots

**Table 1.2** Carbon (C) allocation into different compartments of the arbuscular mycorrhizal (AM) plant-soil system in studies assembling C budgets of the whole plants[a]

| Reference | Plant–fungal partner combination | | | Isotope | Length of labeling period | Length of chase period | Recently fixed C allocation (% of total) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Host plant species | AM fungal species as reported | Current AM fungal name | | | | Aboveground respiration | Shoot | Roots[b] | Substrate[c] | Belowground respiration[d] | AM fungus | | Note |
| | | | | | | | | | | | | AM fungal mycelia | AM fungal respiration | |
| Paul and Kucey (1981) | *Vicia faba* | *Glomus mosseae* | *Funneliformis mosseae* | $^{14}C$ | 48 h | 96 h | 2 | 40–47 | 18–19 | 0.5 | 28–31 | 1 | 3 | A |
| Kucey and Paul (1982) | *Vicia faba* | *Glomus mosseae* | *Funneliformis mosseae* | $^{14}C$ | 48 or 8 h | 96 or 116 h | 1–2.3 | 41.7–52 | 16.8–29 | | 22.1–37.9 | 0.8–0.9 | 2.8–3.3 | A |
| Snellgrove et al. (1982) | *Allium porrum* | *Glomus mosseae* | *Funneliformis mosseae* | $^{14}C$ | 30 min | 48 h | 2.3–6.3 | 49.7–60.8 | 15.7–27.1 | 2.1–5.3 | 9.7–23.1 | | | A |
| Harris et al. (1985) | *Glycine max* | *Glomus fasciculatum* | *Rhizophagus fasciculatus* | $^{14}C$ | 16 h | 68 h | 4–6.3 | 51–61.2 | 9.9 | 1.3–1.8 | 14.6–16.3 | 2.7–2.8 | 4.7–13.7 | B |
| Jakobsen and Rosendahl (1990) | *Cucumis sativus* | *Glomus fasciculatum* | *Rhizophagus fasciculatus* | $^{14}C$ | 16 h | 80 h | 2.5 | 54.1 | 13.2 | 2.3 | 27 | 0.8 | | A |
| Calderón et al. (2012) | *Sorghum bicolor* | *Glomus clarum* | *Rhizophagus clarus* | $^{14}C$ | 3 h | 24 days | 5 | 47.9 | 28.9 | 6.3 | 11.9 | | | A |

The studies vary in terms of symbiotic partner combinations, plant age, labeling pulse or chase periods, and presence or absence of *Rhizobia* for leguminous hosts

A—carbon allocation into the different compartments as reported by the authors, B—carbon allocation into the different compartments calculated by us from values provided by the authors

[a]Only including studies where all the relevant measurements were made and properly reported

[b]Including nodules and intraradical mycelium for dually colonized leguminous hosts

[c]Including extraradical AM fungal mycelium

[d]Including rhizobial and fungal respiration if the latter is not explicitly provided

photosynthetically fixed C, C allocated to shoots 40–61%, C allocated to roots 10–29%, C allocated to substrate 1–6%, and C allocated specifically to AM fungal mycelium 1–3%; AM fungal respiration reaching 3–14%; and belowground respiration in total reaching 8–38% (Paul and Kucey 1981; Kucey and Paul 1982; Snellgrove et al. 1982; Harris et al. 1985; Jakobsen and Rosendahl 1990; Calderón et al. 2012).

Based on summary of all available literature on the magnitude of C fluxes in AM symbioses, it seems that the average C expenditure of the AM symbiosis may well be under 10% of the plant C budget (see Table 1.1 for more details). For comparison, in ectomycorrhizal symbioses, the magnitude of C allocated to fungal partner oscillates (apparently) around 3–36% of C fixed by photosynthesis (Bryla and Eissenstat 2005 and references therein). Very low (0.4% of the total C fixed by the plant) loss of plant photosynthate to its associated mycorrhizal fungus was, in contrast, reported for mycorrhizal green orchid *Goodyera repens* by Cameron et al. (2008).

The reported values on C allocation to AM fungi range widely. Here, the low number of publications dedicated to mycorrhizal C costs, especially in comparison with the quantity of literature concerning nutritional benefits of mycorrhizas, do not allow to properly uncover the determinants of plant C allocation to AM fungi. However, it seems that the choice of model host plant, AM fungal species and/or their combinations (Pearson and Jakobsen 1993; Lerat et al. 2003; Lendenmann et al. 2011), developmental stage of the symbiosis (Wright et al. 1998), environmental conditions (Slavíková et al. 2017), size and setup of the pots, and the duration of the isotope labeling/chase periods all strongly affect the outcome of quantification of C allocation to the AM fungi (see also Tables 1.1 and 1.2).

The exploration of mycorrhizal C cost has formerly been restricted by the available methodologies. Using $^{14}C$ radioisotope to directly trace the C fluxes from plant to mycorrhiza and to the soil was subject to strict health and radiosafety regulations (Schuur et al. 2016). Commercial availability of C sources enriched by stable $^{13}C$ isotope in the recent decades together with customization of the necessary mass spectrometry instrumentation made the direct C tracing much more available. However, despite the fact that the isotopic pulse-chase labeling enabled significant advances in assessing the C transfers within the plant–soil systems, it still only provides information with regard to the fate of recently fixed plant C, thus inevitably covering only a short period within the plant and/or fungal life cycles (Johnson 2008). This may be particularly short-sighted with respect to the mycorrhizal symbioses in trees and other long-living plants that could accumulate C reserves over long periods of time.

Further, the estimates of the mycorrhizal C costs based on incomplete C budgets (Pang and Paul 1980; Koch and Johnson 1984; Pearson and Jakobsen 1993; Heinemeyer et al. 2006; Drigo et al. 2010; Lendenmann et al. 2011) should be regarded with caution. This is because the gaseous C losses from shoots or roots/soil may reach a significant share of the plant C budget and thus should not be neglected (Lendenmann et al. 2011; Slavíková et al. 2017). Ignoring these C pools automatically leads to overestimation of the mycorrhizal C costs, which obviously was the case in some of the previous studies, although not the study by Jakobsen

and Rosendahl (1990) reporting the highest C costs of AM symbiosis ever (Table 1.1). Provided the rapidity of C fluxes between the plant, AM fungi, and the soil (Johnson 2008), it is sometimes very challenging to distinguish the C allocation to the root biomass, intra- and extraradical AM fungal mycelium and the soil/substrate, and to separate root and hyphal respirations (Heinemeyer et al. 2006). To this end, comparing mycorrhizal and nonmycorrhizal plants seems inevitable, although it is now widely accepted that this may be a source of many artifacts (Smith and Smith 2012). Moreover, depending on the balance between net costs and benefits of the symbiosis, mycorrhizal phenotypes appear to cover a whole continuum of plant responses to AM fungal colonization ranging from positive to neutral to negative (Johnson et al. 1997; Klironomos 2003). For some combinations of symbiotic partners and environmental conditions, mycorrhizal C costs may simply outweight the growth benefits conferred to plants (Johnson et al. 2015), and it may not be possible to produce nonmycorrhizal and mycorrhizal plants of the same size and mineral nutrition (Peng et al. 1993; Graham et al. 1996; Lendenmann et al. 2011). Here, the solution to compare physiology of mycorrhizal and nonmycorrhizal plants may be in using P fertilization to produce mycorrhizal and nonmycorrhizal plants of the same size (Brown and Bethlenfalvay 1987; Baas and Lambers 1988; Slavíková et al. 2017). Another possibility is using plants with a split-root system (Douds et al. 2000).

Peripheral importance has been so far dedicated to fungus-to-plant C transfers, despite they have been shown as a significant component of the overall C budget (at least) in the orchid mycorrhizas. Yet, because up to 10% of plant species are at least partially mycoheterotrophic and receive a net C gain from their fungal symbiont for at least a part of their life (Leake 2005), they should be taken seriously. Clear demonstration of the fungus-to-plant C flux, although much lower than the C flow in opposite direction, was shown by Cameron et al. (2008) who quantified the bidirectional C fluxes by using $^{14}C$ labeling in green orchid *Goodyera repens* associated with fungus *Ceratobasidium cornigerum*. In ectomycorrhizas, the transfer of amino acid-C from fungus to plant has also been demonstrated (Abuzinadah and Read 1989), although importance of this mechanism for bulk C transfer from fungus to plant is probably low. Yet it may potentially have some impact on the C economy of the mycorrhizal symbiosis (Taylor et al. 2004) and thus should be incorporated in the assessments of mycorrhizal C cost. Such an "up-flow" of C may occur even in arbuscular and ericoid mycorrhizal associations, but have not been demonstrated as yet (Johnson 2008).

## 1.3 Mechanisms of C Transfer Between the Symbiotic Partners

Although it has been demonstrated many times that there is a fast and directed C transfer between the plants and the AM fungi (e.g., Johnson et al. 2002b; Dilkes et al. 2004; Olsson and Johnson 2005; Kiers et al. 2011), the molecular mechanisms

of such a transfer still remain elusive—no single gene responsible for mycorrhiza-directed C efflux from the plant cells specifically at the symbiotic interface has been identified as yet. This is in contrast to a wealth of knowledge on genes involved in the movement of sugars within a plant. The sugar fluxes from plant mesophyll (or sugar reserves elsewhere) to the rhizosphere are obviously coordinated by complex network involving many genes such as sucrose transporters, monosaccharide transporters (MSTs) and the SWEET translocator family (for more details see Doidy et al. 2012; Garcia et al. 2016). Indirect evidence makes the periarbuscular interface a hot candidate for site of exchange (trading) of C against the mineral nutrients, although unequivocal experimental proof for this is largely missing (Garcia et al. 2016). On the other hand, it seems well established that it is monosaccharides (particularly the glucose) that are the major form of C taken up by the AM fungi from the plant (Pfeffer et al. 1999; Schüβler et al. 2006; Nehls 2008; Helber et al. 2011). Thus, it seems that complex carbohydrates such as sucrose are cleaved at the plant–fungus interface, either by plant or fungal invertases (Casieri et al. 2013). Proton-ATPase activity on the plant membranes at the symbiotic interface indicates an active membrane transport mechanism (Krajinski et al. 2014), although direct coupling of this activity with C efflux has not yet been established. It seems, however, that the SWEET transporters are currently the hottest candidates for explaining the AM-directed C flux (Garcia et al. 2016).

On the fungal side, monosaccharide transporters were identified in ectomycorrhizal (Garcia et al. 2016 and references therein) as well as in AM fungi (Schüβler et al. 2006; Helber et al. 2011). First glomeromycotan MST (GpMST1) was described by Schüβler et al. (2006) in *Geosiphon pyriformis* interacting with autotrophic cyanobacterium *Nostoc punctiforme*. Its functional analogue in a "true" AM fungus appears to be the RiMST2 gene in *Rhizophagus irregularis*, which remains the only MST transporter proved so far to directly mediate sugar uptake by the AM fungus at the symbiotic interface with its plant host (Helber et al. 2011; Garcia et al. 2016).

## 1.4 Common Mycorrhizal Networks

Due to low host-mycobiont specificity in AM mycorrhizas, under most situations, the same AM fungal genotype at any given field site usually colonizes several plant individuals belonging to the same or different species. At the same time, the plant roots typically host multiple AM fungal species (Helgason et al. 2002; Vandenkoornhuyse et al. 2003), increasing the chances of sharing the mycobiont with the neighboring plant. The resulting "common mycorrhizal networks" (CMN) allow redistribution of mineral nutrients taken by the hyphae from the soil, water and the C costs between the involved plants (Merrild et al. 2013; Toju et al. 2013; Weremijewicz and Janos 2013; Prieto et al. 2016; Workman and Cruzan 2016) and also provide highways for transfer of signaling compounds between the plants (Johnson and Gilbert 2015). It is thus possible that one plant could feed the AM

hyphal network with C, whereas another plant would derive most of the benefits (mostly nutritional) of the shared association without providing much C to the fungus in return (Walder et al. 2012; Walder and van der Heijden 2015)—effectively resulting in "unfair" redistribution of the symbiotic C costs in the community. This could result in supporting the weaker competitor (e.g., a seedling vs. adult plant) or in strengthening the competition for resources, facilitated by the CMN (Newbery et al. 2000; Kytoviita et al. 2003; McGuire 2007; Weremijewicz and Janos 2013; Johnson 2015; Weremijewicz et al. 2016).

The big remaining question is, particularly with respect to the AM symbioses, whether any C could be transferred from the AM fungus to the plant. Although such a transfer has been postulated several years ago (Bidartondo et al. 2002; Simard and Durall 2004), current evidence for such a C transfer pathway is still equivocal, marked with a number of unanswered questions (Courty et al. 2011; Field et al. 2015) and opposing a strong experimental evidence that the fungus operates efficient control mechanisms (such as lipid/trehalose valve) ensuring the C to remain in the fungal tissues (Fitter et al. 1998; Pfeffer et al. 1999, 2004). This is in contrast to a well-established evidence about C transfer from the mycorrhizal fungus to its plant host in orchids in general and in achlorophyllous orchids in particular (Cameron et al. 2008; Barrett et al. 2010). What seems to be the rule, however, is that the mycorrhizal fungus obtains the C mainly or exclusively from a neighboring green plant rather than from the soil organic matter. This further reinforces the CMN as a key supply link to the mycoheterotrophic plants as well as in redistribution of C among ectomycorrhizal trees or shrubs (Selosse and Roy 2009; Deslippe and Simard 2011; Klein et al. 2016).

## 1.5 Food Chains

A substantial amount of C fixed by a plant is transported belowground shortly after photoassimilation and subsequently, within hours to days, detectable in the soil micro- and macrobes including the mycorrhizal fungi (Dilkes et al. 2004; Bahn et al. 2009; Mencuccini and Hölttä 2010). The mycorrhizal hyphae, who are responsible for diverting of up to several percent of plant photosynthetic production belowground (Table 1.1), then function as a specific channel for C flow from plant (leaves) down to soil (Kaiser et al. 2015), dictating who gets a share and who does not (Drigo et al. 2010; Schrey et al. 2015). The C from the mycorhizal hyphae can pass onto the other members of soil biota as hyphal exudates. These could be produced either in an unspecific manner in form of organic acids or exoenzymes produced to the soil environment to manipulate nutrient availability in the immediate vicinity of the hyphae (Valentine et al. 2013; Sato et al. 2015) in a similar way as root exudates (Kuzyakov 2010; Bird et al. 2011; Philippot et al. 2013). Alternatively, hyphal exudates could be targeted to "hypersymbiotic" microbes associated with the hyphae and fulfilling specific and complementary functions to that of the hyphae themselves (Hodge et al. 2010; Cheng et al. 2012; Jansa et al. 2013; Taktek

et al. 2015; Zhang et al. 2016). Further, the C from the living or dead AM fungal hyphae/spores could also pass onto other soil biota by grazing/parasitism caused by a wide range of biotrophic microbes (Fitter and Garbaye 1994; Klironomos and Kendrick 1996; Rousseau et al. 1996; Crowther et al. 2012), and the mycorrhizal necromass is becoming a food source of soil saprotrophes (Treseder and Allen 2000). Worth mentioning remains the possible involvement of AM hyphal products such as glomalin in formation of recalcitrant soil organic matter—although the genesis and structure of this elusive compound is not entirely clear (Gadkar and Rillig 2006), it has been shown to correlate tightly with the presence/activity of AM fungi as well as with soil aggregate stability (Wright et al. 2006; Hammer and Rillig 2011; Fokom et al. 2013) and thus deserves a mention at this place—as potentially an important components of mycorrhizal C budget and also as a potential food source for specialized microbes.

## 1.6 Conclusions, Future Research Directions

The survey of literature on C fluxes in mycorrhizal plants (Table 1.1) offers few interesting insights: First, mycorrhizal C costs in terms of share of C fixed by the plant and allocated to the AM fungal symbiont is usually below 10%. This is an important observation since the maximum values (~20% of plant C budget) rather than the mean value (<10%) are most often cited in the secondary literature. Second, there is no observable increase in frequency of reports on C costs of mycorrhizal plants throughout the years. Since about 35 years, the papers appear sporadically, use different model systems and analytical approaches and our understanding of the determinants of the magnitude of C fluxes in mycorrhizal plants thus remain rather rudimentary, although we know lot more now than when Jack Harley published his "Problems of mycotrophy" in 1975 (Harley 1975). Third, not always have all the relevant compartments of the experimental system been included in the observations—most importantly, the aboveground respiration could represent 10 or more percent of the plant's C budget and ignoring it could easily lead to overestimation of mycorrhizal C costs (Slavíková et al. 2017). Fourth, there is a large variation in the mycorrhizal C costs assignable to functional differences between symbiotic partners, environmental conditions, duration of the pulse-chase labeling, etc. (Table 1.1). It needs systematic efforts now to disentangle the determinants of magnitude/direction of C fluxes in mycorrhizal plants, which could eventually result in uncovering such important phenomena like the simultaneous bidirectional C fluxes in green orchid mycorrhizas (Cameron et al. 2008). The role of CMN and direction of C transfers in more complex plant–fungal communities should carefully be addressed in mycoheterotrophic plants, including achlorophyllous AM hosts, orchid, and Monotropoideae. To the best of our knowledge, there is also nearly no quantitative information on the magnitude of C fluxes between the partners in ericoid mycorrhiza, with a notable exception of a (qualitative) study by Grelet et al. (2009). Particularly, manipulation of light intensities in

model experiments is still rare, although they would directly be relevant to the C source strength of the mycorrhizal plants (Konvalinková and Jansa 2016).

It is also surprising how little is still known about the molecular mechanisms of C transfer between the symbiotic partners, despite the current wealth of high-throughput techniques and advanced tricks to study molecular design of living organisms. It seems like there is insufficient exchange of information and concepts between the molecular geneticists and the physiologists, although both are frequently approaching the same system and asking closely related questions. In this regard, it would be important to explore the possibilities of spatially explicit in vivo measurements of C transfers between symbiotic partners using $^{11}$C-positron tomography (Wang et al. 1989) and possibly couple these with reporter genes or microRNA-based techniques to manipulate/visualize gene expression of specific genes.

Last but not least, the fact that ectomycorrhizal (and possibly also other mycorrhizal) fungi under field setting almost invariably rely on recently fixed C rather than on saprotrophy (Talbot et al. 2008; Lindahl and Tunlid 2015) is surprising given the ease of culturing some of these fungi on sugar-containing media in vitro (e.g., Hughes and Mitchell 1995; Midgley et al. 2004). It is hard to believe that the fungi would not use this capacity to obtain C saprotrophically at least under some circumstances. Indeed, more research in this direction is certainly warranted, particularly scrutinizing specific ecosystem scenarios conducive for expression of the saprotrophic capacity of the fungi—such as cold and wet periods, extensive period of darkness (polar nights), vegetation dormancy, or like.

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# Chapter 2
# Basic and Applied Research for Desert Truffle Cultivation

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**Abstract** This chapter summarizes the latest basic and applied advances in desert truffle research carried out to improve our knowledge of the biodiversity, physiology, biotechnology, and cultivation of these hypogeous and edible fungi. ITS-rDNA sequences in phylo-geographic studies and host plant and soil pH characteristics have been the key to describing eight new desert truffle species. The production of desert truffle mycorrhizal plants has been improved by using β-cyclodextrin and bioreactors for mycelium culture and native beneficial bacteria (PGPR and MHB) to increase seedling survival and mycorrhization. Some fungal enzymes have also been characterized in *Terfezia claveryi* ascocarps. The presence of alkaline phosphatase both in mycelia and ascocarps indicates that this enzyme plays an important role during the life cycle of *T. claveryi*, while acid phosphatase might be involved in a process that takes place during the ascocarp stage. Numerous desert truffle plantations have been established in Spain in the last 10 years. A high density of mycorrhizal plants combined with a proper irrigation are two important factors to stimulate ascocarp production. The combination of a high rate of intracellular colonization together with the fine-tuned expression of fungal and plant aquaporins could result in a morpho-physiological adaptation of this symbiosis in drought conditions. Moreover, desert truffle sylviculture is proposed for improving truffle production and for conserving the natural areas where desert truffle grow.

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## 2.1 Introduction

The first plantation of the desert truffle *Terfezia claveryi* was established in 1999 in south-east Spain (Murcia) (Honrubia et al. 2001), since when most of the data related to the biotechnological aspects of the production of mycorrhizal plants and plantation management practices have been compiled in three publications of Springer (Morte et al. 2008, 2009, 2012). More recently, additional information on desert truffles related to soil properties (Bonifacio and Morte 2014), the types of mycorrhiza (Roth-Bejerano et al. 2014), cryptic and new species (Bordallo and Rodriguez 2014), the benefits conferred on plants (Kagan-Zur et al. 2014a), ascocarp enzymes (Pérez-Gilabert et al. 2014), and cultivation (Morte and Andrino 2014; Honrubia et al. 2014) have been published by our group in the first international and monographic book devoted to desert truffles by the same publisher (Kagan-Zur et al. 2014b). However, the increasing demand for this crop, in Spain and in other countries, has prompted a search for new strategies to increase ascocarp production in the field, to improve the production protocol of mycorrhizal plants, and to advance our knowledge of the biology and biodiversity of these desert truffles.

The present chapter describes all the experiments carried out to obtain these objectives and the last results obtained.

## 2.2 Edible and New Species of Desert Truffles

The edible hypogeous ascomata of fungi belonging mostly to the Pezizaceae family are known as "desert truffles" due to their habitat, typically arid and semi-arid ecosystems, mostly in the Mediterranean region (Morte et al. 2009; Zambonelli et al. 2014), where they constitute an important economic resource for local populations. Species of *Terfezia* and *Tirmania* have a long history of culinary and medical use because they are rich in nutrients and bioactive compounds (Shavit 2014).

Recently, some new species of desert truffles have been identified (Bordallo et al. 2012, 2013, 2015; Kovăcs et al. 2011). The ITS-rDNA sequence, host plant, and soil pH seem to be the key to describing new desert truffle species. *Terfezia* species (or their host) seem to be able to adapt to a wide range of soil pH values (high or relatively low), edaphic conditions, and texture (Bonifacio and Morte 2014). *Terfezia canariensis* has been described as belonging to the claveryi group. Five new *Terfezia* species—*T. pini, T. pseudoleptoderma, T. albida*, *T. grisea*, and *T. cistophyla*—have recently been proposed as forming part of the previously single leptoderma–olbiensis cryptic group, and another two species, *T. eliocrocae* from alkaline soils and *T. extremadurensis* from acid soils, have also been proposed as new species (Fig. 2.1). In desert truffles, as in the case of mycorrhizal fungi, the preference-specificity factor of the host is regarded as an

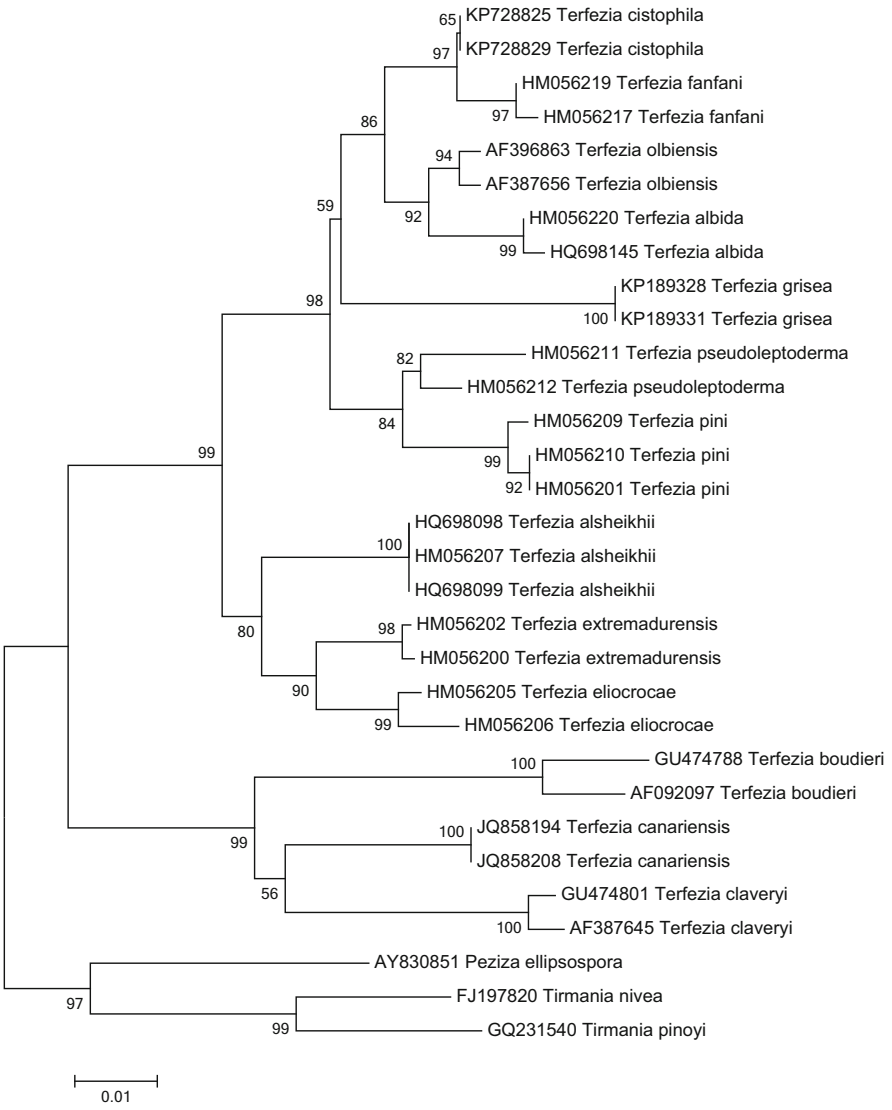


**Fig. 2.1** The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 400 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4

important factor for understanding their life cycle. The difficulty of sampling desert truffles implies their slow discovery (Bordallo and Rodríguez 2014). However, using new tools, such as molecular biology and phylo-geographic studies, should allow us to identify differences among cryptic species.

However, not all hypogeous species belonging to the desert truffle genera are edible fungi. So, what makes a desert truffle edible? Apart from their taste, the nutrients, and antioxidant compounds they contain, a medium-large size and an easy to clean peridium are essential if they are harvested and marketed. For example, due to its small size and pubescent peridium that accumulates soil debris, *Picoa lefebvrei* (Pat.) Maire is not marketed despite it being especially rich in natural antioxidants (Murcia et al. 2002) and very tasty, much more so than many edible *Terfezia* species.

At least, 14 edible desert truffles have been identified as being regularly consumed by people (Table 2.1), all of which have characteristic host plants and soil pHs that define their mycorrhizal symbiosis and ecology (Table 2.1).

**Table 2.1** Edible species of desert truffles, their host plant species, and soil pH requirements

| Edible desert truffles | Host plants | Soil pH |
|---|---|---|
| *Choiromyces magnusii* | *Cistus ladanifer* | Acid |
| *Mattirolomyces terfezioides* | *Citrus* spp, *Prunus* spp<br>*Helianthemum ovatum*<br>*Robinia pseudoacacia* | Alkaline–neutral |
| *Picoa juniperi* | *Helianthemum* spp | Alkaline |
| *Picoa lefebvrei* | *Helianthemum* spp | Alkaline |
| *Terfezia arenaria* | *Tuberaria guttata* | Acid |
| *Terfezia boudieri* | *Helianthemum lippii*<br>*Helianthemum salicifolium*<br>*Helianthemum sessiliflorum* | Alkaline |
| *Terfezia canariensis* | *Helianthemum canariense* | Alkaline |
| *Terfezia claveryi* | *Helianthemum almeriense*<br>*Helianthemum canariense*<br>*Helianthemum guttatum*<br>*Helianthemum hirtum*<br>*Helianthemum ledifolium*<br>*Helianthemum salicifolium*<br>*Helianthemum violaceum* | Alkaline |
| *Terfezia fanfani* | *Tuberaria guttata* | Acid |
| *Terfezia leptoderma* | *Helianthemum salicifolium*<br>*Tuberaria guttata* | Acid |
| *Tirmania nivea* | *Helianthemum salicifolium*<br>*Helianthemum lippii* | Alkaline |
| *Tirmania pinoyi* | *Helianthemum salicifolium*<br>*Helianthemum lippii* | Alkaline |
| *Tuber lacunosum* | *Tuberaria guttata* | Acid |
| *Tuber oligospermum* | *Pinus* spp, *Quercus* spp, *Cistus* spp | Acid–alkaline |

Among these fungal species, two species of desert truffle have been successfully cultivated and reported, *Terfezia claveryi* Chatin in Spain (Honrubia et al. 2001; Morte et al. 2008, 2009, 2010, 2012) and *Terfezia boudieri* Chatin in Tunisia (Slama et al. 2010) and Israel (Khagan-Zur, pers. com.). More recently, mycorrhizal plants with *Picoa lefebvrei* and *Tirmania nivea* have been planted in 2014, and with *Terfezia arenaria* in 2015, in Spain, but fruiting ascocarps have not yet been obtained.

## 2.3 Production of Desert Truffle Mycorrhizal Plants

The increasing demand for desert truffle mycorrhizal plants for desert truffle cultivation has prompted research into new strategies to help the transition from experimental scale to medium-large scale cultivation. The first step in this process is the selection and production of suitable mycorrhizal seedlings of good quality and adapted to different cultivation sites.

For the mycorrhizal synthesis, both seedlings and micropropagated plants of *Helianthemum* species, together with *Terfezia* spores and mycelium, have been used (Morte and Andrino 2014; Morte et al. 2008, 2009). The system for mycorrhizal plant production from *Helianthemum* seeds and *Terfezia* spores is the most used because it is cheaper than using micropropagated plants and mycelium as inoculum. However, each of these systems presents its own strengths and weaknesses.

### *2.3.1 Plant Production*

For this purpose, a suitable host plant species should be chosen, taking into account edaphic and bioclimatic conditions; if possible, it is better to use a perennial rather than an annual species.

Most of the plant species reported as host plants for experimental desert truffle mycorrhization are perennial and annual species from *Helianthemum* genus, belonging to the Cistaceae (Morte and Andrino 2014). Many *Helianthemum* species show erratic seed germination, and seed scarification is necessary to increase germination rates. Moreover, high mortality of the germinated seedlings is common during the first 2 months after germination in nursery conditions (Morte et al. 2012). Micropropagation techniques have been used for plant production since they improve seed germination and plant survival (Morte et al. 2008). *Helianthemum almeriense* Pau has been successfully micropropagated by the photomixoautotrophic (PM) method (Morte and Honrubia 1992, 1997), and the same plant was used as a model to improve *Helianthemum* propagation by photoautotrophic (PA) micropropagation (Morte et al. 2012). When cultured in the absence of sucrose, this plant increased its survival rate during acclimation using

a PA system. At the same time, substituting agar by perlite, as physical support, contributed to a significant reduction in plant losses during acclimation. In addition, the absence of sucrose reduced the presence of microbial contamination during the cultivation vessel phase (Morte et al. 2012). This method permitted us to grow a large volume of *H. almeriense* seedlings with germination rates of around 80–90% and very satisfactory results.

To ascertain the most suitable moment for plant transplantation from in vitro to ex vitro conditions in order to prevent plant losses, the probability of plant survival was estimated based only on a chlorophyll meter SPAD-502 measurements. The maximum survival rate for *H. almeriense* was established at 28 SPAD-502 units, or its equivalent in total chlorophyll content, 1.6 mg/g leaf (Morte and Andrino 2014).

In addition to plant micropropagation, we tried to improve plant production from *Helianthemum* seeds by reducing the high mortality of the germinated seedlings that normally occurs during the first 4 weeks after germination in nursery conditions (Morte et al. 2008). It was realized that the early inoculation of *Helianthemum* seedlings with *Terfezia* was not sufficient to enhance the low survival rates in nursery conditions. This led us to wonder if the use of other microorganisms present in the rhizosphere of the mycorrhizal association *Helianthemum* x *Terfezia*, such as plant growth-promoting rhizobacteria (PGPR), could help in one or more stages of mycorrhizal plant production system. For this purpose, 64 native bacterial colonies were isolated from mycorrhizal roots of *H. almeriense* with *T. claveryi*, mycorrhizosphere soil, and peridium of *T. claveryi* in order to evaluate their effect on the mycorrhizal plant production (Navarro-Ródenas et al. 2016). Based on a phylogenetic analysis of the 16S rDNA partial sequence, the 64 colonies were gathered in 45 different strains from 17 genera, the largest genera being *Pseudomonas* (41% of the isolated strains), *Bacillus* (12% of the isolated strains), and *Varivorax* (8%). All bacteria were characterized phenotypically and by their PGPR traits, including auxin and siderophore production, phosphate solubilization, and the presence of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Table 2.2). Only bacterial combinations with several PGPR traits and *Pseudomonas fluorescens*, strain 5, which presents three different PGPR traits, showed a positive effect on plant survival and growth. Particularly relevant were the bacterial treatments involving auxin release, which significantly increased the root–shoot ratio and the mycorrhizal percentage (Table 2.3). Moreover, *Pseudomonas mandelii* strain 29 was able to considerably increase the mycorrhization ratio but not plant growth, thus being considered as a mycorrhiza helper bacterium (Table 2.3). Among these bacteria, the fluorescent pseudomonads complex was the most abundant and significant in terms of the effects on PGPR traits in the *Terfezia* × *Helianthemum* symbiosis. The use of some of these bacteria at different stages of nursery plant production helps overcome some of the current bottlenecks of desert truffle plant production at semi-industrial scale. The benefits would include increased survival rates and mycorrhization, reduced production time, and, ultimately, greater plant quality (Navarro-Ródenas et al. 2016). Therefore, the mycorrhizal roots, mycorrhizosphere soil, and peridium of desert truffles must be regarded

**Table 2.2** Characterization of plant growth-promoting traits

| Strain n° | Organisms identified | Phosphate solubilization[a] | IAA production[b] | Siderophore production[c] | ACC desaminase |
|---|---|---|---|---|---|
| 1 | *Pseudomonas* sp. | ++ | – | + | – |
| 2 | *Paenibacillus* sp. | – | – | – | – |
| 3 | *Bacillus thuringiensis* | – | – | – | – |
| 4 | *Achromobacter* sp. | – | – | – | – |
| 5 | *Pseudomonas fluorescens* | ++ | + | – | + |
| 6 | *Microbacterium paraoxydans* | – | + | – | – |
| 7 | *Pseudomonas* sp. | – | ++ | ++ | – |
| 8 | *Bacillus atrophaeus* | – | – | – | – |
| 9 | *Pseudomonas* sp. | – | – | – | – |
| 10 | *Pseudomonas* sp. | ++ | – | + | – |
| 11 | *Bacillus megaterium* | ++ | – | – | – |
| 12 | *Sphingomonas* sp. | – | – | – | – |
| 13 | *Rhizobium radiobacter* | – | – | – | – |
| 14 | *Acinetobacter lwoffii* | – | – | – | – |
| 15 | *Flavobacterium* sp. | – | – | ++ | – |
| 16 | *Novosphingobium panipatense* | – | + | – | – |
| 17 | *Bacillus simplex* | – | – | ++ | – |
| 18 | *Stenotrophomonas rhizophila* | – | – | – | – |
| 19 | *Arthrobacter* sp. | – | – | – | – |
| 20 | *Sinorhizobium meliloti* | – | – | – | – |
| 21 | *Pseudomonas* sp. | ++ | – | ++ | – |
| 22 | *Variovorax paradoxus* | – | – | – | – |
| 23 | *Variovorax paradoxus* | – | – | – | – |
| 24 | *Phyllobacterium bourgognense* | – | – | – | – |
| 25 | *Pseudomonas* sp. | – | – | – | – |
| 26 | *Microvirga* sp. | – | + | – | – |
| 27 | *Pseudomonas* sp. | – | – | – | – |
| 28 | *Pseudomonas moraviensis* | – | – | + | – |
| 29 | *Pseudomonas mandelii* | ++ | – | – | – |
| 30 | *Pseudomonas* sp. | – | – | – | – |

(continued)

**Table 2.2** (continued)

| Strain n° | Organisms identified | Phosphate solubilization[a] | IAA production[b] | Siderophore production[c] | ACC desaminase |
|---|---|---|---|---|---|
| 31 | *Pseudomonas* sp. | – | – | – | – |
| 32 | *Pseudomonas* sp. | – | – | – | – |
| 33 | *Pseudomonas* sp. | – | – | + | – |
| 34 | *Pseudomonas brenneri* | +++ | – | + | – |
| 35 | *Rhodococcus* sp. | – | – | – | – |
| 36 | *Flavobacterium* sp. | – | – | – | – |
| 37 | *Phyllobacterium ifriqiyense* | – | – | – | – |
| 38 | *Variovorax paradoxus* | – | – | – | – |
| 39 | *Rhizobium galegae* | – | – | – | – |
| 40 | *Pseudomonas* sp. | ++ | + | – | – |
| 41 | *Arthrobacter nitroguajacolicus* | – | – | – | – |
| 42 | *Pseudomonas* sp. | + | – | +++ | – |
| 43 | *Arthrobacter* sp. | – | +++ | – | – |
| 44 | *Pseudomonas* sp. | – | – | – | – |
| 45 | *Variovorax paradoxus* | – | – | – | – |

Modified from Navarro-Ródenas et al. (2016)
[a]+ values <300 μg/mL, ++ values >300–550 μg/mL, +++ values >550 μg/mL
[b]+ values <50 μg/mL, ++ values >50–100 μg/mL, +++ values >100 μg/mL
[c]+ values <20 μg/mL, ++ values >20–60 μg/mL, +++ values >60 μg/mL

as environments enriched in bacteria which can increase the quality of the plant in the desert truffle plant production system at semi-industrial scale.

### 2.3.2 Fungal Inoculum Production

Mycorrhizal plants have been successfully produced by using both desert truffle spores and mycelia (Morte et al. 2008). Mature spores, obtained by blending truffles, are the most commonly used due to the slow and erratic growth of the mycelium in vitro. Working with spore inoculum, the spore solution can be applied directly to the plants or using perlite as a carrier, whereby the spores adhere to the pores and cavities within (Andrino et al. 2012; Morte et al. 2012). Using such a carrier technique allowed us to use 40% fewer of spores (Morte and Andrino 2014). However, the problem with spore inoculation techniques is that pests, pathogens, and other mycorrhizal fungi can still contaminate the plants (Iotti et al. 2016).

**Table 2.3** Summary of the effects of the different bacterial treatments on plant bioassays at different stages of mycorrhizal plant development

| | Treatment | Stages | | | |
|---|---|---|---|---|---|
| | | i | ii | iii | |
| Control | Species | Survival | Growth | Root/shoot ratio | Mycorrhization |
| 5 | *Ps. fluorescens* | * | N | ** | ** |
| 15 | *Flavobacterium* sp | N | N | * | N |
| 29 | *Ps. mandelii* | N | N | N | *** |
| 44 | *Arthrobacter* sp | N | N | *** | ** |
| 5+7 | *Ps. fluorescens*+*Pseudomonas* sp | *** | N | * | * |
| 34+7 | *Ps. brenneri*+*Pseudomonas* sp | N | N | * | * |
| 15+41 | *Flavobacterium* sp+*Pseudomonas* sp | *** | N | N | *** |
| 44+21 | *Arthrobacter* sp+*Pseudomonas* sp | ** | N | N | N |

*N* absence of significance (modified from Navarro-Ródenas et al. 2016)
*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, level of significance with regard to the control

Therefore, it is more advisable to use mycelium than spores whenever possible and profitable.

Desert truffle mycelia have been grown successfully on MMN (Modified Melin-Norkrans) medium. Desert truffle mycelium can be used directly from the plates as inoculum for in vitro mycorrhizal synthesis (Morte et al. 1994; Morte and Honrubia 1995, 1997) and from liquid fermentation for both in vitro and in vivo inoculation trials (Morte et al. 2008). However, only fungal strains well adapted to in vitro conditions should be used to produce mycelium by liquid fermentation in a bioreactor. In this sense, we have obtained a mycelium biomass (grams of dry weight/liter medium) of 0.41 g/l for *Picoa lefebvrei* (Santiago-Marín 2015), 0.30 g/l for *Terfezia claveryi* (Arenas 2014), and 1.16 g/l for *Terfezia olbiensis* (Morte et al. 2004). Similar values have been obtained for other ectomycorrhizal fungi, like *Pisolithus microcarpus* (0.48 g/l) by Rossi et al. (2002). However, other ectomycorrhizal fungi presented more vigorous growth in a bioreactor than desert truffles, like *Lactarius quieticolor* (3.26 g/l) and *Rhizopogon roseolus* (8.5 g/l) (Chávez et al. 2014). Therefore, greater effort is needed in order to increase these mycelium biomasses to ensure the continuous production of mycorrhizal plants.

Fungi produce a wide variety of secondary metabolites. In a wide generalization, Hanson (2008) considered that extracellular metabolites isolated from the culture filtrate may be associated with the combative relationship of the organism with its environment, while those isolated from the mycelium may have a protective role. Some of these substances are able to inhibit the development of their own populations (Trinci and Whittaker 1968). Thus, the erratic growth of the mycelium of *T. claveryi* may be caused by compounds produced by the fungus itself. To check

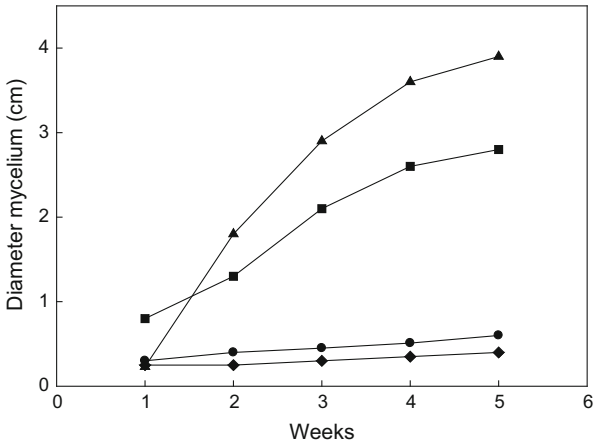


**Fig. 2.2** Effect of adding different types of cyclodextrins (CDs) to the culture medium on the mycelial growth (cm) of *T. claveryi*. (*circle*) control; (*diamond*) γ-CD; (*square*) α-CD; and (*triangle*) β-CD (From López-Nicolás et al. 2013)

this, we tested the effect of several cyclodextrins (CDs) on *T. claveryi* mycelium. CDs are non-reducing cyclic glucose oligosaccharides that are produced as a result of the transformation of starch by certain bacteria. Two characteristics of CDs, the existence of a hydrophobic cavity and the presence of two hydrophilic hydroxyl rings, allow them to form inclusion complexes in water with a variety of organic guest molecules, such as volatile compounds (López-Nicolás et al. 2009) and phenols. CDs are able to stimulate the mycelium growth of the desert truffle *T. claveryi*, increasing fourfold the values of colony diameter, growth rate, and colony fresh weight after cultivation (López-Nicolás et al. 2013). The increase in mycelium growth observed when CDs are added to the culture medium is probably due to the formation of an inclusion complex and not to the CDs being used as a carbon source. β-CD (8 mmol $l^{-1}$) was seen to be the most effective natural CD to stimulate the mycelium growth of the *T. claveryi* (Fig. 2.2). The inner diameter of the hydrophobic cavity of β-CD, a structure formed by seven molecules of glucose, leads to a more favorable interaction between the CDs and the different molecules present in the culture medium that would otherwise hinder the correct growth of this desert truffle (López-Nicolás et al. 2013).

### *2.3.3 Mycorrhizal Plant Production*

For the production of desert truffle mycorrhizal plants, four in vivo and two in vitro inoculation options were designed, the time required for each of them ranging between 4 and 8.5 months, depending on the type of plant propagation system and inoculum source used (Fig. 2.3). The photoautotrophic *Helianthemum* micropropagation system allowed this time to be reduced to 12 weeks with respect to photomixotrophic system since fungal inoculation is carried out at the moment plants are transferred from in vitro to ex vitro conditions, so that

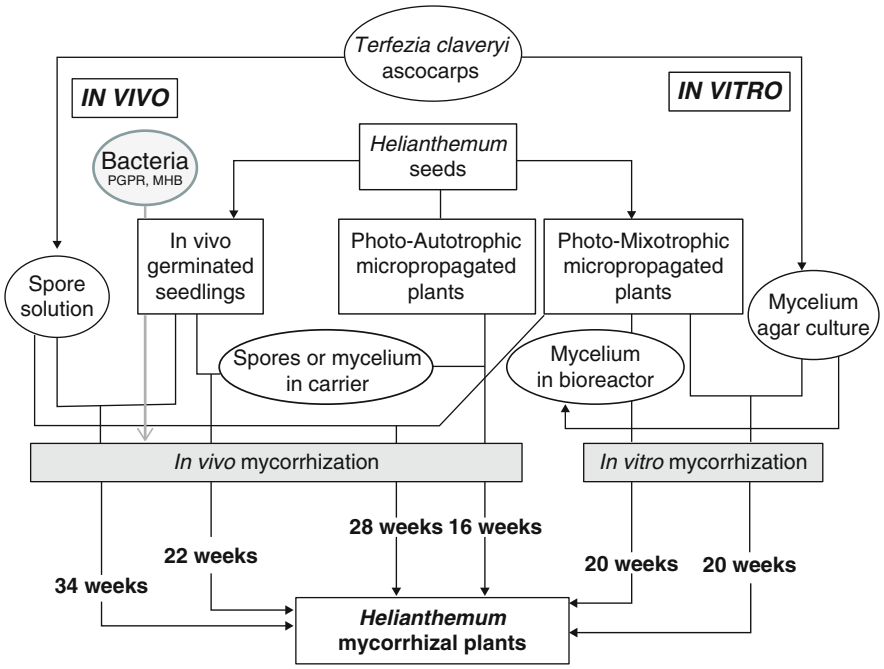


**Fig. 2.3** In vivo and in vitro ways for production of desert truffle mycorrhizal plants and time required for each of them. *PGPR* plant growth-promoting rhizobacteria, *MHB* mycorrhiza helper bacteria

plant acclimatization and mycorrhization occur at the same time (Morte and Andrino 2014).

The last and most recent improvement to this protocol was the use of native bacteria (PGPR or MHB) in the mycorrhizal plant production system starting from *Helianthemum* seeds and desert truffle spores. This system is subdivided into three different stages: (i) seed germination, which includes seed germination itself and the development of true leaves for about 4 weeks; (ii) plant growth, which includes shoot elongation, plant hardening, and the development of secondary fine roots; and (iii) inoculation and mycorrhization, which includes shoot and root development and mycorrhization. The use of a combination of bacteria that includes four PGPR traits (Table 2.3), strain 5 (*Ps. fluorescens*) and strain 7 (*Pseudomonas* sp) at stage (i) and the strain 29 (*Ps. mandelii*) at stage (iii), increased seedling survival and growth (stage i) and mycorrhization percentage (stage iii), respectively. Our next objective will be the use of these bacteria in each one of the plant production routes to improve plant quality and reduce plant production time.

## 2.4 Advances in Understanding Desert Truffle Enzymes

The symbiosis between *T. claveryi* and *H. almeriense* is an ectendomycorrhiza (Gutiérrez et al. 2003; Navarro-Ródenas et al. 2012a) that is well adapted to drought conditions in poor soils. In order to understand these adaptations as well as the process of the fungal fruit-body formation, some enzymes involved in the primary and secondary metabolisms were studied during the different stages of the fungal life cycle.

*Phosphatases* have traditionally been classified as alkaline (ALP) or acid (ACP). Two peaks of activity were observed when phosphatase was measured in the crude extract of *Terfezia claveryi* ascocarps, one with maximum activity between pH 9.5 and 10.0, corresponding to ALP and another, of lower activity, with a maximum at pH 4.5 (Navarro-Ródenas et al. 2009). These results indicate that *T. claveryi* ascocarps contain both ACP and ALP, the latter being the main one. In addition to the optimum pH, ALP and ACP from *T. claveryi* ascocarps differ in their optimum temperature, around 45 °C for ALP and 35 °C for ACP. The thermostability of both enzymes at their respective optimum temperatures is also different: ALP activity decreases slightly with time at 45 °C, so that 25% of the initial activity is lost after 1 h (Navarro-Ródenas et al. 2009) while at 35 °C, in the same period, ACP loses 60% of its initial activity. This temperature sensitivity was one of the reasons that impaired ACP purification despite the various approaches assayed. A single ALP isoform was extracted and partially purified by precipitation with polyethylene glycol, a protocol that allowed elimination of ACP and most of the lipids and phenolic compounds. The gentle extraction method used, without sonication and with a buffer of high ionic strength and without the addition of detergent, indicates that the partially purified ALP is a soluble enzyme (Navarro-Ródenas et al. 2009).

The response of ACP and ALP to certain inhibitors also differed, which represents a useful tool for measuring each activity independently in a crude extract. Tartrate, a classical inhibitor of ACP, when present in the reaction medium at 1 mM, produced a 20% inhibition of *T. claveryi* ACP but had only a limited inhibitory effect (5.9%) on ALP (Navarro-Ródenas et al. 2009). At the same concentration, orthovanadate produced 70% inhibition in ALP and 80% inhibition of ACP. Kinetic analysis of the effect of orthovanadate confirmed that it is a competitive inhibitor of *T. claveryi* ALP.

Our group also reported the presence of ALP in *T. claveryi* mycelium, where it was seen to respond to water stress and could be used as an indicator of the metabolic activity present (Navarro-Ródenas et al. 2011, 2012a). The presence of ALP both in mycelia and ascocarps indicates that this enzyme must play an important role during the life cycle of *T. claveryi*, while ACP might be involved in a process that takes place during the ascocarp stage.

Two oxidoreductases (tyrosinase and lipoxygenase) were also isolated and characterized from *T. claveryi* ascocarps (Pérez-Gilabert et al. 2001a, b, 2005a, b).

Although the physiological role of most of these enzymes is not clear, their activity may affect the flavor, color, and texture of their ascocarps.

*Tyrosinase* (EC 1.14.18.1) is a copper-containing bifunctional monooxygenase, which uses molecular oxygen to catalyze the oxidation of monophenols to their corresponding *o*-diphenols (monophenolase or cresolase activity) (Pérez-Gilabert et al. 2001a) and their subsequent oxidation to *o*-quinones (diphenolase or catecholase activity) (Pérez-Gilabert et al. 2001b).

*T. claveryi* tyrosinase, extracted both from mature and immature ascocarps, is one of the few fully latent tyrosinases which have been characterized to date. However, activity could only be detected from the enzyme extracted from ascocarps if an activating agent such as SDS or trypsin was added to the reaction medium (Pérez-Gilabert et al. 2001a, b). The use of SDS as an activating agent is interesting since this detergent is known to inactivate many enzymes.

Both cresolase and catecholase activities of tyrosinase have been localized in the peridium, hypothecium, and the ascogenic hyphae of the gleba (Pérez-Gilabert et al. 2001a, b), which seem to be the most metabolically active tissues in the truffle ascocarp. This co-localization confirms the bifunctional character of this enzyme.

*Lipoxygenases* (LOXs) are non-heme iron-containing dioxygenases that catalyze the insertion of molecular oxygen into polyunsaturated fatty acids containing one or more 1,4 *Z,Z*-pentadiene systems, yielding the corresponding hydroperoxides. These hydroperoxides are subsequently metabolized *via* several secondary pathways giving rise to molecules, the so-called oxylipins, which have a wide range of biological functions (Brash 1999; Brodhun and Feussner 2011). LOXs are present in a wide variety of plant and animal tissues. Some plant LOXs are constitutive, whereas others are expressed by wounding and by fungal pathogens (Oliw 2002).

Due to the high proportion of polyunsaturated fatty acids present in *T. claveryi* ascocarps, lipid rancidity is one of the main factors limiting the storage life of this fungus, since lipid peroxidation gives rise to unpleasant odors and tastes, leading to consumer rejection. Enzymes such as LOX can accelerate the spoilage caused by oxidative rancidity. Hydroperoxides produced by this enzyme decompose to form volatile aroma compounds, which are perceived as off-flavors (Gordon 2001). In addition, the free radicals formed during lipid oxidation may also lead to a reduction in nutritional quality by reacting with vitamins, especially vitamin E, which is lost from foods during its action as antioxidant.

LOXs are classified according to the positional specificity of their products. Linoleic acid represents 45.4 % of total fatty acids in *T. claveryi* ascocarps, while linolenic acid represents 5.8% (Murcia et al. 2003). When the substrate specificity of the purified LOX was investigated, the highest relative activity was obtained using linoleic acid (100%), followed by linolenic acid (91%) and γ-linolenic acid (32%) (Pérez-Gilabert et al. 2005a). So, the specificity of purified LOX was characterized using linoleic and linolenic acid at the pH optimum of this enzyme (pH 7.0) and at pH 10.0, at which values a single peak corresponding to the 13-hydroperoxide was obtained with both substrates (Pérez-Gilabert et al. 2005a).

Thus, LOX from ascocarps can be considered a 13-LOX, similarly to the lipoxygenases from *P. ostreatus* (Kuribayashi et al. 2002) and *Gäumannomyces graminis* (Su and Oliw 1998). Although there is little information on fungal LOX and its physiological role (Brodhun and Feussner 2011), the synthesis of a single specific hydroperoxide from free fatty acid substrates is related to the formation of biological mediators of signaling molecules (Brash 1999). Although several authors have studied the effect of plant oxylipins in arbuscular mycorrhiza (León Morcillo et al. 2012), the effect of fungal LOX on mycorrhizal symbiosis needs to be clarified.

## 2.5 Desert Truffle Plantations and Sylviculture

The cultivation of desert truffles is a very new commercial activity, with only 16 years of history. This cultivation is quite complicated by the species themselves and by the climatology of the cultivated areas, so it is a challenge both for basic and applied researchers to make the practice sustainable and profitable.

The first step in the establishment of a desert truffle plot is to choose suitable host plants and fungal species that are well adapted to the environmental conditions and soil characteristics. Moreover, high quality mycorrhizal plants, with certified mycorrhization levels, should be selected (Honrubia et al. 2014). All the cultivation practices necessary for desert truffle plantation management and sylviculture to improve wild production were well documented by Honrubia et al. (2014). Desert truffle fructification usually occurs 1–3 years after plantation, depending on mycorrhized seedling quality, site suitability, season and framework of the plantation, as well as management practices, mostly irrigation and weed elimination.

Numerous plantations have been established in Spain with the *Helianthemum* species *H. almeriense*, *H. violaceum*, *H. hirtum,* or *H. lipii* as host plants and *T. claveryi*, *Picoa lefebvrei* or *Tirmania nivea* as desert truffles (Fig. 2.4). Moreover, experimental results are available for the cultivation of *Terfezia boudieri* in Tunisia (Slama et al. 2010) and Israel (Kagan-Zur, pers.). All the species of *Terfezia* cultivated until now are typical of alkaline or basic soils. For the cultivation of acid soil species, we recently established, in April 2016, an experimental plot of 500 plants with the species of *Cistus salviifolius* and *Cistus ladanifer* mycorrhizal with *Terfezia arenaria*, a highly prized and widely consumed species. This plantation is located in the province of Cáceres (Extremadura, Spain), where we hope to apply our experience gained with species suited to alkaline soils.

There are two important factors that need to be taken into account to stimulate ascocarp production: a high density of mycorrhizal plants and adequate irrigation. As regard the first factor, a successful plantation frame was 1 × 1 m in rows separated by 2 m, which gave the first ascocarps after 2 years (Fig. 2.4). The small size of these shrubs allows to arrange them closer and thus optimizing the cultivated field. This means a plantation of around 8000 plants/ha, which, while very expensive to establish, could be amortized after 5 years of cultivation if production is

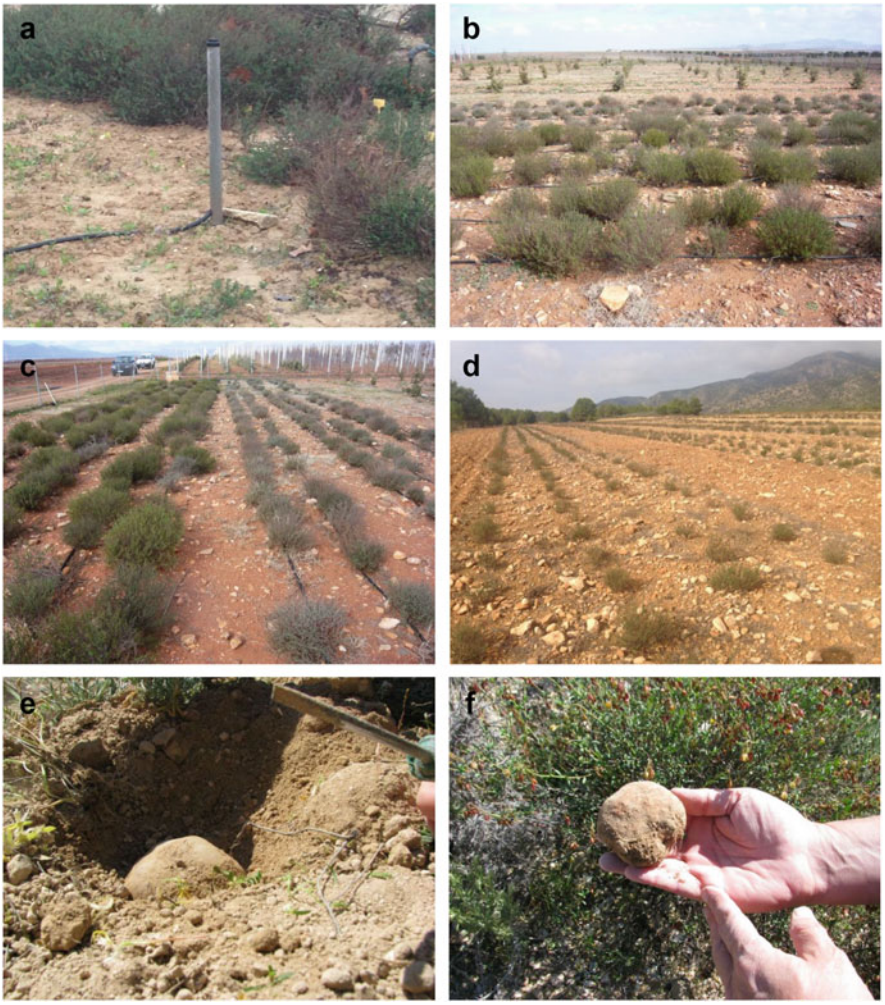


**Fig. 2.4** Irrigation system can be provided by sprinklers (**a**) or drippers (**b**, **c**) in plantations of *H. almeriense* and *H. violaceum* with *T. claveryi* in Murcia (Spain) (**a**, **b**, **c**, **d**). Production of *T. claveryi* ascocaps in spring (**e**, **f**)

adequate (200–450 kg/ha). Adequate irrigation involves the amount of water needed and the time at which it is applied. After following *T. claveryi* production for 10 years in an orchard established in 1999, we observed a statistical correlation between the amount of precipitation during autumn (September, October, and November) and *T. claveryi* truffle production the following year (Morte et al. 2012). Therefore, it is essential to irrigate during these months if rainfall is not enough or does not occur. We estimated an irrigation of 50 l/ha/month in the region of Murcia according to the soil characteristics (loamy clay soil) in order to keep soil

matric potential at around −75 and −100 KPa. The irrigation system can use drippers or by sprinklers (Fig. 2.4).

In field plots in Murcia (Spain), drought stress significantly affected the mycorrhizal colonization percentage, which was 70% in non-irrigated mycorrhizal plants and 48% in irrigated mycorrhizal plants. However, no significant differences in plant growth were observed between non-irrigated and irrigated mycorrhizal plants before and after drought stress (Morte et al. 2010). Under drought stress, stomatal conductance was more sensitive to water stress than photosynthesis. There was a high degree of stomatal closure under water deficit and low radiation conditions, which improved water use efficiency in the plants grown under drought conditions (Morte et al. 2010).

The molecular base of this drought tolerance can be explained by the expression patterns of some aquaporin genes isolated from *H. almeriense* and from *T. claveryi* (Navarro-Ródenas et al. 2012b, 2013). Some of these aquaporins were subjected to fine-tuned expression only under drought-stress conditions. A beneficial effect on plant physiological parameters was observed in mycorrhizal plants compared with nonmycorrhizal ones. Moreover, stress induced a change in the mycorrhizal type formed, which was more intracellular under drought stress. The combination of a high rate of intracellular colonization and the fine-tuned expression of aquaporins could result in a morpho-physiological adaptation of this symbiosis to drought conditions (Navarro et al. 2013).

An alternative to desert truffle cultivation is to use suitable ecosystems in open forests managed in order to maintain and increase the productivity of these areas, in what is called *desert truffle sylviculture*, for which strategies were well defined in Honrubia et al. (2014). The sustainability of the desert truffle ecosystems implies a compromise between exploiting all the resources they harbor and respecting all the interests involved and those that may arise as social demands change. Only by producing (through the exploitation of resources), conserving (following criteria of sustainability), and improving (as regards the biodiversity and multifunctionality of the space in question) will ecosystem management offer guarantees for the future development of rural zones (Honrubia et al. 2014).

A successful example of this desert truffle sylviculture in natural production areas has been carried out in Abu Dhabi (UAE), where it was possible to stimulate the production of *T. boudieri* and *T. nivea* by sprinkler irrigation and spore inoculation of areas in the presence of *H. lippii* plants. In addition, the area was fenced in order to prevent the consumption of truffles by animals (Gouws et al. 2014).

However, more studies on mycorrhizal plant water relations and photosynthetic parameters are necessary if we want to control desert truffle fruiting in the face of global climate change. Future efforts of our group will be directed at deepening our knowledge of these subjects, so that, step by step, we will ultimately domesticate the cultivation of the desert truffle. The recent release of the sequencing and assembly for *Terfezia claveryi* genome, by the Joint Genome Institute and proposed by Dr. F Martin in collaboration with our group from the University of Murcia, may help to reveal the factors and enzymes required for the establishment and

maintenance of its interesting symbiosis, the formation of fruit bodies, and how climate change might affect the biology of this fungus.

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# Chapter 3
# The Role of Arbuscular Mycorrhizal Fungi and the Mycorrhizal-Like Fungus *Piriformospora indica* in Biocontrol of Plant Parasitic Nematodes

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**Abstract** Fungal root symbionts have long been known to provide benefits to their plant hosts in terms of nutrient acquisition and growth promotion. The arbuscular mycorrhizal fungi (AMF) are ubiquitous symbionts of plants that help procure nutrients and protect plants from both abiotic and biotic stresses, including plant parasitic nematodes. Recently, the discovery of another group of mycorrhizal-like fungi belonging to the basidiomycete order Sebacinales have also been shown to colonize roots and assist their hosts in acquisition of nutrients as well as providing protection from a wide variety of both abiotic (drought, salinity, and temperature) and biotic (microbes, insects, and nematodes) stresses. *Piriformospora indica* is one such beneficial root symbiont discovered from the Thar Desert of Western India. It had been shown to enhance uptake of nutrients such as nitrogen, phosphorous, and potassium as well as some micronutrients and to alter plant hormones to promote plant growth and defense. It also recently has been shown to antagonize nematode growth and development. These fungi offer promise for the biocontrol of plant parasitic nematodes.

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## 3.1 Introduction

Mycorrhiza is a combination of two classical Greek words, "mushroom" and "root." Mycorrhiza represents a symbiotic association of the underground mycelia of fungi with plant roots without harming the plant. Mycorrhizal fungi are responsible in improving growth of host plant species due to increased nutrient uptake, production of growth promoting substances, and tolerance to biotic and abiotic stresses (Sreenivasa and Bagyaraj 1989). The Arbuscular Mycorrhizal Fungi (AMF) are widely distributed in natural and agricultural environments and have been found associated with more than 80% of land plants, liverworts, ferns, woody gymnosperms and angiosperms, and grasses (Smith and Read 2008). Recently, Basidiomycete fungi belonging to the order Sebacinales, including *Piriformospora indica* as well as *Sebacina* spp., have been shown to colonize the roots of a variety of agricultural crops and to provide similar benefits to plants in terms of growth promotion, nutrient acquisition, and protection from abiotic and biotic stress (Varma et al. 2012; Gill et al. 2016).

Plant parasitic nematodes (PPNs) represent one of the largest sources of uncontrollable biotic stress experienced by plants, causing as much as US$173 billion in annual losses of crops worldwide (Elling 2013). They influence nearly all crops to some degree. The majority of crop damage is caused by the tylenchid nematodes, root-knot nematodes (RKN), and cyst nematodes (Bird 2004). The most damaging nematodes have sedentary endoparasitic lifestyles (Hussey and Roncadorl 1982; Vercauteren et al. 2002). The two main sedentary nematodes are the cyst nematodes (*Heterodera* and *Globodera*) and the root-knot nematodes (*Meloidogyne*) (Baum et al. 2007). In sedentary nematodes, the J2 larval worm stage invades the plant near the tip of a root and infects through the epidermal and cortex tissue and migrates to the developing vascular cells. The J2 nematodes inject their secretions into and around the plant cells to form the large feeder cell(s) (Caillaud et al. 2008). The feeding cells of cyst nematodes merge through the breakdown of neighboring cell walls to form the feeding structure known as the syncytium, through which the nematodes feed throughout their development (Ali et al. 2015). Feeding cells of root-knot nematodes (giant cells) form by repeated nuclear division in the absence of cell division (Abad et al. 2003). On the formation of feeding cells the juvenile nematode rapidly becomes sedentary because of their somatic muscles atrophy. The juveniles feed, enlarge, and molt three times to the adult stage. The large feeding cells formed by these nematodes plug the vascular tissue of the plant increasing susceptibility to water stress (Grundler and Hofmann 2011). Female sedentary endoparasites enlarge considerably into a saclike shape and are capable of laying large numbers of eggs. They are typically laid outside the nematode in a gelatinous egg mass, but in cyst nematodes most eggs are retained inside the female body which becomes melanized to encase and protect the eggs. Both types of nematodes have the same basic feeding strategy, but many cyst nematodes have an obligate sexual cycle (Cotton et al. 2014), whereas common species of RKN can reproduce largely by parthenogenesis (Ritz and Trudgill 1999).

## 3.2 Control of Plant Parasitic Nematodes

One of the main methods for control of PPN has been the use of resistant crop varieties. However, known resistance alleles are limited, breeding resistant varieties require large time and resource investments, and many PPN have already evolved to overcome plant resistance. Other agricultural practices such as crop rotation and the use of organic amendments have also been employed with some success (Timper 2014). Nematicides were once widely used to control PPN, but these chemicals are often associated with harmful environmental and health effects. For example, methyl bromide, one of the most important chemical fumigants used to control nematodes and other pests, affects a wide range of organisms, including beneficial microorganisms and humans, and is a chemical that contributes to the depletion of the Earth's ozone layer (Carpenter et al. 2001). In recent decades, concerns about the environmental and health hazards of using chemical nematicides and limited availability and durability of resistant crop varieties have led to increased interest in development of biological control agents, including fungi, as a component of crop protection (Grosch et al. 2005). Root symbionts such as AMF can compete with plant pathogens for nutrients and space by producing antibiotics, by directly parasitizing pathogens, or by inducing resistance in the host plants (Schouteden et al. 2015). Thus, these microbes have great potential for the biocontrol of nematodes and other soil-borne pathogens (Berg et al. 2007).

## 3.3 Role of AMF in Biocontrol of Nematodes

The biocontrol effect of AMF on soil-borne pathogens has been observed in a wide range of plant species and against many pathogens, many of them soil-borne fungi causing root rot or wilting (Azcon Aguilar and Barea 1996; Harrier and Watson 2004). However, they have also shown potential against both necrotrophic and biotrophic aboveground pathogens (Fritz et al. 2006) as well as nematode pests (Veresoglou and Rillig 2012; Schouteden et al. 2015). AMF have been shown to control PPN in a variety of temperate agricultural crop plants (Pinochet et al. 1996) such as tomato and carrot (Sousa et al. 2010), soybean (Oyekanmi et al. 2007), as well as tropical crops such as banana (Hol and Cook 2005). Although there are many research reports on the biocontrol effect of AMF, their actual use as biological control agents in the field is still not a routine agricultural practice (Salvioli and Bonfante 2013). This is partially due to variability in performance, depending on the AMF isolate, pathogen, plant species, and environmental conditions (Dong and Zhang 2006; Veresoglou and Rillig 2012; Salvioli and Bonfante 2013; Bajaj et al. 2017).

## 3.4 Mechanism of Biocontrol of Nematodes by AMF

The potential modes of action of AMF against nematodes include direct effects of AMF on the pathogen such as competition for space or nutrients or inhibition or indirect plant-mediated responses. The latter includes enhanced or altered plant growth, morphology and/or nutrition, biochemical changes associated with plant defense mechanisms, and changes in plant root exudates that promote antagonistic microbiota that leads to increased tolerance to nematodes (Whipps 2004; Schouteden et al. 2015). However, it has been observed that a threshold level of AMF colonization is a pre-requisite for many these plant responses (Cordier et al. 1998; Slezack et al. 2000). AMF also have the ability to induce systemic resistance against plant parasitic nematodes in roots (Elsen et al. 2008). The different mechanisms cannot be considered as completely independent from each other, and biocontrol probably results from a combination of these mechanisms (Vierheilig et al. 2008; Cameron et al. 2013). In addition, the relative importance of a specific mechanism can vary depending on the specific AMF–pathogen–plant interaction.

## 3.5 Increased Nutrient Uptake

The mutualistic relationship of AMF with plants increases the uptake of water and mineral nutrients, such as P, N, Ca, Cu, Mn, S, Zn, and Fe (Parniske 2008; Bajaj et al. 2014; Balliu et al. 2015), and in exchange the fungus receives photosynthetic carbon for their survival from their host (Gianinazzi et al. 2010). AMF protect the plant from both biotic and abiotic stresses (Chadha et al. 2015; Bajaj et al. 2015). Nematode damaged plants frequently show impaired water uptake through roots and deficiencies of N, B, Fe, Mg, and Zn, particularly. Cotton fields with better nutrient status were able to tolerate higher populations of when infested with *Rotylenchulus reniformis*, the sedentary semi-endoparasitic nematode, in their roots (Pettigrew et al. 2005). Cotton plants which were colonized with AMF, also showed increased Zn uptake, which contributed to tolerance against *Meloidogyne incognita* by reducing the detrimental nutrient deficiency imposed by RKN (Kantharaju et al. 2005). Regression analysis of nematode population densities against the mineral content in rice also revealed a positive correlation between the migratory ectoparasitic *Helicotylenchus* spp. and Mg. However, a negative correlation was observed between the migratory endoparasitic nematode *Pratylenchus zeae* and Zn or Fe, and between *Meloidogyne incognita* and Mg and Ca (Coyne et al. 2004). These observations indicate that the nutrient status of the host plant can affect PPN population densities in both positive and negative ways.

## 3.6 Altered Root Morphology

In addition to increased nutrient uptake, mycorrhiza-colonized plants have enhanced root growth and branching (Gamalero et al. 2010; Gutjahr and Paszkowski 2013). Increased root growth may help the plant to counterbalance suppression of root growth caused by PPN. For example, this ability of AMF was observed in the banana tree where decreased root branching caused by the migratory endoparasitic nematodes was overcome by colonization with the Glomeromycete *Funneliformis mosseae* (Elsen et al. 2003).

## 3.7 Competition for Nutrients and Space

The PPN and fungi share similar physiological requirements and ecological niches. Thus, there can be competition for nutrients and space between these two groups of organisms, especially when critical nutrient sources such as carbon are limited (Vos et al. 2014). Several studies have demonstrated nutrient competition between AMF and fungal pathogens with respect to carbon (Hammer et al. 2011; Vos et al. 2014), but there is not much evidence for direct competition with nematodes (Jung et al. 2012). Similarly, since AMF and PPN both reside in and derive their nutrition from roots, they may also compete for space (Jung et al. 2012). The suppression of growth by PPN could be because the arbuscules of mycorrhiza are formed in the cortex, the same region where migratory PPN feed. This is not the case for cyst nematodes which feed on syncytia, with the feeding cells confined within the endodermis and thus less affected by AMF (Schouteden et al. 2015).

## 3.8 AMF-Induced Systemic Resistance

Systemic biological control of several pathogens has been reported to result from indirect effects resulting from changes in the host plant (Shoresh et al. 2010; Vos et al. 2012a; Song et al. 2011). Recently, it has been reported that the induction of systemic plant defense responses by AMF occurs because MAMP (microbe-associated molecular patterns) are conserved between beneficial and pathogenic fungi (Zamioudis and Pieterse 2012). Thus, AMF may be considered as putative pathogens by plants (Paszkowski 2006). When the plant's pattern recognition receptors recognize MAMP, a MAMP-triggered immunity response (MTI) is activated which forms the first line of defense of the plant, inhibiting invasion of other pathogens (Jones and Dangl 2006). The systemic nature of the mycorrhiza-induced resistance was observed in banana colonized by *G. intraradices* against the migratory burrowing nematode *R. similis* (Elsen et al. 2008). On other hand, in

*Ammophila arenaria*, no systemic resistance against *P. penetrans*, a lesion nematode, was observed after colonization by native AMF (De la Peña et al. 2006).

## 3.9 Altered Roots Exudates

The symbiosis of plants with AMF often changes the biochemical composition and level of production of roots exudates. This, in turn, impacts the hatching, mobility, chemotaxis, and host localization by nematode juveniles (Vos et al. 2012a, b). Changes in root exudates could involve compounds such as sugars and organic acids (Hage-Ahmed et al. 2013), amino acids (Harrier and Watson 2004), flavonoids and strigolactones (Steinkellner et al. 2007), plant hormones, and phenolics (McArthur and Knowles 1992). There is ample evidence that root exudates can alter the rhizosphere microbiome (Lakshmanan et al. 2014). While few studies address this topic, it is possible that root exudates induced by AMF and other root symbionts promote rhizosphere communities antagonistic to nematodes (Vos et al. 2012a, b). The level of colonization and the particular symbiont involved also impacts root exudates in the rhizosphere (Kobra et al. 2009; Lioussanne et al. 2008), and it is believed that a threshold level of colonization is also required for this mechanism of biocontrol (Paulitz 2000; Chatterton and Punja 2011).

## 3.10 Role of *Piriformospora indica* in Biocontrol of Nematodes

*Piriformospora indica,* a Basidiomycetes of the order Sebacinales, is an endophytic symbiotic fungus which was isolated from rhizosphere of the xerophytic woody shrubs from the Thar deserts of Rajasthan, India (Verma et al. 1998; Varma et al. 2013, 2014). It has plant growth promotional activity while providing benefits of biotic and abiotic stress tolerance to the host plant (Gill et al. 2016). It also protects the plants from pathogens and herbivores (Verma et al. 1998; Deshmukh et al. 2006; Daneshkhah et al. 2013; Bajaj et al. 2015). Like AMF, it has an extensive range of hosts, colonizing members of the bryophytes, pteridophytes, gymnosperms, angiosperms (both monocots and dicots), and orchids. In the majority of plant species investigated, there are two distinct phases in the colonization of plants by *P. indica*. In initial stages of infection, *P. indica* acts as a biotroph, but later on acts as a necrotrophic, killing some cells of the plant root through apoptosis (Zuccaro et al. 2011) and essentially forming a saprophytic association (Deshmukh et al. 2006) However, in orchids, the fungus forms a symbiotic association with the plant that promotes root growth (Ye et al. 2014). *Piriformospora indica* increases nutrient uptake, particularly of phosphorus (Singh et al. 2000; Malla et al. 2004), and improves plant growth and stress tolerance by inducing phytohormones (Gill

et al. 2016; Siddhanta et al. 2017). Studies have revealed that it also can enhance the production of plant secondary metabolites (Bagde et al. 2010, 2014; Das et al. 2012, 2013; Kumar et al. 2012; Prasad et al. 2008, 2013; Bajaj et al. 2014).

## 3.11 Biotic Stress Tolerance

*P. indica*-infested plants are more resistant to biotic stresses. In barley infected with macroconidia of the necrotrophic fungal pathogen *Fusarium culmorum*, *P. indica*-infested plants were more tolerant to the devastating effect of *F. culmorum* root disease (Harrach et al. 2013). Root and shoot fresh weights were reduced only twofold in *P. indica*-colonized plants, compared with the 12-fold in controls with *F. culmorum* alone. Similar results were observed for the root pathogen *Crocus sativus*, which shows a hemibiotrophic nourishment strategy (Waller et al. 2005). These results show that *P. indica* exerts beneficial activity against major crop pathogens that cause enormous worldwide economic losses. Deshmukh et al. (2006) reported comparable biological activities of the treatments in terms of biomass increase and protection against biotrophic stress of *Blumeria graminis*, powdery mildew fungus in barley. Colonization of barley roots with *P. indica* induced systemic resistance against the biotrophic leaf pathogen. Analysis of a number of *Arabidopsis* mutants showed that jasmonate signaling is important for *P. indica*-induced resistance (Stein et al. 2008). A subset of defense-related genes are expressed earlier and more strongly induced by leaf pathogens in root endophyte-colonized barley plants than in control plants (Molitor et al. 2011). Hence, the mechanisms of *P. indica*-induced resistance seem to be similar to the well-characterized induced systemic resistance described for plant growth-promoting rhizobacteria-colonized plants (van Wees et al. 2008).

## 3.12 Biocontrol of Nematodes by *P. indica*

Daneshkhah et al. (2013) reported that colonization of *P. indica* on *Arabidopsis* roots in vitro antagonized the infection and development of cyst nematodes. In other fungi, this antagonistic activity can be elucidated by production of secondary fungal metabolites and enzymes such as chitinases that feature toxicity against parasitic nematodes (Shinya et al. 2008). Endophytic fungi are able to produce large amounts of toxic chemicals in vitro (Vu 2005), some of which may have direct nematicidal activity. Further studies are needed to determine if *P. indica* produces compounds with direct toxicity to nematodes, although its genome sequence showed few genes with known functions in fungal secondary metabolism (Zuccaro et al. 2011). Daneshkhah et al. (2013), however, noted that cell-wall extracts of *P. indica* alone significantly decreased nematode infection and development. *P. indica* may also impact production of plant secondary metabolites that deter

nematodes. *P. indica* root colonization affected $J_2$ infection, especially during the biotrophic phase. In this phase, the expression of *MYB51*, a plant gene involved in the biosynthesis of antimicrobial indole glucosinolates (Clay et al. 2009), is induced in roots of *P. indica*-treated plants (Jacobs et al. 2011). In roots inoculated with *P. indica,* it was observed that expression of *CBP60g* and *SID2*, markers of the salicylic acid-mediated signaling pathway, were upregulated (Jacobs et al. 2011). Therefore, salicylic acid-mediated signaling may also be involved in significant inhibitory effect on *H. schachtii*, since salicylic acid was revealed to inhibit growth of *H. schachtii* (Wubben et al. 2008).

## 3.13 Case Study: *Piriformospora indica* Antagonizes the Soybean Cyst Nematode in Planta

Field soil was collected from an agricultural field with no soybean cyst nematode infestation, mixed with 30% sand and autoclaved twice. Mycelium of *P. indica* at concentrations of 0% (w/w), 2.5% (w/w), and 5% (w/w) was thoroughly mixed with soil and placed into clay pots in a controlled greenhouse trial in order to analyze its possible effects on growth, development, and pest resistance towards the SCN (Bajaj et al. 2015, 2017).

Root colonization was observed by staining the roots with lactophenol cotton blue, and intracellular chlamydospores of *P. indica* were observed confined to the root cortex. Levels of root colonization ranged from 45% to 50% in 2.5% and 5% *P. indica* treatments at 8 weeks after planting. No colonization was observed in 0% *P. indica* treatment. Soybean showed a positive interaction with *P. indica*, as demonstrated by increased shoot biomass and shoot length of inoculated plants as compared to control plants. However, the overall biomass of colonized roots was lower than that of the uncolonized control roots. *P. indica* not only induces development of the vegetative aerial part of the plant, but also is responsible for early maturation with respect to flowering in soybean (Table 3.1).

The number of SCN eggs per cc soil, a common screening measure of SCN severity in agricultural fields, was significantly lower in the *P. indica* amended pots. There was a decrease of 29.7% in the 2.5% *P. indica* treatment and 36.7% in the 5% *P. indica* treatment. Egg density per cc soil was also significantly reduced between the 2.5% to the 5% *P. indica* treatments. Egg density calculated as number of eggs/cc soil/gram root wet or dry weight also showed a trend, although not significant, of decreasing egg density with increasing *P. indica* in soil.

**Table 3.1** Effects of *P. indica* on soybean

| *Piriformospora indica* colonization in *Glycine max* | | | | | |
|---|---|---|---|---|---|
| Increased flowering and pods | Decreased root volume | Decreased egg density of SCN | Increased nutrient uptake | Increased hormones | Increased metabolism in plants |

Although the mechanism of nematode inhibition in this study is unknown, *P. indica* may either directly inhibit nematode development, as discussed above, or may control plant responses that impact nematode colonization and development. One possible plant response is altered carbon-partitioning in the plant. The fungus has been shown to control expression of a *Nicotiana* attenuate homolog of Hsl-Pro-l, a locus initially identified as having a role in resistance to the beet cyst nematode (*H. schachtii* Schmidt) (Cai et al. 1997), but now thought to be involved in more generalized responses to both abiotic and biotic stresses and in repartitioning of carbon resources within the plant (Schuck et al. 2012). Enzymes such as sucrose synthases and invertases may also impact development of cyst nematodes by altering plant sink strengths and changing systemic sugar partitioning to decrease syncytial sugar levels. Higher sugar levels in roots were shown to contribute to enhanced nematode development (Cabello et al. 2013) and have major nutritional value for this obligate parasite. Thus, allocation of sugars to shoot growth over root growth could impact the availability of sugars to root cyst nematodes. In contrast to AMF, *P. indica* decreased root growth and branching. However, decreased root growth may also reduce infection by the SCN (Bajaj et al. 2015) by lowering the number of potential sites for nematode infection (Schouteden et al. 2015).

## 3.14 Conclusions

AMF and other beneficial fungi such as *P. indica* confer many of the same benefits to plant hosts including improved nutrient uptake, increased plant growth, enhanced abiotic and biotic stress tolerance, induced systemic resistance against pathogens, and production or induction of plant protective secondary compounds or root exudates. However, there may also be significant differences in the mechanisms of nematode antagonism by these two groups of fungi. Instead of increasing root growth and proliferation like AMF, *P. indica* causes cell death in roots and directs resources to shoot growth, leading to smaller root:shoot ratios. Reduced root growth and volume may promote biocontrol of nematodes both by diverting sugars on which juvenile nematodes feed from root to shoot and also by providing less root surface area for infection. While further studies are needed to investigate nematode inhibitory compounds produced by AMF, to our knowledge, the majority of known mechanisms of AMF protection against nematodes are plant mediated. In contrast, compounds and cell-wall components of *P. indica* have been shown to directly inhibit infection and development of nematodes in roots. Both groups of fungi offer promising avenues for successful biocontrol of PPN.

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# Chapter 4
# Mycorrhizal Fungi Under Biotic and Abiotic Stress

**Manoj Kumar, Ram Prasad, Vivek Kumar, Narendra Tuteja, and Ajit Varma**

**Abstract** Mycorrhizal fungi are associated with host plant roots which complement and augment plant growth, productivity, and immunity; nevertheless, current work by scientists shows that mycorrhiza also provoke so-called induced systemic tolerance (IST) to abiotic and biotic stresses. As we discuss here, the mycorrhiza also upsurge nutrient uptake and transport from soils, thus reducing the need for chemical fertilizers and avoiding the buildup of nitrates and phosphates in the agricultural soils. A decrease in fertilizer use would reduce the effects of contamination of water from fertilizer run off and leaching lead to savings for farmers. Abiotic stresses (such as soil salinity, drought, heat, cold, mineral deficiency) have become main threats to the universal agricultural production. These stress in alone and/or in combination control the plant growth, development, maturity, and productivity by causing physiological disorders, ion toxicity, and nutritional and hormonal disparities. Some precious soil microbes like mycorrhizal fungi inhabit the rhizosphere and develop a symbiotic and mutualistic relationship with the roots of most host plant species. Mycorrhiza can considerably enhance resistance of host plants to varied abiotic and biotic stresses. In this chapter, we highlight the importance of mycorrhizal fungi alleviation of various stresses and their beneficial effects on plant growth expansion and production. Though these stresses can negatively affect the mycorrhizal fungi, there are many reports which exhibit better growth, performance, and production of mycorrhizal plants under stress conditions. These positive consequences are explained by increased host plant nutrition, higher potassium, nitrogen and phosphate in plant tissues and a better osmotic modification by buildup of well-matched solutes such as proline, glycine betaine, or soluble sugars. Mycorrhizal inoculated plants also increase photosynthetic, physiological, biological, and water use efficiency under various stresses.

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## 4.1 Introduction

Being sessile in nature, plants have a greater chance to interact with their immediate environment. In particular, conditions with abiotic stress factors like salinity, drought, cold, heat, nutrient imbalances, and metals can severely impact growth and development of plants and finally decrease their overall yield to about 70% (Auge 2001; Saxena et al. 2013). In order to avoid stresses and minimize their potential impacts, plants may bring several modifications in their morphology and/or structure/ultrastructure (Souza et al. 2012). Alternatively, plants may adopt several stress tolerance strategies through modulating their physiology and biochemistry to limit stress accrued damages or to facilitate the repair of damaged systems (Sharifi et al. 2007). Notably, adoption of above-mentioned strategies by plants can be modulated to achieve improved plant productivity/yield by externally applying chemicals and other sustainable efforts (Siqueira et al. 1990). Among the sustainable efforts, the association of soil microbiota with plant roots can also be exploited and implied to improve plant growth and productivity under normal and stressful environment (Talavera et al. 2001; Sailo and Bagyaraj 2005; Marulanda and Barea 2009). Arbuscular mycorrhizal fungi (AMF) are microscopic filamentous fungi that colonize the roots and their rhizosphere simultaneously and spread out over several centimeters in the form of ramified filaments (Toth et al. 1990; Xie et al. 2014). Mycorrhizal fungi are the most extensively studied fungal symbionts which are associated with approximately 90% of all land plants and has been reported to significantly contribute multiple benefits to its host plants (Muthukumar et al. 2014; Prasad et al. 2017). The AM fungi are of great ecological implication as they can form beneficial symbiosis with the most terrestrial plants (Xie et al. 2014) and also with a few wetland or swamp plants (Xie et al. 2014). Literature is full on the role of AM fungi in improving plant growth, metabolism, and eventually bring high crop/plant productivity under normal and stressful environment (Abdel Latef and Chaoxing 2011; Gavito and Azón-Aguilar 2012; Impa et al. 2012; Beltrano et al. 2013; Gholamhoseini et al. 2013).

Indeed, survival necessitates the ability to rapidly adapt to changes in the environment, especially those which represent long term or chronic changes. Whenever possible, one of the easiest ways to counteract such stresses is to relocate to a more suitable niche. However, such a strategy is obviously restricted in a short-term period and is not achievable with stationary organisms such as plants. Consequently, plants have developed a variety of strategies to cope against biotic stresses such as herbivory or parasitism and abiotic stresses such as salinity, drought, heat, or toxic metal contamination (Khan 1974; Parida et al. 2002; Sowinski et al. 2005; Kumar et al. 2010). Among abiotic stresses, soil salinization is probably one of the most important in the world (Sharifi et al. 2007; Aroca et al. 2013; Talaat and Shawky 2014). In addition, high temperature and low precipitation leading to salt accumulation at the soil surface affect the establishment, growth, and development of plants and even more as salinity increases. The delay in root growth can be caused by too low soil water potential and salt cell toxicity (Beltrano et al. 2013).

The cell toxicity causes cell death and also root necrosis in very sensitive plant genotypes. In tally to all these harmful effects on roots, shoot growth is also influenced and as a result the root/shoot ratio is troubled (Maggio et al. 2007). Overall, salinity leads to many deleterious effects on plants and that at different life stages. To counteract this problem, many strategies were proposed to overcome salt detrimental effects such as searching for new salt tolerant crops, genetically engineering plants, removing excessive salt accumulation in groundwater and desalinizing water for irrigation. Although these strategies appear efficient, yet they are costly and out of reach for developing countries that are the most affected.

In this context, the ecosystem services rendered by soil biota in maintaining soil quality, plant health, and soil resilience are extremely pertinent (Gianinazzi and Gianinazzi Pearson 1988). In particular, soil microorganisms that form mutually beneficial relationships with plant roots have become a target of increasing interest in agricultural research and development because they offer a biological alternative to promote plant growth and reduce inputs in sustainable cropping systems (Gianinazzi et al. 2008). The ubiquity of AM fungi at the interface between soil and plant roots makes them a key functional group of soil biota which by their nutritional and non-nutritional activities profoundly influences ecosystem processes that contribute to the ecosystem services in agro-ecology. Our aims in this chapter are to highlight the key role that the mycorrhizal symbiosis can play as an ecosystem service provider to guarantee plant productivity and quality under abiotic and biotic stress conditions. The appropriate management of various stresses rendered by mycorrhiza will impact on natural resource preservation and utilization with an apparent net reward for human society.

## 4.2 Mycorrhiza and Abiotic Stresses

The abiotic stresses such as soil salinity and drought cause widespread losses to agricultural production. On the other hand, depletion of mineral, water stress, soil salinity and alkalinity, presence of heavy metals, or high temperature are serious problems in many parts of the globe, particularly in the arid and semi-arid regions (Evelin et al. 2009). It is forecasted that two-thirds of the cultivable agriculture land may vanish in Africa, a third in the Asia, and one-fifth in the South America by the year 2025. It is also predicted that the arable land area per occupant or resident on the planet will be lessened to 0.15 ha in the year 2050 (http://www.un.org/esa/sustdev/documents/agenda21/french/action12.htm). In countries like United States and Spain, one-third of the country part is undergoing desertification, which is an alarming situation.

The potential and probability of mycorrhizal fungi to improve the plant tolerance in abiotic stress conditions has been recognized since long (Smith and Read 2008), and their manipulation and application in perpetual and lasting agricultural systems will be of incredible and remarkable importance for soil quality and crop production under severe edapho-climatic conditions (Lal 2009). Among more current

examples of the employment of valuable and advantageous soil microbes to augment crop tolerance against several abiotic stress conditions. Studies on the beneficial effect of co-inoculated bacteria and mycorrhizal fungi from arid environments on plant growing under drought stress (Marulanda-Aguirre et al. 2008; Marulanda and Barea 2009) emphasize the interest of influencing autochthonous or natural mycorrhizal fungal isolates from the dry regions for re-vegetation of the degraded land zones to enhance soil quality, productivity, and to fight against desertification in the Mediterranean ecosystems. This is justified by quoting an example of an indigenous drought tolerant strain of *Glomus intraradices* associated with innate and local bacterium reduced by 42% the water required for production of *Retama sphaerocarpa* (Marulanda et al. 2006). In another report (Bouamri et al. 2006; Porras-Soriano et al. 2009), the mycorrhizal fungi alleviate stress salinity in olive tree plantations in Spain or in arid North African region, where palm grove yields are considerably influenced by water drought and soil salinity conditions.

Alternative area where mycorrhizal fungal inoculation has become a potential and probable tool for increasing plant tolerance to the environmental stress conditions is in the re-vegetation of naturally or industrially deliberately metal contaminated soils. There are several examples in the literature to demonstrate and exemplify this role of mycorrhizal symbiosis, though the fundamental mechanisms are not hitherto fully understood (Khade and Adholeyavan 2009). Occurrence of mycorrhizal fungi in Ni hyper accumulating plant species found naturally on the metal rich soils proposes potentials of using heavy metal hyper accumulating plants along with mycorrhizal fungi for phytoremediation approaches and tactics (Turnau and Mesjasz-Przybylowicz 2003; Gamalero et al. 2009). Additionally, in another reports conducted by Lugon-Moulin et al. (2006) and Nziguheba and Smolders (2008), many phosphate fertilizers are a chief source of soil pollution by Cd in agricultural systems which again pleads for the lessening of crop dependence on phosphate fertilizers. Rivera-Becerril et al. (2002) and López-Millán et al. (2009) reported that mycorrhizal fungi, through their mycelium network, not only enhance inorganic phosphate uptake by plant roots but they also have a buffering consequence on the Cd uptake, decreasing the toxic effect of Cd on plant growth and production.

## 4.3 Mycorrhiza and Drought Stress

Drought, also commonly known as water deficiency or water stress, is the absence of satisfactory water table for normal plant development and growth (Subramanian and Charest 1998, 1999; Marulanda and Barea 2009). The unobtainability or inaccessibility of water to the root zone, stern transpiration rate or accelerated generation of reactive oxygen species (ROS), and consequent initiation of oxidative stress in plants can be key reasons of drought stress effects in plants (Auge 2001; Bárzana et al. 2012). Symbiosis of plant with mycorrhiza can progress the overall plant growth by enhancing root thickness and length, leaf area, plant biomass, and

nutrient uptake and transport under mild to severe drought condition (Davies et al. 2002). Enhancement of plant growth by mycorrhizal inoculation can was attributed to the formation of widespread hyphal networks and excretion of glomalin, which in turn augment water and micro- and macronutrients uptake, thus improving soil structure (Gholamhoseini et al. 2013).

Participation of mycorrhizal symbiosis in numerous biochemical and physiological processes including (1) direct uptake and transport of water and nutrients by mycorrhizal fungi, (2) augmented osmotic regulation, (3) enhanced gas exchange and water use effectiveness, and (4) better defense against oxidative damage has also been reported in literature (Mittler 2002; Marulanda et al. 2007; Ruíz-Sánchez et al. 2010). Mycorrhizal symbiosis resulted in superior and better leaf water potential, enhanced gas exchange, augmented stomatal conductance and transpiration and photosynthetic rates in mycorrhizal inoculated plants under drought conditions (Morte et al. 2000; Mena-Violante et al. 2006).

Mycorrhizal fungi can also alter water regulation in the plant through alteration in hormonal balance signaling or by motivating osmolytes in the mycorrhizal plants (higher strength or amount of photosynthetic by products and solvable sugars in the leaf symplasm) compared to non-mycorrhizal plants (Porcel and Ruiz-Lozano 2004). In this regard, role of abscisic acid (ABA) has also been proposed as one of the non-nutritional instrument by which mycorrhizal symbiosis stimulates stomatal conductance and other physiological characters in drought stressed exposed plants (Porcel et al. 2006; Ruiz-Lozano et al. 2006). Recently, in *Zea mays* plants colonized by mycorrhiza *Glomus intraradices*, an augmented expression of two aquaporin genes (GintAQPF1 and GintAQPF2) was described in both root cortical cells holding arbuscules and extra radical mycelia under the drought stress (Moussa and Abdel-Aziz 2008; Li et al. 2013). Porcel et al. (2007), Li et al. (2013) and Rapparini and Peñuelas (2014) reported that mycorrhizal hyphal growth was also found to be connected with water absorption area.

## 4.4 Mycorrhiza and Nutrients Stress

Insufficiency of mineral micro- and macronutrients has been reported to affect plant growth by persuading changes in the plant growth pattern, chemical composition, and antioxidant defense capacity ultimately rendering plant susceptibility to diverse stress aspects (Hajiboland 2012). Remarkably, mycorrhizal infection can progress the uptake of micronutrients and other macronutrients having low mobility including Fe, Cu, and Zn (Baslam et al. 2013). The mycorrhizal inoculated lettuce plants with higher accessibility of nitrogen and phosphorus in soil revealed decreased content of micro- and macronutrients in the tissues (Azcón et al. 2003). Mycorrizal colonization also enhanced all the micro- and macronutrients when plants were fertilized with a low level of both phosphorus and nitrogen (Baslam et al. 2013; Ortas and Ustuner 2014; Xie et al. 2014). In another report, Yaseen et al. (2012) recorded highest macro- and micronutrients (Ca, K, Mg, P, Fe, and Si) uptake of

chickpea in mycorrhizal inoculated plants. Development of widespread hyphal network in the soil improves effects of exceptionally low pH through improved uptake of phosphorus (Muthukumar et al. 2014). About 80% of the total phosphorus acquired by mycorrhizal *Medicago truncatula* was delivered by the extra radical mycelium of the mycorrhizal fungi connected with those plants (Smith et al. 2000). Also, Rohyadi (2008) perceived an upsurge in phosphorus uptake and transport in maize colonized by mycorrhiza *G. margarita* under acidic conditions and advocated that augmented phosphorus levels in mycorrhizal maize tissues could be owing to the better assessment of soil by mycorrhizal fungal hyphae (Muthukumar et al. 2014).

## 4.5 Mycorrhiza and Biotic Stresses

To check the spread of pests (pathogenic bacteria, fungi, virus, and nematodes) causing great yield losses in common cultivated crops, usual agriculture practice has been using huge quantities of harmful pesticides. Along with this, scientists are working on the plant breeding programs in order to obtain disease resilient plants. Nevertheless, the pesticides are often only partly or moderately effective against potential soil-borne diseases. Furthermore, they are disadvantageous and unfavorable to human health and also to the environment and as a result an ever cumulative number of pesticides are being taken off the market. Additionally, disease resistance obtained by plant breeding plans is often due to single plant genes, which can be quickly weakened by evolving biodiversity in the pathogenic agents. Balancing tactics have therefore to be developed to guarantee durable tolerance of plants to the potential pathogens.

Several studies have confirmed the beneficial effect of mycorrhizal fungi in encouraging plant tolerance to biotic stress triggered by soil-borne potential pathogens interrelating with many plant species. This has been constantly demonstrated among a number of pathogenic fungi or Oomycetes such as *Fusarium*, *Rhizoctonia*, *Verticillium*, *Thievalopsis*, *Aphanomyces*, *Phytophthora*, and *Pythium*, as well as the nematodes from the genera *Heterodera*, *Meloidogyne*, *Pratylenchus*, and *Radopholus* (Harrier and Watson 2004; Whipps 2004; Hao et al. 2009). Most of the research work has been carried out under very precise and planned conditions at the early stages of plant growth, but a few studies conducted in field or in greenhouse under actual production conditions approve these results (Bødker et al. 2002; Newsham et al. 1995; Torres-Barragan et al. 1996; Utkhede 2006). It would not be possible to write here all the research papers published on this topic. In its place, we have selected to exemplify this by results attained for tomato crop which is one of the most extensively grown vegetables all over the world. Moreover, this crop is susceptible to many bacteria, insects, fungi, and nematodes causing noteworthy reduction in fruit yield (34%) under current production practices, as reported by (Engindeniz 2006). Though the tomato plant is not highly approachable to mycorrhizal fungi in terms of plant growth (Smith et al. 2009), but

it clearly reimbursements from mycorrhiza inoculation when challenged by plant root pathogens such as *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Rhizoctonia solani*, *Phythophthora parasitica*, or *Meloidogyne incognita*. In this case, root colonization by mycorrhizal fungi can principally lessen root infection and disease seriousness caused by the potential pathogens, which results in enhanced fresh plant weight (up to 198%) and fruit yield (14.3%) compared to the pathogen infected mycorrhizal non-inoculated plants.

## 4.6 Mycorrhizosphere: A Biocontrol Zone

The mycorrhizosphere is zone covered and explored by mycorrhiza and has been hypothesized to constitute an environment conducive to microbes hostile to soil-borne potential pathogen survival and proliferation. Certainly, co-inoculation of the non-mycorrhizal species *Dianthus caryophyllus* with the mycorrhizal species *Tagetes patula* in the presence of *G. intraradices* evidently lessened the disease caused by *F. o. dianthi* in the *D. caryophyllus* in a way clearly unconnected to plant nutrition which proposes a lessening in pathogen growth and development within this mycorrhizosphere (St-Arnaud et al. 1997). Furthermore, a decrease in the number of infection loci of tomato roots pre-colonized with the *G. mosseae* and inoculated with *P. nicotianae* zoospores concludes that pathogen may be disturbed prior to root infiltration and penetration in mycorrhizosphere (Vigo et al. 2000).

Mycorrhizosphere influenced by the rhizobacteria–mycorrhiza–root tripartite association also presents precise characteristics, in which each character influences the other actor's growth, development, and health. Remarkably through the discharge of glycoproteins such as glomalin, mycorrhiza fungi favor the establishment of aggregates which provide stable microsites which are favorable to root and microbe establishment (Rillig and Mummey 2006). The mycorrizal extra radical network also establishes specific microsites which also favor the growth of some bacteria. Amongst the plant growth promoting rhizobacteria (PGPR) (Bowen and Rovira 1999), phosphate solubilizing and nitrogen fixing bacteria have been shown to synergistically interrelate and cooperate with mycorrhizal fungi, increasing phosphate and nitrogen availability to plant and promoting its growth and development and probably favoring its capability to counter pathogen impact on plant growth and production (Johansson et al. 2004; Barea et al. 2005; Artursson et al. 2006; Lioussanne et al. 2009).

## 4.7 Mycorrhiza and Phytophagous Insects

The mycorrhizal standing of the plant can also manipulate insect and herbivore performance, but the magnitude and direction of the effect depend upon the feeding mode and lifestyle of the insect pathogen (Hartley and Gange 2009;

Koricheva et al. 2009). Many different researches cover an abundant range of mycorrhizal–plant–insect interactions under controlled or natural/field conditions. Upon a wide-ranging review of published data, Hartley and Gange (2009) established that, generally, mycorrhizal fungi have strong negative effects on rhizophagous insects, but the effects on insects feeding on shoot are weaker and more inconstant. Regardless of this inconsistency, few general patterns appear: generalist insects are frequently adversely influenced by mycorrhizal fungus, whereas specialist insects may often take advantage. Moreover, aphids usually perform better on mycorrhizal plants while the leaf chewing insects are generally negatively affected by the association.

Above-mentioned patterns may arise from differential impact of nourishing and defense aspects in insect pathogen. While the common insects are sensitive to the plant defense mechanisms and the specialist herbivores are likely to be able to evade the defenses of their host plant and remain unnoticed. As a result, the common or generalists insects may be affected by the improved defense capacity of mycorrhizal plants, while the specialists will evade the defenses and may get benefit from improved nutritious status of host plant. The negative effect on leaf chewer insects is likely related to their vulnerability to jasmonate or jasmomic acid dependent defenses (Peña-Cortés et al. 2004) potentiated in mycorrhizal inoculated plants. Additionally, mycorrhizal fungi can also have an impact on herbivores by influencing the performance and function of their predators and parasitoids: for example, in tomato plant, the volatile blends released by mycorrhizal plants can be more attractive to aphid parasitoids compared to those from non-mycorrhizal plants (Guerrieri et al. 2004).

## 4.8 Conclusions

We can conclude that mycorrhizal fungi are significant for improving plant tolerance to abiotic and biotic stresses, but also respond to various types of stresses individualistically of their host plant. The stresses affect the richness and community composition of mycorrhizal fungi. A change in diversity of mycorrhizal fungi will feed back into host plant community and will lead to corresponding changes in variety and leading plant species, and these responses will become sturdier with the climate changes, agriculture practices, and plant invasions. Mycorrhizal fungi are proficient in adapting to the abiotic and biotic environment which may or may not increase their mutualistic performance. Impact of ecological and evolutionary responses of mycorrhizal fungi to abiotic and biotic stresses is likely to become even more imperative for both natural and cultivable agricultural systems in the face of biotic stresses and climate changes, such as incursion by non-native species. Substantial progress has been made in understanding the role of mycorrhizal symbiosis in bestowing drought confrontation to plants, but dissimilar aspects still necessitate attention for unknotting new and unique metabolites and hidden metabolic pathways. The collected biochemical, physiological, and molecular data

based on classical means and methods will benefit from the various "omic" techniques and procedures and their combinations. An in depth examination using the progressive methodologies could help to clarify and explain the mechanisms of drought avoidance and/or tolerance induced by mycorrhizal symbiosis and to distinguish the abiotic and biotic stresses induced processes of the protective mechanisms regulated by mycorrhizal symbiosis.

Increasing our information on modifications of the plant physiology in mycorrhizal fungi, as well as in the biology of the potential insect attackers, is indispensable in order to define markers of stimulated resistance and to generate analytical models for the outcome of particular mycorrhiza-insect interactions. Additional challenge ahead is to decode the connections in plant responses to abiotic and biotic stresses. The experimental indications point to common controlling nodes in signaling pathways governing responses to abiotic and biotic stresses, and those nodes could be the target of biotechnological approaches or tactics for optimization of plant protection by mycorrhizas. Lastly, it is significant to consider mycorrhizal fungi in a multitrophic context, as the influence of the symbiosis on plant interactions may be modified by other organisms in the ecosystem.

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# Chapter 5
# Role of Arbuscular Mycorrhizal Fungi (AMF) in Salinity Tolerance and Growth Response in Plants Under Salt Stress Conditions

**Mahesh Borde, Mayura Dudhane, and Mohan Kulkarni**

**Abstract** Salt stress is a major agricultural problem all over the world, which has an effect on the functioning of growth and physiology of crop plants. Salinity in soil reduces plant growth by decreasing net photosynthesis rate and stomatal conductance; it also inhibits antioxidant enzyme index. Arbuscular Mycorrhizal Fungi (AMF) are one of the root symbionts which play a vital role in enhancing the crop plant growth and help the host plant in developing tolerance against abiotic stress factors like salt, drought, etc. This chapter aims to evaluate the beneficial role of AMF on plant growth and physiological performance under salinity stress conditions. AMF help to enhance the plant growth, by increasing plant biomass, photosynthetic activity water potential, and selective uptake of nutrients under salinity stress condition. AMF reduce the adverse effect of salt stress by increasing antioxidant defense mechanism in response to salinity stress conditions and promote salinity tolerance in crop plants. AMF mitigate the salt-induced deleterious effects by virtue of maintaining the osmotic balance by regulating the $Na^+$ ad $K^+$ ratio. AMF also help in maintaining osmotic adjustment in host plants by inducing the synthesis of various osmolytics like proline, glycine betaine, etc. Thus, tolerant AMF species can be used as bioinoculant to improve agricultural productivity under salinity stress conditions.

## 5.1 Introduction: Effect of Soil Salinity on Growth of Plant

Soil salinity is a major problem in numerous parts of the world in arid and semiarid regions (Giri et al. 2003; Al-Karaki 2006). Also, 7% of the earth's land surface has become saline prone (Ruiz-Lozano et al. 2001). Excessive application of chemical

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fertilizers, use of ground water for irrigation, flood irrigation practices, and no rotation of crops are the major reasons for increasing agricultural soil salinization. Agricultural productivity is affected, if the fertile soil is transformed into saline soil, as increased soil fertility decreases up to 20% of crop productivity (Porcel et al. 2012; Munns and Gilliham 2015). Increased soil salinization of arable land results in the loss of 30% of agricultural land within next 25 years and up to 50% within next 40 year (Porcel et al. 2012; Abdel-Fattah et al. 2014). High salt accumulation in soil decreases soil porosity, soil aeration, and water conductance which results in water deficit condition to the plant and causes physiological drought (Mahajan and Tuteja 2005). Salinity injuries could decrease photosynthetic rate, reduce antioxidant enzyme activities, reduce stomatal conductance, induced ion deficiencies, affect membrane stability index, and reduce relative water content of the plants (Talaat and Shawky 2012). In addition to these factors, some other factors like physiological drought, ion toxicity, ion imbalance, and soil compaction may cause growth reduction. Salinity adversely affects the normal growth of plant by causing injury of foliage, nutrient deficiencies, lowering soil properties, nitrate content, inhibiting carbonic anhydrase, and nitrate reductase activities. Salt stress destroys the PSII reaction center and disrupts electron transport in the photosynthetic apparatus which reduces the net photosynthetic rate (Sheng et al. 2008; Dudhane et al. 2011; Talaat and Shawky 2012).

Also, soil salinity affects plant growth through toxic effects of $Na^+$ and $Cl^-$ ion, which leads to denature enzyme structure, damage to cell organelles, decreased photosynthesis, respiration, and disturbing osmotic imbalance leading to physiological drought, as well as nutrient imbalance in the plant. So, collectively many such effects due to salt stress ultimately lead to reduced plant growth as well as loss in agricultural productivity (Adiku et al. 2001; Ramoliya et al. 2004; Evelin et al. 2009).

In saline soil, plant cells take up large amount of dissolved salts which results in plasmolysis of plant cells, and these cells start to collapse, affecting the morphological parameters of the plants like leaf expansion and reduction in fresh and dry matter and content of leaf and root tissue (Hernandez et al. 1995). There are some reports which show that the low level of salinity does not affect the plant growth of legumes, but plant weight, fresh, and dry matter content decreases significantly (Parida and Das 2005). The shoot and root length and dry biomass content of *Trifolium alexandrinum* were also decreased after salinity stress condition (Shokri and Maadi 2009). Many studies have been carried out on variety of plants to understand the detrimental effect of osmotic stress created by soil salinity. Salinity is also responsible for oxidative damage to the plant through the generation of reactive oxygen species (ROS) (Ahmad et al. 2010). In response to salt stress, plants accumulate different types of salt stress proteins and osmolytes like proline, glycine betaine, and malonaldehyde to protect the plant from osmotic shock. These biochemical constituents are accumulating in higher concentration after salt stress exposure.

## 5.2 Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AMF) is special type of symbiotic fungus which forms symbiotic, nonpathogenic association with the terrestrial plant root. The AMF are widely distributed in saline land of terrestrial ecosystem (Yamato et al. 2008). This type of plant fungus symbiotic partnership is found in a wide range of plants including angiosperm, gymnosperm, and pteridophytes; mosses; etc. (Bago et al. 2000). This symbiotic association improves water and nutrient uptake of host plant and protects the plant from various biotic and abiotic stresses (Gupta et al. 2000; Zuccarini and Okurowska 2008). AMF are soil fungi belonging to a recently new approved phylum, Glomeromycota, with a presumed origin at least 460 million years ago. AMF are obligate symbionts that require plant hosts to complete their life cycle. In soil, their spores germinate during favorable conditions and recognize compatible host roots to activate their symbiotic relationship (Schubler et al. 2001; Prasad et al. 2017).

AMF are effective to plants performance against different salinity stress conditions. Under 100 mM NaCl salt stress, AMF-inoculated *Allium sativum* plant showed increased morphological parameters like leaf area, plant fresh weight, and dry weight (Borde et al. 2010). The increase in biomass of tomato plant inoculated with *Glomus mosseae* was observed under moderately saline condition (Ghazi and Al-Karaki 2001). Several studies have been carried out to investigate the role of AMF-inoculated plant in adaptation against salt stress. The positive cumulative effects like nutrient uptake, photosynthetic ability, and biochemical and physiological performance on plant growth due to mycorrhizal inoculation mitigate the salt tolerance (Borde et al. 2011). Under salt stress condition, several researchers showed that the AMF-inoculated plant increased their fitness than non-AMF-inoculated plant (Sannazzaro et al. 2007; Zuccarini and Okurowska 2008; Borde et al. 2011). Mycorrhization improved higher biomass and fruit yield in tomato plant under salt stress (Al-Karaki 2000). Many studies on various plants have demonstrated that mycorrhizal fungi help the plant under salinity stress conditions by enhancing photosynthetic ability, uptake of various nutrients, increasing antioxidant defense, and increasing osmolyte accumulation which results in improved plant growth and tolerance under salinity stress condition (Table 5.1). Increase in the growth parameters of the host plants have been observed in all these studies (Sharifi et al. 2007). In various greenhouse experiments, different AMF species like *Glomus fasciculatum, Glomus mosseae, Glomus etunicatum*, and *Glomus intraradices* were tested for improvement of growth parameters like shoot length, root length, fresh and dry weight, number of nodules, leaf number, pod number, etc. (Kaya et al. 2009).

**Table 5.1** Effects of AMF on plant performance under salinity stress conditions

| Sr No. | Plant name | Effect of AMF | Reference |
|---|---|---|---|
| 1. | *Solanum Lycopersicon* | Enhanced chlorophyll content | Hajiboland et al. (2010) |
| | | Increased antioxidant activity | He et al. (2007), Ghorbanli et al. (2004), Qun et al. (2007) |
| 2. | *Lactuca sativa* | Increased in growth, minerals content | Kohler et al. (2009) |
| 3. | *Piper nigrum* | enhanced chlorophyll content | Kohler et al. (2009) |
| 4. | *Citrus sinensis* | Uptake of potassium and calcium | Wu et al. (2010) |
| 5. | *Oscimum basilium* | Increased in proline accumulation | Shekoofeh et al. (2012) |
| 6. | *Phragmites australis* | Higher osmolytes accumulation | Al-Garni (2006) |
| 7. | *Cajanus cajan* | Higher osmolytes accumulation | Garg and Manchanda (2009) |
| 8. | *Zea Mays* | Higher osmolytes accumulation | Sheng et al. (2011) |
| | | Increased water potential and photosynthetic activity | |
| 9. | *Glycine max* | Increased CAT activity | Ghorbanli et al. (2004) |
| 10. | *Trifolium alexandrinum* | Increased plant biomass | Shokri and Maadi (2009) |
| 11. | *Acacia nilotica* | Increased Potassium | Giri and Mukerji (2004) |
| 12. | *Sesbania aegyptiaca* | Increased in chlorophyll content and potassium | Giri et al. (2007) |
| 13. | *S. grandiflora* | Decreased in potassium | Wu et al. (2010) |
| 14. | *Cucurbita pepo* | Enhanced relative water content | Colla et al. (2008) |
| 15. | *Vigna radiate* | Higher plant growth | Rabie (2005) |

## 5.3 Salinity Tolerance by AMF Through Nutrient Uptake and Ionic Balance

AMF may increase the tolerance of plants to salinity stress by providing nutrient uptake, and the selective absorption of ion leads to ion balance during stress condition (Asghari et al. 2005; Wu et al. 2010; Hammer et al. 2011). Also, AMF protect the plants by activating some enzymes (Giri and Mukerji 2004; Rabie and Almadini 2005) and alleviate water stress (Sheng et al. 2008). AMF have been shown to promote plant growth; enhance nutrient uptake such as Nitrogen, Phosphorus, Magnesium, and micronutrients from the soil; improve soil structure and also able to enhance plant tolerance under different abiotic and biotic stresses such as drought and salinity; and protect host plants against pathogens (Wu et al. 2014; Hameed et al. 2014; Hashem et al. 2014b; Evelin et al. 2012; Sikes et al. 2009). Crop plants inoculated with AMF have been shown to enhance the plant growth and agricultural yield and maintain the osmotic and ionic adjustment to a normal level so that crop plants will grow well under salinity stress conditions (Hameed et al.

2014). This AMF association improves absorption of water and nutrient uptake, solubilizes the complex into existing forms, and enhances the nutrient profile and growth profile of host plant. AMF also help in nutrient cycling in soil, root architecture, and enables to provide essential nutrients to host plant under the salinity stress. AMF play a key role in the regulation of ion and membrane transport proteins that control the ion homeostasis of the host plants (Ramos et al. 2011).

Increase in AMF colonization leads to Phosphorus (P) uptake in *Pennisetum glaucum* plants which indicates that alkaline phosphatases are probably involved in P acquisition, and there are possibilities that more than one acid phosphatase might be responsible for the transporting P, thus leading to increased P uptake under salt stress condition. A good amount of K/Na ratio is also considered to be beneficial for maintaining ion balance in the cytoplasm. The ability of the plant to tolerate stress mainly depends upon the amount of P accumulated.

Excess of sodium chloride (NaCl) causes damage to nearly about 20% of 230 million irrigated agricultural land in and around the world (Munns and Tester 2008). Higher concentration of $Na^+$ (>40 mM) have damaging impact on plant growth for the most part due to hyperosmotic stress (water deficit under strongly negative water potential), over absorption and imbalance of ion (Munns and Tester 2008). During salinity stress, ions like potassium ($K^+$) and sodium ($Na^+$) play an important role in contribution towards the strength of an ion and osmotic pressure (Serrano and Rodriguez Navarro 2001). Increased salinity in the irrigated agricultural land causes crop plant to decrease the concentration of $K^+$, $Ca2^+$, $NO_3^-$ and also increase the concentration of inorganic phosphate (Pi). Whereas, the concentration of $Na^+$ and $Cl^-$ ions increases to such an extent that it leads to ionic injuries and osmotic and nutritional imbalance (Bothe 2012).

Exposure of crop plants to salt stress results impedes uptake of essential mineral elements. Increased $Na^+$ concentration within the root zone directly influences the uptake of several essential elements like $K^+$ and $Na^+$ that share antagonistic relationship with $K^+$ (Kohler et al. 2009). Under salinity stress, AMF inoculation decreases the uptake of $Na^+$ and increases uptake of $K^+$ ion concentration, as compared to non-AM plants, suggesting AMF-induced preferential loading of $K^+$ than $Na^+$ into the root (Hu and Schmidhalter 2005). Excess amount of $Na^+$ and $Cl^-$ ions in saline soil disturbs ionic balance in soil solution and hampers its original potential, therefore uptake, transport, and utilization of essential nutrients are affected by salt stress (Roberts et al. 1984). Hammer et al. (2011) found that AMF can selectively take up elements such as $K^+$ and $Ca^+$, which act as osmotic equivalents by avoiding uptake of toxic $Na^+$ ion, which lower the $Na^+$ ion concentration in plant cell under salinity stress condition. This could make AMF important for salinity stress alleviation for their host plant.

Restricted uptake of potassium and phosphorous is observed by Kohler et al. (2009) and Wu et al. (2010). AMF inoculation to Citrus plants under salinity stress considerably mitigated the deleterious impact on the uptake of essential elements like phosphorous, potassium, and calcium (Wu et al. 2010). AMF inoculation under salinity stress condition in Lettuce plants contributed significantly to growth maintenance by mediating enhanced uptake of essential mineral elements as compared

to the non-inoculated Lettuce plant (Kohler et al. 2009). AMF inoculation maintaining higher K/Na ratio is one of the strategies of AMF to mitigate stress-induced deleterious changes in plants (Wu et al. 2010; Tomar and Agarwal 2013).

Optimal concentration of $K^+$ in plant cell is essential for several important metabolic processes (Tomar and Agarwal 2013). Under salinity stress condition, AMF inoculation showed selective absorption of P, K, and Ca ion over deleterious $Na^+$ and maintaining lower Na/K ratio (Ahmad et al. 2014).

## 5.4 Salinity Tolerance by AMF Through Plant Physiological Response

Shi et al. (2002) and Shi and Guo (2006) found that salt stress could decrease photosynthetic ability and induce physiological drought in plants, which leads to a decrease in crop production. AMF are known to survive stressed soil and participate in the plant growth and development and improves the plant tolerance against biotic and abiotic stresses (Abd-Alla et al. 2000), by regulating the physiological and biochemical process of plants (Evelin et al. 2009). Recently, many researchers have reported that AMF could enhance ability of plants to cope with salinity stress (Yano-Melo et al. 2003; Rabie 2005; Jahromi et al. 2008) by improving plant nutrient uptake (Cantrell and Linderman 2001; Asghari et al. 2005) and ion balance (Giri et al. 2007), protecting enzyme activity (Rabie and Almadini 2005; Giri and Mukerji 2004), and facilitating water uptake (Berta et al. 1990; Ruiz-Lozano and Azcón 1995).

Rabie (2005) suggested that AMF protect the host plants against the detrimental effects of salinity stress. AMF showed higher growth performance in *Cajanus cajan* plants than non-AMF plants at all levels of irrigation. Also, there are reports of modifications of plant physiological performance of plants i.e., osmotic modifications (Rao and Tak 2002) and photosynthesis (Sheng et al. 2008; Borde et al. 2011). Biological remediation, such as the application of AMF to saline soils as bioinoculants, could alleviate salt stress in plants (Evelin et al. 2009; Porcel et al. 2012). This may be the result of a more efficient mineral uptake (Evelin et al. 2012), ion balance (Giri et al. 2007), protection of enzymatic activities (Patel and Saraf 2013), and/or facilitation of water uptake (Aroca et al. 2007).

Amount of chlorophyll pigments declines when affected by salinity stress. This reduced chlorophyll content caused by salinity stress was confirmed by Doganlar et al. (2010), Rasool et al. (2013), Datta and Kulkarni (2014), and Alqarawi et al. (2014) in *Solanum lycopersicum, Cicer arietinum, Glycine max*, and *Ephedra alata,* respectively. In *E. alata*, Alqarawi et al. (2014) demonstrated that chlorophyll content reduced considerably with increasing salinity levels. Reduced chlorophyll contents under stress is attributed to increased activity of chlorophyllase causing degradation of pigments and hence resulting in reduced photosynthesis and affect growth. Compared with stressed plants, AMF-inoculated plants maintained higher

contents of chlorophyll pigments. The fact that AMF colonization significantly increased chlorophyll content in many plants is supported by the findings of Hajiboland et al. (2010) for *Solanum lycopersicum* L. and Aroca et al. (2013) for lettuce. In pepper, inoculation of AMF increased chlorophyll content under normal as well as salt-stressed conditions (Kaya et al. 2009). Enhancement in chlorophyll pigments due to AMF is because of enhanced mineral uptake especially magnesium, an important component of chlorophyll molecule (Sheng et al. 2008). Higher chlorophyll contents in AMF-inoculated plants contribute to greater photosynthetic activity leading to maintain normal growth. Hence, it is clear that AMF inoculation enhances chlorophyll contents and also mitigates the negative impact of salinity to some extent. AMF symbiosis enhanced chloroplast functioning and photosynthetic ability of garlic plant under saline stress condition (Borde et al. 2010; Colla et al. 2008; Sheng et al. 2008).

Many researchers have reported that AMF inoculation could enhance the ability of plants to cope up with salinity stress (Talaat and Shawky 2011; Abdel-Fattah and Asrar 2012; Cekic et al. 2012). AMF colonization enhanced relative water content in *Zucchini* leaves (Colla et al. 2008), water potential and photosynthesis of maize plants (Sheng et al. 2008), and chlorophyll concentration in the leaves of several plant species, i.e., *Sesbania aegyptiaca, Sesbania grandiflora,* and *Lotus glaber* (Colla et al. 2008) under salinity stress condition. Generally, salinity stress has negative effect on AMF colonization; yet some reports have shown improved growth and productivity of mycorrhizal plants under saline conditions (Dudhane et al. 2011; Talaat and Shawky 2011; Cekic et al. 2012).

## 5.5 Salinity Tolerance by AMF Through Antioxidant Defense Response

Besides soil salinity, the increase in the content of salt in soil solution leads to imbalance in nutrients and ion. Due to this imbalance in ion and nutrients, the level of ROS generation in the plant increases considerably. Salinity hinders plant health and therefore to understand the various mechanisms that enable plant to overcome salt-induced stress and growth is essential. Plant cope up salt stress by increasing the production of some osmolytes and antioxidant enzymes which protect the plant cell from oxidative damage (Rai et al. 2011). Plants have evolved specific protective mechanisms, involving antioxidant molecules and enzymes in order to defend themselves against oxidants (Jiang and Zhang 2002; Núñez et al. 2003/2004). When pathogens attack the plant, plant reacts to the attack by activating its defense mechanism such as POD and CAT which are involved in cell wall strengthening or by their role as antioxidant (Mehdy 1994). Superoxide dismutase (SOD) is the basic antioxidant enzyme that converts superoxide to oxygen and hydrogen peroxide ($H_2O_2$) (Alscher et al. 2002). Besides SOD, CAT is also involved in scavenging $H_2O_2$ by decomposing and converting it into water and oxygen. Therefore, SOD

and CAT are treated as the main components which respond and regulate the antioxidant activities by controlling concentration $O_2^-$ and $H_2O_2$ produced in the plant during stress (Van Breusegem et al. 2001).

Oxidative types of stresses are induced by salt stress in plant (Hajiboland et al. 2009). Antioxidant enzymes, being the best defense mechanism, help plant to fight against oxidative damage induced by stress. The production of ROS during various environmental stresses which also include salinity stress is considered as a major factor responsible for damaging the crop plant (Hernandez et al. 1995). The accumulation of ROS has serious impact on plants, especially the disturbance it causes in the metabolism of the plant through oxidative damage (Jiang and Zhang 2001). In order to reduce the damage caused by ROS, plants have their own protective mechanism which reduces the frequency of oxidative damage (Abdel Latef 2010). Detoxification of ROS is done by the enzymes which include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione reductase (GR) (Ahmad et al. 2008, 2010; Liu et al. 2014; Ahanger et al. 2014).

To reduce the adverse effect of stress, plant itself induces some antioxidant enzymes, but mycorrhizal association in plant helps to enhance host's antioxidant defense mechanism. These enzymes are continuously generated in mitochondria, peroxisomes, and cytoplasm of the plant.

Antioxidant defense system is positively associated with salt stress defense response of mycorrhizal colonized plants. It was also found that higher antioxidant enzyme activities in AMF plant can be correlated to plant growth improvement under salt stress (Alguacil et al. 2003; Zhong Qun et al. 2007).

Zhong Qun et al. (2007) reported that in tomato, the activities of SOD, CAT, and POD were observed to be higher in salinity-stressed AMF-inoculated plants as compared to control salt-stressed seedlings. Similar observations were reported in maize by Tang et al. (2009). Also, the increase in the activity of CAT resulted by inoculation of AMF has been reported in soybean (Ghorbanli et al. 2004). Under salinity stress condition, stomatal closure increases the consumption of the NADPH.

Under saline conditions, plant initiates antioxidative defense mechanism, to protect from harmful effect of ROS. In another study, low (50 mM) NaCl and moderate (100 mM) NaCl caused significant increase in shoot SOD activity in mycorrhizal plant as compared to non-mycorrhizal *Pennisetum glaucum* plant (Borde et al. 2011). Enhanced SOD activity in mycorrhizal plant could help in beneficial effect of mycorrhizal colonization (Borde et al. 2011). The increase in POD activity in mycorrhizal plant detoxifies the harmful effect of ROS (Borde et al. 2011). Whereas Catalase involves in decomposition of $H_2O_2$ in peroxisomes of mycorrhizal *Pennisetum glaucum* shoot under moderate and high salinity stress condition.

Different AMF fungal species showed varying extent of antioxidant enzyme activity in maize crop. The salinity stress-affected plant showed increase in SOD activity in root rather than shoot. Mycorrhizal plants showed higher SOD activity than non-mycorrhizal maize crop (Ruiz-Lozano et al. 2013). This increase in SOD activity in mycorrhizal maize crop helps to cope up with oxidative stress. In maize

plant, CAT activity is not evident in salt stress condition, suggesting that the AMF symbiosis does not affect the CAT activity under salt stress condition. (Ruiz-Lozano et al. 2013).

Under salt stress, the osmolytes contribute to cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins (Ashraf and Foolad 2007). Compatible solutes are normally present in very small amount in plant when plant grows under normal condition. But, the concentration of these osmolytes increases rapidly after salt stress exposure. Accumulation of these osmolytes under salt stress varies with host plant as well as AMF species (Rabie and Almadini 2005). Accumulation of compatible solutes in the host plant is referred as positive physiological index under salt stress. These osmolytes help to maintain osmotic balance and membrane integrity and also this acts as a main reservoir of energy and nitrogen for use by plants under salt stress (Ashraf and Foolad 2007).

## 5.6 Salinity Tolerance by AMF Through Proline Accumulation

Proline is the most common osmolytes in plants, which plays an important role in increasing adaptability of plant under salinity stress conditions. It is one of the organic solutes synthesized by plant in response to drought and salinity stress and play an important role in maintaining osmotic adjustment of cell to ameliorate the salt stress effect. Proline is synthesized by two enzymes like pyronine-5 carboxylate synthase (P-5 Cs) and pyronine-5 carboxylate reductase (P-5 Cr) (Kishor et al. 1995). Proline is an important organic compound which participates in osmotic adjustment during abiotic stress conditions (Kishor et al. 1995).

AMF increase proline accumulation in plants subjected to drought stress (Smith and Read 2008). Proline is one of the osmolytes which accumulates in less salinity tolerance species, which modulates the salt stress through osmotic adjustment, plays a multiple role in plant stress tolerance (Yoshiba et al. 1995), protects macromolecules during dehydration (Sanchez et al. 1998), and serves as a hydroxyl radical scavenger (Alia et al. 1995). Thus, AMF are considered to act as bio-ameliorators of saline soils (Singh et al. 1997). Sannazzaro et al. (2007) showed that *G. intraradices* inoculation in two genotype of *Lotus glaber* affect polyamine and Proline accumulation under salinity stress. Datta and Kulkarni (2014) showed increase in proline content in mycorrhiza-inoculated *Glycine max* and *Cyamopsis tetragonoloba legumes* under salinity stress conditions.

Some researchers have reported that enhanced concentration of osmolytes in mycorrhizal plants under salt stress (Feng et al. 2002; Al-Garni 2006; Sharifi et al. 2007; Garg and Manchanda 2009), no effort has been made to compare it with reference to protect ultra structural damage. In view of the defensive mechanism of the osmolytes on the ultrastructure of cells, it is assumed that AMF-mediated

increase in osmolytes concentrations might reduce ultrastructural damage of cells under salt stress. Higher concentration of osmolytes in mycorrhizal over non-mycorrhizal plants under salt stress has been reported in *Phragmites australis, Lotus glaber, Cajanus Cajan,* and *Z. mays* (Al-Garni 2006; Sannazzaro et al. 2007; Garg and Manchanda 2009; Sheng et al. 2011). These are the compatible solutes like free proline and glycine betaine which contribute to osmoregulation through maintaining the cell water content (Ahmad et al. 2008; Ahanger et al. 2014). AMF association with plant improved plant growth by enhancing physicochemical characteristics of rhizospheric soil (Asghari et al. 2005; Ahanger et al. 2014) and enhancing mobilization and uptake of several essential macro- and micronutrients in soil through modifying the root architecture (Ahanger et al. 2014; Hameed et al. 2014).

The proline accumulation is significantly higher in AMF inoculated garlic shoot than non-AMF shoot under low to moderate saline condition, whereas root proline accumulation was higher in mycorrhizal plants than non-mycorrhizal plants. But, overall more proline accumulation is observed in root than shoot because the roots are the primary sites for salinity stress condition (Borde et al. 2011). In Fenugreek plant, the non-mycorrhizal plant showed significantly higher proline accumulation than mycorrhizal plant under saline stress. The lower accumulation of proline in mycorrhizal fenugreek plant leads to reduced salt stress effect (Sannazzaro et al. 2007; Evelin et al. 2013). *Arachis hypogea* showed more proline accumulation in mycorrhizal plant than non-mycorrhizal plant under salt stress. Mycorrhizal *Arachis hypogea* plant adjusts better osmotic balance under stress condition than non-mycorrhizal plant (Al-Khaliel 2010). The alteration of higher proline accumulation under mycorrhization may be due to the enhancement in the activity of proline synthesizing enzymes and reduction in catabolizing ones or its restricted incorporation during protein synthesis (Ahmad et al. 2010). Increased proline accumulations due to AMF are in support by the findings of Shekoofeh et al. (2012) for *Ocimum basilicum*.

To conclude this chapter, salinity is considered to be among the most damaging stress faced by the plants when survival and productivity are in concern. The results conclude that the AMF alleviate the detrimental effect of salinity through improved plant growth by increasing the physiological activities in plants such as photosynthetic ability, relative water content, selective uptake of nutrient, and maintaining the ionic balance of plants. One of the salinity tolerance mechanisms triggered by AM inoculation is increased in the antioxidant enzyme activity of plants like POD, SOD, and CAT which scavenge the ROS and alleviate the salinity stress. Another mechanism of salinity tolerance by AMF colonization increased the non-antioxidative mechanism of plant mainly by accumulating the osmolytes such as Proline; this maintains the osmotic adjustment of plants under salinity stress condition. This cumulative effect increases the physiological performance and tolerance of the plants under salinity stress condition. For this reason, the application of AMF inoculum is more likely to give economical benefits when performed on high-valued crops.

Future studies need to be focused on detailed understanding of the key factors that can be used to overcome the problem of salinity and tolerance of salinity by inoculating the plants with AMF with reference to AMF species producing various antioxidants, proline accumulation, increasing P and $K^+$ uptake, and increasing the plant growth through changing the physiological status of plants under salinity stress conditions. Study also need to be done for expression and characterization of certain stress-related genes, finding links and manipulation of various metabolic pathways that AMF fungi trigger to overcome the problem of salinity stress.

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# Chapter 6
# Arbuscular Mycorrhizal Technology Based on Ecosystem Services Rendered by Native Flora for Improving Phosphorus Nutrition of Upland Rice: Status and Prospect

**Dipankar Maiti, Neha Nancy Toppo, Mukesh Nitin, and Binit Kumar**

**Abstract** Upland ecology is predominantly rainfed and drought prone having nutrient-poor, well-drained, acidic soils. Direct-seeded rice (*Oryza sativa* L.) is the major crop beside maize (*Zea mays* L.), pulses and oilseeds in this ecology. Small portion of uplands with assured irrigation is also grown with vegetables. Nearly one-sixth of world's rice land is under uplands (about 20 million ha) of which (upland rice area) almost two-thirds is in Asia (IRRI 1975; Gupta and O'Toole 1986). Upland farmers, particularly in Asia, are generally resource poor having small land holdings, and majority of them practice subsistence farming. Despite its (uplands) disadvantaged natural conditions for field crops leading to poor productivity, soil microbial health is comparatively less disturbed due to minimum use of modern agrochemicals. This makes the ecology suitable for manipulating beneficial soil microbial resources in favour of crop production. Poor phosphorus (P) acquisition by crops is one major constraint of this ecology. On the other hand, the aerobic soil conditions support arbuscular mycorrhizal (AM) activities which are known to facilitate P acquisition in associated plants. The present article deals with avenues of harnessing ecosystem services rendered by AM fungi (AMF) for improving P nutrition of upland rice under rice-based cropping systems.

## 6.1 Introduction

Sustainable agricultural systems are fast gaining global significance in view of the growing demand for ecologically sound and economically viable cultivation methods that reduce the pressure on the environment due to modern agricultural practices. Excessive application of agrochemicals intended for maximizing yield is

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detrimental in the long run both in terms of degradation of environment and negative impacts on sustainable productivity issues. Judicious integration of microbial inoculant application in the forms of bio-fertilizers and biopesticides with that of synthetic chemicals, in the cultivation systems, is the only route to mitigate such adverse impact on the environment leading to harnessing optimum productivity on long-term basis (sustainable agriculture).

Arbuscular mycorrhiza fungi (AMF) are ubiquitous soil inhabitants, forming symbiosis with about 83% of dicotyledonous and 79% of monocotyledonous plants (Wilcox 1991) including most of the agriculturally and horticulturally important crop plants (Smith and Read 1997; Harrier and Watson 2003; Goltapeh et al. 2008; Prasad et al. 2017). AMF benefit host plant principally by increasing uptake of relatively immobile nutrients like phosphorus (P) (Bagyaraj et al. 2015). Phosphate ions (Pi) remain untrapped by plant unless being intercepted through root system, unlike relatively mobile nutrients like nitrogen (N) which becomes available to plant by means of mass flow. Extra-radical mycelial (ERM) network of AMF extends in the soil beyond the phosphate depletion zone (Marshner 1995; Smith and Read 1997; Johansen et al. 1993; Li et al. 1991a) that quickly develops around roots due to plant uptake of the mobile P present in the soluble/labile P pool. The new pool of labile P (adsorbed on soil particles) beyond P depletion zone is intercepted by an increase in the absorbing area via the external hyphae (ERM) of AMF connected to colonized plant roots and help in acquisition of this P fraction from soil by plant. In exchange, AMF obtain carbon (C) from the colonized plant. AMF colonization also provides additional benefits to the host including (1) improved drought resistance through enhanced tolerance of water stress (Abdelmoneim et al. 2014; Maiti et al. 2009a; Davies et al. 2002; Auge et al. 1994), (2) increased resistance to soil borne diseases (Whipps 2004; Huang et al. 2003; Lingua et al. 2002), (3) increased tolerance to salinity (Salim et al. 2013; Mohammad et al. 2003; Feng et al. 2002) and (4) metal toxicity (Lin et al. 2007; Hildebrandt et al. 2007; Diaz et al. 1996). AMF have also been noted to increase uptake of macronutrients other than phosphorus (P) including nitrogen (N), potassium (K) and also certain micronutrients like zinc (Zn), magnesium (Mg), copper (Cu), iron (Fe) and manganese (Mn) (Fattah 2013; Gosling et al. 2006; Vosatka et al. 2006; Smith and Read 1997; Li et al. 1991b). There is also emerging evidence that AMF reduce nutrient loss from soils by enlarging nutrient interception zone and preventing nutrient loss after rain-induced leaching events (Cavagnaro et al. 2015). Beside these, AMF have been shown to play an important role in maintaining soil aggregate stability (Srimathi et al. 2014; Ambriz et al. 2010; Degens et al. 1996; Tisdall 1991) by building up macroporous structure of soil that allows penetration of water and air and prevents soil erosion (Miller and Jastrow 1992). AMF produce an extracellular glycoprotein called glomalin, which supports hyphae to stick to soil, promoting formation of stable soil aggregates (Wright and Upadhyaya 1996). Thus, AMF are used in soil/land reclamation and revegetation (Wu et al. 2002; Requena et al. 2001; Miller and Jastrow 1992) and bioremediation and help mankind by promoting supply of protective (antioxidants) nutrient components to human beings through agricultural products

(Gianinazzi et al. 2010). In addition, AMF provide ecological advantages by influencing microbial and chemical environment of the mycorrhizosphere in favour of plant growth (Johansson et al. 2004).

A sustainable agricultural system essentially involves natural processes to achieve adequate levels of productivity and food quality while minimizing environmental pollution (Siddiqui and Pichtel 2008; Prasad et al. 2015). This includes minimum use of soluble mineral fertilizers and limiting the usage of synthetic pesticides crucial for crop protection against pests and diseases (Gosling et al. 2006). AMF can compensate for lower inputs of P fertilizers provided a high species diversity and an effective, active AMF community is encouraged and maintained in the soil via sound management practices so as to maximize benefits from AM association without conflicting with other beneficial farm management/agricultural practices. Therefore, the biological management of the key issue of poor P acquisition, through AMF technology, has gained considerable importance so as to accrue benefits (1) via the native or indigenous mycorrhizal fungi or (2) by the application of commercial inoculums containing exotic/non-native isolates. Both approaches have certain advantages and disadvantages. Under ecologically sound soil system with least microbial disturbances which (microbial disturbances) occur as a result of intensive agriculture, maintaining microbial diversity almost intact with efficient strains, exploitation of native population is more advantageous because:

(1) It is very much cost-effective, particularly under rainfed agroecosystems with lower cropping intensity where inoculated population is crashed down due to long fallow disorder (Thompson 1987) and off-season soil desiccation (Maiti et al. 1996) necessitating repeated inoculum application
(2) Native population are more adapted to the soil system ensuring minimum rejection by resident population which is usually encountered by exotic strains/species
(3) It warrants no ecological threat of unintentional introduction of undesirable contaminants to the ecology (Schwartz et al. 2006)

An introduced inoculum with exotic microbial source may even depress yield if the native AMF population is effective (Kahiluoto and Vestberg 1998; Izaguirre-Mayoral et al. 2000; Klironomos 2003). Commercial AMF inoculants containing efficient exotic strains are, however, more effective for plantation crops and reclamation of degraded lands.

Upland ecology, predominantly grown with direct-seeded rice as major crop, is constrained by poor P acquisition under drought-prone condition (Fageria et al. 1982). However, aerobic soil conditions favour AM activities which facilitate P acquisition by colonized plants. Further, the low-input agriculture, practiced by the resource poor upland farmers, allows less disturbance of soil microbial community in terms of both population and diversity including that of AMF (Toppo et al. 2013). This situation provides ample scope for manipulating AM activities to improve P nutrition of upland rice and associated crops. Moreover, uneven distribution of monsoon rain, as a consequence of climate change, in recent years, has led to

unprecedented drought spells which further accentuated poor P nutrition under rainfed agroecosystems. Long-term experiment (1999–2009) data showed that native AM-aided P use efficiency of upland rice was higher in drought years with below-average rainfall over that of normal rainfall years (Maiti et al. 2013). This further justifies importance of AM technology under rainfed ecology. Possible avenues of exploiting native AMF flora in favour of improved P nutrition of crops under rainfed ecology with special reference to direct-seeded upland rice have been discussed in the present review.

## 6.2 Native AMF-Based Technology

Continuous attempts are ongoing worldwide to trap renewable ecosystem services rendered by vast and diverse groups of beneficial microbes harbouring in the environment. Majority of them are having niche in soil, the essential medium for agriculture. Unidirectional focus on gearing up productivity, to satisfy need of the time, during the last decade, through use of synthetic agricultural chemicals has deteriorated soil microbial health posing threat to sustainability of higher productivity. This situation has prompted the agriculturists, in recent time, to think of judicious integration of synthetic agrochemicals' use and trapping natural ecosystem services. Ecologies like uplands in India are less disturbed in terms of soil microbial diversity and, hence, more suitable for exploiting native beneficial microbes through practice of such 'microbe-supportive' crop culture methods. With this presumption, several potential crop components of upland rice and rice-based cropping systems of uplands were evaluated to identify most suitable native AMF-supportive components. The identified components were validated through on-farm trails in farmers' fields in participatory mode and were integrated to develop native AM technology for upland rice with in-built, eco-friendly native AMF-aided higher P use efficiency. The crop culture options evaluated were (1) cultural management, (2) rice-based cropping systems/rotations and (3) development of farmers' friendly method of AMF inoculum production of native origin and application.

### *6.2.1 Cultural Management Options*

Among several, two cultural management options (agro-practices), viz. tillage (Jasper et al. 1991) and P application dose (Habte and Manjunath 1987), are having higher influence on native AMF activities in soil. Based on these observations, scientists attempted to manipulate these two agro-practices in favour of AM without compromising their direct effects on productivity.

**Optimization of Tillage Schedule for Upland Rice** Off-season tillage is agronomic recommendation, especially in uplands, for reducing weed and soilborne pest infestation. Repeated off-season tillage (OST), however, imposes soil disturbance-induced (SDI) deleterious effects on established mycelia network of AMF in soil-reducing colonization in subsequent crops (Jasper et al. 1991; McGonigle and Millar 1993a). Uplands in Asia are vulnerable to this effect due to lack of judicial planning or scheduling of OST which is normally done by upland farmers whenever off-season rain is received to attain proper soil moisture for tillage, using bullock or tractor drawn plough. Farmers also have very short window of getting soil conditions favourable for OST under this rainfed ecology. In such situation where OST cannot be totally ignored and also imposes deleterious effects on mycorrhizal activities in soil, efforts were made to optimize OST schedule under this ecology to minimize SDI effects. The initial results obtained under fixed plot experimentation in India revealed that a minimum space of 13 weeks between two OST would minimize SDI effects with most suitable OST schedule of one after harvest (rice) followed by one summer tillage (Maiti et al. 2011a).

**Optimization of P Application Dose for Upland Rice** High soil P availability limits AMF activities in soil (Habte and Manjunath 1987) and AMF-mediated P acquisition (Richardson et al. 2011) because it (high soil P) reduces formation of AMF-crop symbiosis mainly by lowering (1) growth rate of infection units, (2) production of secondary external hyphae, (3) spore germination and (4) effectiveness of AMF inocula (Kahiluoto et al. 2000, 2001). High P concentration in plant root, at the same time, reduces colonization due to reduced root membrane permeability resulting in decreased loss of AMF favouring metabolites (Graham et al. 1981; Smith and Read 1997; Vierheilig 2004). Under abundant soil P, direct uptake pathway is preferred by plant (Balzergue et al. 2011; Nagy et al. 2009), and reduced AMF colonization is observed (Sally et al. 2011). This necessitated optimization of P fertilizer dose under AMF inoculation to improve AMF efficacy and maintaining P economy without compromising productivity. Enhanced benefits through optimizing soil P were demonstrated in various crops (Habte and Manjunath 1987; Shukla et al. 2011). For upland rice under AMF-supportive rice-based crop rotation and AMF inoculation, about 33% reduction of recommended P dose was worked out to be optimum to achieve higher grain yield (+30.4%) and P economy to the tune of savings of about 10 kg $P_2O_5$/ha(Maiti and Barnwal 2012).

### *6.2.2 AMF-Supportive Cropping System and Rotation Options*

Soils used for agricultural productivity usually have low AMF diversity (Menendez et al. 2001; Verbruggen and Kiers 2010), distribution (Fontenla et al. 2001) and population compared to natural ecosystems. The reasons are adverse effects of agricultural operations (Oehl et al. 2005) like soil disturbance-induced (SDI)

deleterious effects of tillage on established mycelial (AMF) network (Jasper et al. 1991; McGonigle and Millar 1993b) and limiting effects of application of fertilizer(s), particularly P, on AMF activity (Kahiluoto et al. 2000, 2001). This diminishes prospects of reaping benefits from these soil fungi as they need to re-establish their network and activities in soil. Such effects were accentuated under intensive agriculture system over recent past. In Asian and African countries, the problem has further exacerbated under monocropping systems (Sharma et al. 2010). Monocropping tends to select those AMF species which grow and sporulate rapidly. These species offer the least benefit to the plant as they divert more resources to their own growth and reproduction. Moreover, there may be further reduction in the AMF population and activities in soil during no-crop period (Harinikumar and Bagyaraj 2005) or long fallow leading to 'long fallow disorder' (Thompson 1987). Even land cover with non-host (AMF) crop has been demonstrated to be better than long fallow in terms of AMF colonization to subsequent crop (Ocampo and Hayman 1981) and spore population in soil (Kruckelmann 1975). This can be attributed to the previous findings that AM fungal hyphae can make some hyphal growth around the roots of non-host plants without colonizing the roots due to the absence of signals from non-host roots required by AM fungi for successful colonization (Ocampo et al. 1980). Such roots surrounded by AM hyphal growth are more efficient in colonizing host plants than chlamydospores or other inoculum sources. Such unfavourable factors (monocropping and fallow disorder) can be addressed by introducing multi-crop approach with AMF-supportive cropping systems and crop rotations (Ocampo and Hayman 1981). Introduction of AMF-supportive cropping systems including AMF host plants, specific to ecology, would further support healthy soil-AMF environment. Enhanced P nutrition of upland rice under AM-supportive cropping systems and rotations has been elaborately reviewed and discussed by Maiti (2011). The options described in the review included increased AMF colonization and concomitant-enhanced P uptake in (1) direct-seeded upland rice under AM-supportive rice-based cropping systems (Rana et al. 2002) and rotations (Maiti et al. 2006, 2012) and (2) transplanted rice grown in rainfed drought-prone medium lands from seedlings raised in plots pre-cropped with AM-susceptible fodder grasses (Maiti et al. 2008).

### *6.2.3 Developing Farmers' Friendly Method of AMF Inoculum Production of Native Origin and Application*

Commercial inoculums of efficient isolates/strains of AMF are available in market which is useful for plantation, horticultural and cash crops. The use of commercial AMF inoculum, however, has some disadvantages: (1) there is greater propensity of natural soil microflora including native AMF to reject intruders (commercial inoculums), and (2) such inoculums may have possible negative ecological consequences in terms of invasive species introduction as unintended contaminants

(Schwartz et al. 2006). Such constraints of commercial inoculums are more acute in upland ecology where native AMF population of soil has not been much damaged by biologically unsound processes including modern, chemical-based agricultural practices. Moreover, under predominantly monocropped (rice/maize/pulses) upland ecology, soil population crashes under strong ecological stresses like long duration post-crop fallowing, soil desiccation, etc., necessitating frequent use of inoculums which is not cost-effective for low-value field crops like rice. Introduction of exotic species/strain (AMF) through application of commercial inoculums may even depress yield if the native AMF population is effective (Klironomos 2003; Izaguirre-Mayoral et al. 2000; Kahiluoto and Vestberg 1999). Khaliq and Sanders (2000) measured a small (3–4%) reduction in the yield of barley in response to inoculation with a single AMF species. So, AMF inoculums developed from native sources consisting of consortium of AMF species are considered to be more efficient (Oliveira et al. 2005), cost-effective and adaptable to the target ecology. The native biological potential of soils, including that of AMF, is expected to be least disturbed under situations of low input system cultivation as are normally practiced in poorly productive upland ecology, particularly under rice cultivation. There is evidence of diverse (Maiti et al. 1995; Toppo and Maiti 2011) and effective (Maiti et al. 2012) AMF flora in upland soils under rice cultivation.

On-farm technique of inoculum production with AMF (Douds et al. 2005, 2006; Gaur et al. 2000; Gaur 1997; Sieverdin 1991) of native origin (Gaur and Adholeya 2002) has been widely explored and extensively reviewed by Marleen et al. (2011) and Maiti (2011). Based on the knowledge and technology generated, a production protocol of AMF inoculum using native AMF, specifically suitable to upland rice, was developed by Maiti et al. (2009b), and its efficacy in terms of improving P nutrition of rice has been confirmed (Maiti et al. 2011b). The protocol allows the farmers to produce cheaper, soil-based high-volume inoculums of their own through multiplying nucleus inoculums (NI) (starter inoculums) within their farm system. The nucleus inoculums consist of a consortium of spores (AMF) indigenous to the site of application and not always identified to the species level (Gaur and Adholeya 2002). The NI is multiplied on *Sorghum* (*Sorghum bicolor*) roots grown in micro-plots, presterilized (partially) by soil solarization using transparent, thin (1–2 mm) low-density polyethylene (LDPE) film mulching during peak summer months to produce mass inoculums (MI). MI essentially consist of external hyphae (AMF), infected roots, spores and soil (15 cm depth from the solarized plots). The protocol scheme involves production of NI under controlled condition by institutions, supplying to the farmers who can multiply NI in their farm after training and produce mass inoculum (MI). Integration of native AM-supportive crop culture components (AM-supportive rice-based cropping systems and application of AM-inoculum developed from native sources) showed to produce additive effects on native AMF activity, plant growth promotion, P uptake and grain yield of upland rice under rainfed ecology (Maiti et al. 2011b).

**Use of Multifunctional Microbial Consortium** AMF inoculum has been tried in combination with several other beneficial microorganisms to achieve possible

additive or synergistic effects. Benefits of co-inoculation of AMF with (1) N-fixing non-symbiotic bacteria like *Azotobacter chroococcum* (Umakant and Bagyaraj 1998) and *Azospirillum* (Rangarajan and Santhanakrishnan 1995) in mulberry, (2) plant growth-promoting rhizobacteria (PGPR; *Pseudomonas fluorescens*) in *Morus alba* (Rangarajan and Santhanakrishnan 1995), (3) phosphate-solubilizing bacteria (PSB) like *Bacillus licheniformis* in upland rice and (4) PSB (*B. megaterium*) and N-fixer (*Azotobacter*) in mulberry (Baqual and Das 2006) were demonstrated. Co-inoculation of different beneficial microbes has proved to be better because it supports plant growth through various ways and also supports each other's growth.

## 6.3 Innovative Option of Exploiting Native AMF: Use of AM-Responsive Crop Varieties

Possible avenues of exploiting native AMF by agronomic manipulation (adoption of AM-supportive crop culture components) and use of AMF inoculum of native origin have been discussed in the previous chapters. In recent years, researchers are emphasizing on another innovative approach of exploiting native AMF flora through taking advantage of potential of plant species (host) to harness ecosystem services rendered by native AMF. This could be achieved by genetic manipulation of crop varieties for enhanced AM response. The agronomic and genetic manipulations for enhanced mycorrhizal nutrient acquisition and response are mutually inclusive and in combination could exploit (native AMF biodiversity in soil), to the full extent. Mycorrhiza responsiveness has been defined as difference in growth response between mycorrhizal and non-mycorrhizal plants under a given environment (Janos 1988). Siqueira and Saggin (2001) clarified that responsiveness relates to its internal nutrient demand in relation to growth rate under a given environment. Smith and Smith (2011) quantified 'mycorrhizal responsiveness (MR)' in terms of change in plant biomass that results from the symbiosis.

The approach of genetic manipulation (crop) is based on twin attributes of AM symbiosis—lack of host specificity of AMF and variation in AM responsiveness among genotypes of plants. The native AMF flora can be exploited best, provided that (1) the native population remains undisturbed and (2) the crop varieties are highly responsive to AMF both for colonization and P acquisition. Presences of diverse and effective AMF flora, particularly in less biologically disturbed ecology, like uplands, have been confirmed (Maiti et al. 1995). On the second aspect, there is sufficient evidence that plant response to arbuscular mycorrhiza is a variable trait (Smith and Read 1997) and there is scope to exploit the variability to select/breed high mycorrhiza-responsive crop varieties for exploiting the biological potentials of AM in nutrition management of crop plants. AM responsiveness of plants operates at two levels—(1) root colonization and (2) physiological response (including P acquisition) following colonization. Host genotype variations in the extent of

colonization are known in many crop species, including members of the Gramineae (Barker et al. 2002) where high colonization of a genotype does not necessarily translate to high physiological response of the plant (Ravenskov and Jakobsen 1995). Plant species or varieties are reckoned to vary in functional compatibility with AMF where P uptake of one species or a variety may improve more than that of another following colonization by the same AMF species (Burleigh and Bechmann 2002). There have been a few attempts to dissect such functional compatibility or response variation of crop plants genetically, so as to identify the genes that determine/control 'enhanced AM responsiveness'. Such identification holds prospect for use and application in breeding crop plants for high AM responsiveness (Barker et al. 2002), especially for systems of low input cultivation, like the upland rice. The modern molecular tools have widened the scope of identification of such hidden or obscure genes with less time and cost than the conventional methods.

Insights on genetic analysis of AMF symbiosis of crop plants have come from molecular analysis of mycorrhiza-defective mutant of legume hosts—garden pea, vetch, clover and *Lotus japonicus* as model plants. These studies have helped identifying more than 20 loci in different legume species (Marsch and Schultze 2001) which control development of symbiosis, particularly the stages of penetration and post-penetration colonization by the non-host-specific AM fungi (Barker et al. 2002). Characteristically, some of these loci (17 identified till now) have been shown to regulate nodulation development of the legume hosts by their respective host-specific *Rhizobium* strains (Barker et al. 2002). Thus, a concept has emerged that the genetic pathway of AM symbiosis, in part, is shared by other similar plant-microbe interactions including *Rhizobium* symbiosis (Martin 2001). Around 224 genes are affected during AM symbiosis in rice, and 34% of these genes were also found to be associated with mycorrhization in dicots, revealing a conserved pattern of response (Güimil et al. 2005). The likelihood of some of these AM-responsive genes being evolutionary conserved, since the early days of land plant evolution (Simon et al. 1993), has functional importance in plant biology, indicating the possibility of their exploitation in breeding for symbiosis response.

Another area relevant for exploitation of symbiosis is host genes for enhancing plant response related to phosphate (Pi) transporters. Pi transporters of AMF and plant hosts including rice have been cloned and characterized (Versaw et al. 2002). The researches have shown that some of these plant Pi transporter genes are specifically activated in AM symbiosis, for example, the rice phosphate transporter gene OsPT11 was specifically induced during AM infection. This induction is correlated with the degree of AM colonization and is specifically confined to the root system (Paszkowski et al. 2002). Similar evidences have been recorded in potato and tomato plants (Nagy et al. 2005). Among two phosphate transporter genes identified to be involved in AM symbiosis, viz. OsPT11 and OsPT13, the former may be responsible for both AM development and symbiotic phosphate uptake, whereas OsPT13 may act as a sensor to detect the phosphate level appropriate for arbuscule development (Yang et al. 2012). A possibility has thus emerged of exploiting these host-specific or non-specific AM-regulated phosphate

transporter genes for genetic enhancement of nutritional physiology. Rice, in view of its sequenced genome (International Rice Genome Sequencing Project (IRGSP) 2005), seems to be a suitable plant for such model work.

Inter- and intraspecies variation in AMF response (for growth promotion and P acquisition) in cultivars of onion (*Allium fistulosum*) (Tawaraya et al. 2001), other crops (Smith and Read 1997; Koide 1991; McGonigle and Fitter 1990) including cereals like wheat (*Triticum aestivum*) (Hetrick et al. 1995), maize (*Zea mays*), barley (*Hordeum vulgare*) (Gianinazzi-Pearson 1984; Smith and Read 1997) and rice (*Oryza sativa*) (Dhillion 1992; Maiti et al. 1995; Toppo et al. 2015—unpublished) have been observed. Such varietal differences in response suggest that mycorrhizal responsiveness is a genetic character. Sustained research on the genetics of mycorrhiza formation over the last few years has revealed that plant response to mycorrhiza may depend on the genomic backgrounds of the fungus, the plant and their interaction with environment (Franken and Requena 2001). Formal genetic studies with large number of cultivars *of T. aestivum* indicated that 'mycorrhizal responsiveness' genes might exist in different chromosomes of some cultivars (Hetrick et al. 1995). In double haploid mapping populations of barley varieties, differences in mycorrhiza responsiveness have been identified by formal genetic approaches (Langbridge et al. 1995). Using 'near-isogenic lines' (NILs), similar host genotypic variations in colonization and host response have been identified in white clover (Eason et al. 2001). Inter-cultivar variation in P acquisition due to AMF colonization has been reported in double haploid genetic population of *Hordeum vulgare*, and the presence of 'quantitative trait loci' (QTLs) for mycorrhiza responsiveness was indicated (Barker et al. 2002). Subsequently, QTLs for AM responsiveness were identified in crops like *Allium* species (Galvàn et al. 2011) and maize (*Zea mays*) (Kaeppler et al. 2000). Galvàn et al. (2011) identified four genomic regions involved in mycorrhizal responsiveness in onion to *Glomus intraradices*. These QTLs also controlled the plant average performance positively and the number of roots. Response to mycorrhizal fungi in a 'recombinant inbred line' (RIL) population of B73 × Mo17 maize population led to identification of three QTLs to influence growth at low P in the absence of mycorrhiza based on shoot weight. These findings have opened up the possibility to utilize this genetic variability to select/breed high AM-responsive crop varieties to exploit the biological potentials of AMF in managing P nutrition. The knowledge of genetic basis of mycorrhiza response would allow genetic breeding/manipulation of the varieties for increased mycorrhizal response in terms of plant supply of phosphorus in an otherwise phosphorus-deficient soil, simply by getting the plants infected with appropriate mycorrhiza, for the maximum P use efficiency of applied chemical P fertilizers.

Analysis of the completed rice genome sequence resulted in identification of literally tens of thousands of new targets for DNA markers, especially SSRs. In rice (*Oryza sativa*), earlier studies reviewed by McCouch et al. (1997) demonstrated that microsatellite markers are distributed relatively uniformly throughout the genome and detect the level of allelic diversity in cultivated varieties and distantly related species. For mapping, genetic analysis and marker-assisted plant improvement

strategies, a total of 2414 new di-, tri- and tetra-nucleotide non-redundant SSR primer pairs representing 2240 unique marker loci have been developed and experimentally validated for rice which is publicly available (McCouch et al. 2002). This was soon followed by 18,828 class I (di-, tri-, tetra-repeats) SSRs that were released after the completion of the Nipponbare genome sequence in 2005 (Matsumoto et al. 2005). This number is by far the largest number of publicly available SSRs for any crop species. The extremely high density of SSRs (approximately 51 SSRs per Mb) provides a considerable 'tool kit' for map construction and marker assisted selection (MAS) of crop variety for numerous applications (Collard and Mackill 2008). SSR molecular markers are frequently used to assess genetic variation within and between populations (Vigouroux et al. 2005), and there have been many studies describing genetic diversity in a wide range of species (Jamil et al. 2013; Prabhakaran et al. 2010; Herrara et al. 2008; El-Malky et al. 2007; Garris et al. 2005). SSRs have also been used in rice DNA fingerprinting (Rahman et al. 2009; Chakravarthi and Naravaneni 2006). There are lot of evidences where SSRs have already been used to identify several qualitative or quantitative trait loci in rice for successful breeding and selections. Zhang et al. (2001) used 315 DNA markers in a population of 154 double haploid lines of rice and could identify 41 QTLs for drought tolerance. Sharma et al. (2005) using such 178 SSR markers to a $F_2$ genotype population of 208 individuals could map Pi-k (h) gene of rice, which confers resistance to *Magnaporthe oryzae*, the blast disease-causing pathogen. All these evidences are available in the public domain (Gramene literature). Jing et al. (2010) studied QTLs associated with yield-related traits using an 'advanced backcross' population derived from common wild rice (*Oryza rufipogon* L). These markers can be useful in molecular mapping and marker-assisted selection as suggested by Aliyu et al. (2011). In the last few years, a lot of works have been done with these markers for QTL analysis in rice for various traits (Gao and Sun 2013; Sandhu et al. 2013; Zhao et al. 2013; Mararthi et al. 2012; Kebriyaee et al. 2012; Rathi et al. 2011; Wan et al. 2008). However, no such works are still noticed for the identification of the trait of AM responsiveness in terms of enhanced phosphorus nutrition and growth promotion particularly for upland rice. For traits as difficult to evaluate as AM responsiveness, molecular markers allow breeders to track the genetic loci linked to such complex traits and help in their indirect selection. Simple sequence repeats (SSRs) for such analysis are considered the molecular markers of choice, due to high level of polymorphism, high reproducibility, codominance, relative abundance and rapid but simple genotyping assays (Kong et al. 2000). SSRs occur as frequently as once in approximately every 6 kb in plant genomes (Cardle et al. 2000) and are highly preferred due their abundant distribution in the genomes examined till date and their hypervariable nature. Attempts are being made to identify phenotypic plant traits in upland rice varieties most likely associated with AMF-mediated physiological response, which in turn would aid in identification of polymorphic SSR markers between the most significantly different (AM responsiveness) phenotypes (through parental polymorphism), for future usage in QTL analysis for the AM

responsiveness trait in terms of P uptake and growth promotion in the selected upland rice varieties.

## 6.4 Conclusion and Perspective

Poor phosphorus nutrition of rice, grown particularly in upland ecology, is one major constraint for improving productivity. Phosphorus promotes tillering, root development, early flowering and ripening. Without adequate supply of P, plants cannot reach its maximum yield because it reduces panicles/plant, grains/panicle and filled grain number/panicle. Proper P supply also reduces spikelet sterility (Aide and Picker 1996) resulting in further yield increase. Thus, addition of P fertilizers in large amounts for enhancing yield is commonly practiced all around the world (Itao et al. 1999). But the main problem with P fertilizers is its less mobility and fixation with soil complex within a short period of application rendering more than two-thirds unavailable (Sahrawat et al. 2001). This necessitates access of fixed P to plants for promoting P economy and reducing production cost. Biological way (exploiting arbuscular mycorrhiza) of capturing this untapped P in soil thus has gained importance among researchers as an environment-friendly and climate-resilient (Maiti et al. 2013) avenue. The present document reviewed the prospective ways of capturing ecosystem services rendered by native AM fungus for improving P nutrition of upland rice. The future perspectives of exploiting this biological opportunity by means of trapping host plant genetic quantitative trait of high AM responsiveness for P acquisition has also been discussed which (AM-responsive variety) in combination with AM-supportive crop culture components would promote capturing this ecosystem service to the full extent.

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# Chapter 7
# Arbuscular Mycorrhizal Fungi in Redeeming Arsenic Toxicity in Plants

**Surbhi Sharma, Neeraja Singh, and Rupam Kapoor**

**Abstract** Arsenic (As) contamination has transitioned into a global threat, hampering the survival of millions. Chemical fixation/remediation techniques have proved to be inadequate to reduce As toxicity. Use of arbuscular mycorrhizal fungi (AMF) in alleviation of As stress is a reliable and efficient approach. AMF have been reported to be present in As contaminated soils and are known to exert ameliorative role on detrimental effects of As. Although presence of As in soil affects AMF spore germination and colonization, they have been found to occur even in highly contaminated soils. AMF alleviate As toxicity by extending its extraradical mycelium beyond the depletion zone and help in the uptake of various nutrients increasing the biomass of the plant. AMF sequester As in its various fungal structures such as intraradical hyphae, arbuscules and vesicles preventing their translocation to aerial plant parts. Arsenate [As(V)] and inorganic P (Pi) compete for the same transport proteins in root plasma membrane. AMF could decrease As(V) uptake into the roots by suppressing the high affinity As (V)/(Pi) transporters. It thus enhances the P-uptake by circumventing the direct pathway and channelizing P-uptake by mycorrhizal pathway. AMF results in As stress tolerance in plants by enhancing P uptake, biotransformation of As(V), reduced As uptake, sequestration, protection from oxidative damage and improved physiology of plants.

## 7.1 Introduction

One of the toxic metalloids distributed widely in the environment is Arsenic (As) (Huysman and Frankenberger 1990; Phillips 1990; Mahimairaja et al. 2005). The main environmental exposure to As for humans is through contaminated drinking water (Meharg et al. 2009), for instance, in the Indian sub-continent As in drinking water has become a huge problem (Nordstrom 2002). Its entry in the environment can occur by natural activities (rock and soil erosion, volcanic action)

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or industrial and agricultural practices (fertilizers, pesticides, herbicides, mining) (Adriano 2001; Mandal and Suzuki 2002).

Arsenic is generally toxic to plants and is non-essential element. The predominant form of As available for uptake by plants is arsenate [As(V)] and arsenite [As (III)] (Zhao et al. 2009). Once in the cell, As(V) can be readily converted to As(III), the more toxic of the two forms (Gonzalez-Chavez Mdel et al. 2011, 2014). As (V) and As(III) both disrupt plant metabolism, but through different mechanisms. Roots are usually the first tissue to be exposed to As, where the metal inhibits root proliferation and extension. Upon translocation to the shoot, As can severely inhibit plant growth by reducing or arresting biomass accumulation and expansion, as well as compromising plant reproductive capacity through losses in fertility, yield, and fruit production (Garg and Singla 2011). At higher concentrations, As interferes with vital metabolic and physiological processes, which can ultimately lead to cell death.

In its defense, plant launches antioxidant machinery. The various enzymatic and non-enzymatic antioxidants work in congruence to rescue plant from the oxidative stress that As sets in (Sairam et al. 2005; Sharma et al. 2007; Gunes et al. 2009). Other mechanisms such as complex formation with phytochelatins (PCs) and metallothioneins and the subsequent sequestration in the vacuole and transformation of inorganic As into less toxic organic methyl arsenates also contribute (Gonzalez-Chavez et al. 2002) to plant's tolerance to As toxicity (Fig. 7.1)

Arbuscular mycorrhizal fungi (AMF) have multifarious roles, besides providing mineral nutritional; they bequeath host plant with biotic and abiotic stress tolerance (Fig. 7.2). Several studies have highlighted the role of AMF in advocating As tolerance in plants (Covey et al. 1981; Chen et al. 2007; Xia et al. 2007; Hua et al. 2009). However, there are voids in the understanding of functional and structural contribution of AMF in As stress amelioration, more so because the interaction of AMF and plant is species specific in nature.

The present chapter provides a comprehensive review of menace caused due to As contamination globally, As acquisition, and uptake in plants and its detrimental effects. Particular emphasis is given on the mechanisms employed by AMF for alleviation of As toxicity.

## 7.2 Arsenic Menace

Arsenic has been identified as the most prevalent contaminant found in soils (Shaibur et al. 2008). It ranks 20th, 14th, and 12th in the earth's crust, sea water, and human body, respectively (Mandal and Suzuki 2002). However, the established average As content of earth's crust is 2.5 mg/kg (NAS 2000); it is found to be present at a concentration of 45 to 3275 mg/kg (Nagy et al. 2005). Increased concentrations of As in water and soil have become a menace at the global level. The most significant occurrence of As is seen in India, Bangladesh, Nepal, Northern

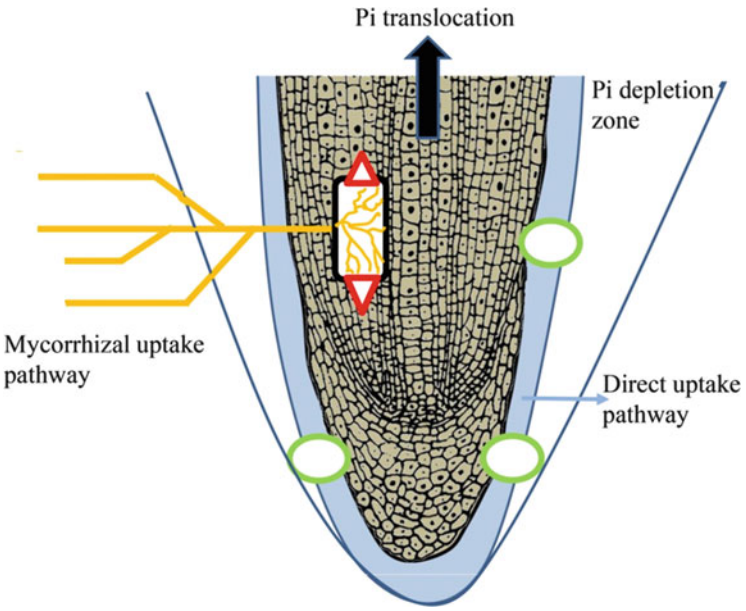


**Fig. 7.1** Schematic representation of inorganic orthophosphate (Pi) uptake pathway in an arbuscula rmycorrhizal root. Rapid uptake of Pi by direct pathway occurs through Pht1 (*green circles*) transporters located at epidermis and root hairs cells. Rate of Pi uptake surpasses the rate of diffusion, resulting in the formation of depletion zone. In the mycorrhizal uptake pathway, extraradical fungal hyphae extend beyond the depletion zone. With the help of fungal transporters located in extraradical hyphae, they help in Pi uptake. Pi reaches the symbiotic interface in the root cortex and to intracellularly present arbuscules. At this interface, Pht1 transporters (*red triangles*) help in Pi absorption in root cortical cells

China, Myanmar, and Vietnam (Das et al. 2004; Patel et al. 2005; Hasanuzzaman and Fujita 2012).

The condition of As toxicity in India is alarming with reports of severe health problems in the populations of West Bengal, Bihar, Jharkhand, and Assam (Acharya 2002; Chakraborti et al. 2008, 2013; Roy et al. 2014; Kumar et al. 2015). Arsenic occurrences in groundwater of Bengal Delta Plain (i.e., Bangladesh and West Bengal) is amongst the largest environmental health disaster encountered recently, where approximately 50 million people are at risk of cancer and other As related diseases due to the consumption of high As contaminated groundwater and food (Singh 2015).

Inorganic As has been categorized by the US Environmental Protection Agency (EPA 1988) and International Agency for Research on Cancer (IARC 1980, 1987) as a class I carcinogen (Hughes 2002). Chronic consumption of As contaminated drinking water leads to cancer of internal organs. Bladder, liver, and kidney are common tumor sites (Smith et al. 1992; Bates et al. 1995). Chronic oral exposure to As causes skin lesions, which are characterized by hyperpigmentation, hyperkeratosis, and hypopigmentation (Yeh et al. 1968; Cebrian et al. 1983).

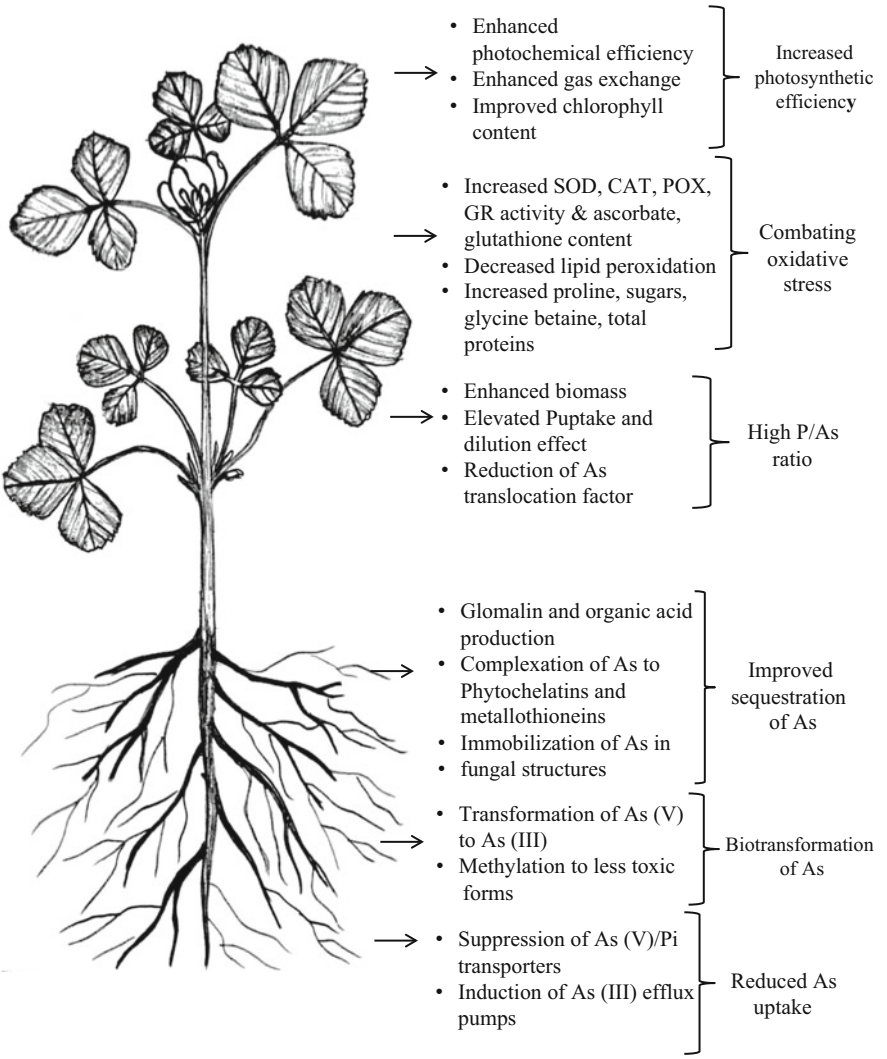


**Fig. 7.2** Schematic representation of arbuscular mycorrhiza in alleviation of arsenic toxicity in plants

## 7.3 Arsenic Uptake in Plants

Arsenic exists in natural systems in both inorganic and organic forms. In inorganic forms, it occurs as trivalent As(III) and pentavalent As(V) state. As (V) predominates in aerobic environments while As(III) predominates in anaerobic environments (Cullen and Reimer 1989). Arsenate can convert to As(III) and *vice versa* based on the redox state and pH of the environment (Zhao et al. 2010).

Certain microorganisms and algae are known to methylate inorganic As(III) present in soil (Bentley and Chasteen 2002). The organic forms of As found in soil are monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and trimethylarsine oxide (TMAO). These organic species generally have low levels (Takamatsu et al. 1982; Huang and Matzner 2006), but can approach higher concentrations too (Abedin et al. 2002). The methylated forms of As(V) can be reduced to more toxic methylated As(III) forms, which pose a great concern as they are commonly found in the environment.

In plants, As accumulation is influenced by its availability in the soil and metabolic as well as physiological properties of the plant (Kumar et al. 2015). Arsenate is an analog of inorganic phosphate (Pi) and, thus, is readily transported across the plasma membrane by phosphate transporters proteins (PHT) (Meharg and Hartley-Whitaker 2002; Smith et al. 2010a; Wu et al. 2011a, b). Under low Pi conditions, As(V) may outcompete Pi for entry into the plant, thus resulting in intensification of Pi deficiency symptoms (Tut and Ma 2003; Catarecha et al. 2007). Arsenate toxicity has been reported to be changed by genetically manipulating the PHT protein concentration (Shin et al. 2004; Gonzalez et al. 2005; Wu et al. 2007).

Arsenite enters root cells through nodulin 26-like intrinsic proteins (NIPS) Meharg and Jardine 2003. These proteins belong to the aquaporin family of major intrinsic proteins (Bienert et al. 2008; Ma et al. 2008). Arsenate shares its transport pathway with Pi while As(III) shares its pathway with silicon (Si). In rice roots, OsNIP2;1/OsLsi 1 Si transporter provides major entry route for As(III) uptake, and OsLsi2 mediates As(III) efflux from exodermis towards stele. Reduction of intracellular As(V) to As(III) occurs by the action of an enzyme arsenate reductase (Bleeker et al. 2006). Arsenite then combines with thiol (–SH) compounds to form As-thiolates, which are then transported and sequestered in the vacuoles (Mukhopadhyay et al. 2000).

Similar to As(III), methylated As species enter roots via the aquaporin channels (Li et al. 2009). Although uptake by roots is much slower for MMA and DMA as compared to As(V) or As(III) (Abedin et al. 2002; Abbas and Meharg 2008), the movement within the plants is considerably greater than the inorganic As species (Marin et al. 1992; Raab et al. 2007; Carey et al. 2010, 2011).

Carey et al. (2010) using X-ray absorption near edge spectroscopy and inductively coupled plasma mass spectroscopy (ICP-MS) demonstrated that xylem and phloem contribute to As unloading in the rice grain. It has been suggested that for the distribution of As in various plant parts and its speciation phloem is an essential prerequisite (Carey et al. 2010; Tiwari et al. 2014). However, molecular components involved in the transportation of As through phloem are not known till date (Tiwari et al. 2014; Kumar et al. 2015).

## 7.4 Impact of Arsenic Toxicity on Plant Growth and Physiology

Exposure to As decreases the germination rate, root, and shoot length, causing chlorosis, increased sterility, and ultimately death in a variety of plant species (Liu et al. 2005; Shri et al. 2009; Smith et al. 2010a). In most of the plants, the limit for As toxicity is 5-20 mg/kg (Mendez and Maier 2008). Roots are the first point of contact with As in the soil; hence, they show higher sensitivity towards As toxicity. With increase in the concentration of As, the seedling growth has been shown to be inhibited in rice plants (Shri et al. 2009). Wheat plants when exposed to high levels of As ($\geq$80 mg/kg) exhibited decrease in biomass and height of the plant (Gulz et al. 2005). Reduction in the shoot and root system of tomato plants at high As concentration has been observed (Miteva 2002). In *Lens culinaris*, decline in plant height, biomass production, root length, and leaf number was seen with increasing the concentration of As in irrigated water (Ahmed et al. 2006). The reproductive development of a plant exposed to As is also greatly affected (Smith and Read 2008). Abnormal another development, fertilization, inhibition of gametogenesis and sporogenesis, and disruption of female gametophyte development have been reported due to As exposure in plants (Spagnoletti and Lavado 2015).

Arsenic stress damages the chloroplast membrane affecting chlorophyll synthesis in plants (Stoeva and Bineva 2003). Activity of protochlorophyllide reductase is inhibited (Stoeva and Bineva 2003); on entering the leaves, As combines with sulfhydrl (–SH) groups of proteins substituting for ferrous ($Fe^{2+}$), zinc ($Zn^{2+}$) ions, and destroying the structure of chloroplast in plants (Li et al. 2007). Low content of large subunit of Rubisco was observed in rice leaves (Andrade et al. 2015). Plasmid DNA encodes large subunit of Rubisco, suggesting that As obstructs chloroplast DNA gene expression (Andrade et al. 2015). In the presence of As stress, the chlorophyll fluorescence Fv/Fm ratio that estimates primary photochemistry ability in photosystem II is also affected (Schreiber et al. 1998). Due to As stress, there is damage to the carbon dioxide ($CO_2$) fixation process resulting in increased internal $CO_2$ concentration in sub-stomatal spaces (Andrade et al. 2015). Reduction in ATP and NADPH production that are required for $CO_2$ fixation reactions in calvin–melvin cycle is caused due to As toxicity, therefore hampering photosynthetic electron transport process in plants (Finnegan and Chen 2012; Andrade et al. 2015). A factor which is linked with heat dissipation in plants is non-photochemical quenching (NPQ). This in the presence of As(III) and As (V) increases, indicating oxidative damage and photo-inhibition of the chloroplast (Andrade et al. 2015). Due to substitution of As(V) instead of Pi in ATP, there is formation of ADP-As which is highly unstable compound (Meharg and Macnair 1994). This results in generation of wasteful reaction cycles that leads to uncoupling of photosynthetic electron transport in thylakoid membrane and respiratory electron transport in inner mitochondrial membrane (Avron and Jagendorf 1959).

## 7.5 Interaction with Phosphorus Uptake

Due to similar chemical properties and electronic configuration, As(V) and P compete for the same uptake carriers in root plasma membrane (Meharg and Macnair 1992; Hartley-Whitaker et al. 2001; Gunes et al. 2009). Therefore, it is imperative to understand their interactions with each other for ascertaining their uptake pathways in plants (Gunes et al. 2009). Plants take up As(V) with the help of P transporters and As(III) in a P independent manner (Wang et al. 2002). Arsenic replaces P in various metabolic pathways; for instance, it competes with P for binding to ATP, resulting in the formation of adenosine diphosphate-As(V) (Trotta et al. 2006). In comparison to As, P binds more efficiently to high-affinity P transporters; thus, high concentration of P in soil solution favors uptake of P than As (Meharg and Macnair 1994; Tu and Ma 2003), As is expelled from the soil solution, and there is increase in P absorption (Alam et al. 2001). Moreover, phosphate is more stable than As(V) over a wide range of pH conditions in soil (Lambkin and Alloway 2003). In a hydroponics study carried out on As hyperaccumulator plants, it was found that in the presence of P the uptake of As in plants is suppressed (Tu and Ma 2003).

## 7.6 Oxidative Damage

Exposure to As results in the production of reactive oxygen species (ROS) that are formed due to valence changes in metal from As(V) to As(III) (Meharg and Hartley-Whitaker 2002). If in a plant the ROS scavenging system does not work efficiently in the presence of As stress causing formation of free radicals, it leads to uncontrolled oxidation, which results in oxidative stress in plants (Srivastava et al. 2005). The ROS include hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($OH^{-}\bullet$), and superoxide radicals ($O_2^{-}\bullet$) (Gunes et al. 2009). These are strong oxidizing agents that result in oxidative damage to biomolecules eventually leading to cell death in plants (Gunes et al. 2009). High levels of As cause lipid peroxidation due to increase in $H_2O_2$ levels resulting in $OH\bullet^{-}$ formation that cause membrane damage in plants. Presence of As results in increased production of malondialdehyde (MDA; biomarker for lipid peroxidation), low shoot growth, and decrease in dry shoot mass (Gunes et al. 2009) in plants. Increase in the level of MDA has also been observed in hyperaccumulator fern species (Srivastava et al. 2005) and in bean (Stoeva et al. 2005). Severe lipid peroxidation in the presence of As occurs due to hydrogen removal from unsaturated fatty acids by ROS resulting in lipid radical production (Garg and Kaur 2013). Due to this, a cascade of cyclic reactions occur that form short chain-like alkanes and lipid acid aldehydes that damage the lipid structure severely (Mishra et al. 2006; Garg and Kaur 2013). Due to As stress in plants, there is increased amount of energy level in the thylakoids surpassing the level that can be dissipated by metabolic pathways of chloroplast; this disrupts the

electron transport process (Stoeva and Bineva 2003; Stoeva et al. 2004). High lipid peroxidation, chloroplast damage, and decreased protein concentration have been observed in *Pteris ensiformis* on exposure to As (Singh et al. 2006). Increase in thiobarbituric acid (TBA) derivatives, which are indicators of oxidative damage due to As exposure, was found in *P. ensiformis* and in white lupine plants (Singh et al. 2006).

Plants have devised various mechanisms in order to combat oxidative stress. These mechanisms are characterized by induction of various enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) and non-enzymatic antioxidants such as ascorbate, glutathione (GSH), and α-tocopherols (Sairam et al. 2005). By conjugation of –SH groups with the electrophile As or by providing substrates for synthesis of PCs (Gupta et al. 2009), GSH protects plants from damage caused due to As and other metalloids (Schulz et al. 2008). In chickpea plants, activity of CAT and APX has been shown to increase, but is insufficient to remove additional $H_2O_2$ formed (Gunes et al. 2009). Decrease in the activity of SOD has been observed in various plant species on exposure to As, especially in maize where expression of SOD genes was initially increased at low levels and ultimately decrease on exposure to high levels of As (Mylona et al. 1998). Concentration of non-enzymatic antioxidants decreases in the presence of As (Hartley-Whitaker et al. 2001). In plants, overproduction of proline acts as a protective mechanism towards As stress (Bohnert et al. 1995).

## 7.7 Phosphorus Uptake Pathways in Plants

### *7.7.1 Direct Uptake Pathway*

The direct uptake pathway of orthophosphate in plants is prevalent in the region behind the root apex (Smith and Smith 2011). In this pathway, Pi is absorbed from the rhizosphere with the help of transporters located in epidermis and the root hair close to the surface of the root. This results in the development of a depletion zone in the rhizosphere (Fig. 7.1) due to faster uptake of Pi and its slow replacement by diffusion from the bulk soil (Gordon-Weeks et al. 2003). It is seen that in the epidermis and root hair cells, there is increase in the expression of genes implicated in encoding high affinity Pi transporters (PiTs) (Gordon-Weeks et al. 2003). This increased gene expression is not seen in case of mature regions of the root probably due to loss of root hair, depletion of Pi in the rhizosphere, and reduced activity of PiTs in the epidermis.

### 7.7.2 Indirect/Mycorrhizal Uptake Pathway

AMF on colonization induce the indirect/mycorrhizal uptake pathway in plants. This pathway overcomes the limitation of direct pathway, and helps in improving plant P nutrition by overcoming the effect of reduced Pi uptake due to the development of depletion zone (Smith and Read 2008). This pathway operates in the region behind the root apex, where direct pathway does not operate (Schnepf et al. 2011). AM fungi form a well-built hyphal network that extends beyond the depletion zone (Fig. 7.1) (Drew et al. 2003). There is deactivation of direct pathway in plants colonized by AMF, due to downregulation of plant PiTs in root epidermis and root hair cells or due to competition for P uptake between root and the hyphal cells in the depletion zone around the root (Schnepf et al. 2008). Plant Pi transporters induced in the presence of AMF has not been described fully. No evidence is available towards the preference of these transporters for Pi or As(V) (Smith et al. 2010a). It has been demonstrated that AM uptake pathway is the dominant route for P uptake even in those plants that do not grow well when colonized by AMF (Jakobsen 1999).

### 7.7.3 Adaptations in AMF for Increased Phosphorus Uptake Under As Toxicity

AMF absorb P even beyond the depletion zone of the rhizosphere, translocating it rapidly to the fungal structures within the roots, and delivering it to the plant root resulting in positive effects on plant growth and biomass (Smith and Read 1997). In AMF, inorganic absorbed P is stored in the form of soluble orthophosphate, polyphosphate granules (Chilvers and Harley 1980), or as soluble polyphosphate. The concentration of inorganic P inside the fungal hyphae has been found to be 1000 times greater than the concentration in soil solution (Gianinazzi-Pearson and Gianinazzi 1986).

Together with the use of 33/32P, contribution of AMF pathway in uptake and transfer of Pi and As(V) could be elucidated (Smith et al. 2010b). By using isotopically labeled P supplied to external mycelium, contribution of AMF in P uptake has been observed in *Hordeum vulgare* (Zhu et al. 2003), *Triticum aestivum*, and few of the AMF non-responsive plants like *Lycopersicon esculentum*. In *Medicago truncatula* (medic) and *Linum usitatissimum* (flax) that show high rate of colonization by the fungus, upto 100% of the P was taken up by AMF pathway (Glassop et al. 2005; Smith and Read 2008). In *H. vulgare*, there was decreased expression of epidermal Pi transporters (*HvPht 1;1* and *HvPht 1;2)* implicated in uptake of As(V) and Pi (Christophersen et al. 2009). In AM plants, increased expression of AM-induced transporter (*HvPht 1;8*) was seen, which is involved in increased Pi uptake by roots thereby activating the mycorrhizal uptake pathway; *HvPht 1;8* expression was unaffected by presence of As (Christophersen et al.

2009). AMF inoculated *M. truncatula* have shown high expression of AM inducible Pi transporter *MtPht 1:4*, leading to enhanced P uptake (Christophersen et al. 2012). Hyphal coils of AMF located intracellularly have been associated in P allocation from fungus to the plant, supported by localized expression of *SORtu; Pht1;3* related with coils formed by *Gigaspora margarita* in roots of potato (Karandashov and Bucher 2005; Glassop et al. 2005).

## 7.8 Effect of As Toxicity on AMF Colonization

It has been shown by various studies that plants inoculated with AMF show increased tolerance towards As toxicity, resulting in improved growth and development (Chen et al. 2007; Smith et al. 2010a; Andrade et al. 2015). Few studies on pure culture of AMF suggest that the magnitude of As(V) toxicity vary among fungal taxa (Spagnoletti and Lavado 2015). Effect of AMF on As remediation in plants depends on various isolates of AMF and species of As (Leyval et al. 1997; Orlowska et al. 2005). Under enhanced metal toxicity, growth of AMF can be entirely inhibited (Weissenhorn et al. 1995). It has been observed that in soils with high contamination of HMs such as As, there is decrease in spore number of AMF species, whereas in moderately As contaminated sites there is increase in diversity and species richness of AMF (Del Val et al. 1999). It has been ascertained by morphological spore identification that there is reduction of AMF species diversity in plants growing in As contaminated sites (Karimi et al. 2011). For instance, Andrade et al. (2015) observed decreased intraradical AMF colonization in the presence of As(III) and As(V) in rice plants. In *H. vulgare* inoculated with AMF and grown on metal spiked soil containing As, Cd, Ni, etc., the sporulation capacity of AMF was greatly affected (Biro et al. 2005).

With increase in As content, there is also increase in mycorrhizal colonization in some plants (Hildebrandt et al. 1999; Audet and Charest 2007). Despite having detrimental effects on growth of AMF in As contaminated sites, there have been instances where AMF is known to grow despite As presence. In *Tagetes erecta*, *Melastoma malabathricum,* and *Pityrogramma calomelanos* inoculated with AMF in the presence of As, there has been successful colonization by the fungus (Jankong and Visoottiviseth 2008). Also, there was increase in fungal structures such as vesicles and arbuscules present in these plants upon colonization. Irrespective of As presence, roots of *M. sativa* have shown extensive colonization by *Glomus mosseae* (Chen et al. 2007). In the presence of As (0, 25, 100 mg/kg) and P (25, 100 mg/kg), increase in hyphal length densities in plants inoculated with AMF have been observed (Chen et al. 2007).

AM fungal strains isolated from As contaminated sites have shown high tolerance towards As resulting in improved growth (Vivas et al. 2003; Bai et al. 2008). Indigenous AM fungi (*Glomus* spp, *Acaulospora* spp) have shown high tolerance towards As by resulting in high levels of AM colonization (Bai et al. 2008). For instance, mine AMF isolates of *Glomus* Spp have been shown to be As resistant as

compared to non-mine isolates (Gonzalez-Chavez et al. 2002). AMF isolate *Rhizophagus intraradices* Br1 growing indigenously on metal contaminates sites confers As tolerance on a variety of plant species in diverse environments and was more effective in transferring As tolerance to maize and tomato (Hildebrandt et al. 2007). Biodiversity of AMF in sites contaminated with As has been analyzed by sequencing the nuclear small subunit ribosomal RNA (SSU rRNA) gene fragments using 454-pyrosequencing (Sun et al. 2016). Of all the AMF genera found, *Glomus* was dominant in the mining area. Due to high sporulation rate, *Glomus* spp show better adaptation in sites contaminated with As and hence resulting in improved ability to recover from As toxicity (Whitfield et al. 2004; Daniell et al. 2001).

## 7.9 Mechanisms Underlying Alleviation of As Toxicity by AMF Colonization

### *7.9.1 Enhanced Phosphorus Uptake and Biomass*

As AMF assist plant uptake of P, it is essential to consider P–As interactions for ascertaining the role of AMF in As uptake in plants (Zhang et al. 2015). When inoculated with AMF, in the presence of As in soil, there is downregulation of P as well as As uptake by direct pathway, whereas there is enhanced expression of indirect pathway which shows selectivity towards P uptake in plants (Glassop et al. 2005), resulting in a higher P/As ratio (Fig. 7.2) (Adriano 2001; Ahmed et al. 2006; Chen et al. 2007; Ultra et al. 2007). Reduction in As translocation factor (TF) and high P/As ratio were seen in *Melastoma malabathricum*, preventing As translocation to the aerial plant parts (Jankong and Visoottiviseth 2008). Therefore, AMF alleviate As toxicity by improving P nutrition and reducing As aggregation to the shoots in plants (Chen et al. 2007). In *Leucaena leucocephala* inoculated with *G. clarum*, there occurs decrease in As translocation factor (<0.99), suggesting sequestration of As in roots of the plants (Xu et al. 2008; Schneider et al. 2013). It has been shown that by inoculation with *G. geosporum,* there is increase in yield of rice pants grown in As contaminated sites (Li et al. 2011b). This increase occurs due to enhanced P/As ratio and decreased grain/straw As content in AMF inoculated plants, resulting in translocation of As from grain to straw mediated by P transporters (Li et al. 2011b).

The enhanced biomass of plant due to AMF colonization accounts for the decreased internal As concentrations, resulting in "dilution effect" observed in mycorrhizal plants (Chen et al. 2007). AMF inoculation enhances P nutrition and plant biomass under As stress and a relative As dilution because P shares chemical properties with As. In *M. malabathricum* plants grown in arsenic (As) contaminated soils, inoculation with AMF resulted in increased surface area of the roots allowing better growth and development of the plant (Jankong and Visoottiviseth 2008). *M. truncatula* and *Allium porrum* when inoculated with a mixture of AMF species

accumulated more P and maintained greater plant growth and development (Jansa et al. 2008). By increasing the P uptake in plants, AMF cause dilution of As (Ahmed et al. 2006; Ultra et al. 2007). It is believed that due to the presence of efficient P uptake mechanism in AMF with high selectivity towards P as compared to that of As, there is increase in the uptake of P in plants (Smith et al. 2010b). In lentil (*L. culinaris*) plants inoculated with *G. mosseae*, and irrigated with 1, 2, 5, and 10 mg As(V) $L^{-1}$, decreased As concentration was found in the pods leading to diminishing As toxicity caused due to consumption of contamination food grains (Ahmed et al. 2006). It has been found that at high concentration of As in soil, maize plants treated with indigenously growing AMF (*Glomus* spp, *Acaulospora* spp.) from As contaminated sites exhibited higher biomass due to enhanced root P as that of non-mycorrhizal plants (Bai et al. 2008). Mycorrhiza growing indigenously has shown to sustain growth of *Pteris vittata* plants by aiding P absorption in As polluted soil (Leung et al. 2006, 2013). Biomass of *G. mosseae* was not influenced by high content of As in soil (200 mg/kg) (Xu et al. 2008). Instead due to high P nutrition resulting from AM, there was increase in shoot biomass and decrease in root As concentration (Xu et al. 2008).

### *7.9.2 Biotransformation of As*

Inoculation with AMF influences speciation and transformation of As in host plant (Zhang et al. 2015).Transformation of As(V) into less toxic organic forms is also one of the strategies employed by AMF to ameliorate As toxicity (Gonzalez-Chavez et al. 2002; Ultra et al. 2007; Jia et al. 2012). Plants do not have the ability to methylate As species; therefore, methylation of As species is carried out by soil microorganisms (Lomax et al. 2012). AMF by releasing substrates in the rhizosphere activate microorganisms that assist in bio-methylation of As in plants (Mukhopadhyay et al. 2002). Presence of methylated As forms has been observed in rhizosphere of *Helianthus annuus* on inoculation with *Glomus aggregatum* (Ultra et al. 2007). Elevated content of DMA in rice grains on inoculation with *R. intraradices* has been reported (Li et al. 2016). Gonzalez-Chavez Mdel et al. (2011) found that high affinity phosphate transporters present on the extraradical mycelium of AMF such as GiPT in *R. intraradices* are responsible for the uptake of As(V) into the fungal mycelium where it gets transformed to As(III) by arsenate reductases. In order to prevent the intracellular toxicity caused by As(III) in the fungal hyphae, a membrane bound As(III) efflux pump is activated (GiArsA/B) (for *Glomus intraradices)* (Liu et al. 2003). This As(III) efflux pump pumps out As(III) in the surrounding medium. Analogous mechanisms of As(V) reduction and efflux of As(III) in the surrounding have been reported in tomato and rice (Xu et al. 2007). GvArsA (for *Glomus versiformis*) and GiArsA have been shown to have higher protein sequence similarity with each other. In GvArsA, there is presence of conserved domains such as ATP binding site, metal binding site, and dimerization interface similar to that seen in ArsA ATPases (Ye et al. 2010; Yang et al. 2011 ). In

bacteria on the inner membrane surface is present ArsA ATPase that functions as efflux pumps (Shen et al. 2003). ArsA ATPases are implicated in transport of As (III) and Sb (III) (Antimony) across membranes in bacteria (Ye et al. 2010). In *Caenorhabditis elegans*, a functional ArsA ATPase, ASNA1, shows a high degree of homology with GvArsA (Tseng et al. 2007). These observations support the concept that GvArsA is involved as one of the components of *G. versiformis* efflux As(III) pump (Gonzalez-Chavez Mdel et al. 2011, 2014). Localization of GiArsA protein is seen in plasma membrane similar to that of bacterial ArsAB ATPase, suggesting that As (III) might be effluxed to periarbuscular space (Gonzalez-Chavez Mdel et al. 2011).

### *7.9.3 Reduced As Uptake*

Decreased uptake of As from soil is one of the strategies employed by AMF to combat As toxicity. Gonzalez-Chavez et al. (2002) suggested that regardless of the plant host genotype for As(V) tolerance, all the AMF strains confer additional As tolerance to the plants. They affirmed that As(V) influx was reduced in plant roots by the suppression of high-affinity As(V)/Pi transporters. It has been found that in *Holcus lanatus* plants inoculated with AMF, there was reduction in As(V) influx due to suppression of As(V)/Pi transporters in plant roots, therefore decreasing As (V) uptake (Gonzalez-Chavez et al. 2002). Mycorrhizal inoculation remarkably inhibited As(V) uptake in rice in short-term affinity uptake system (Li et al. 2011b). Uptake of As(III) and MMA was also lowered in both high and low-affinity transport system in plants inoculated with AMF (Li et al. 2011a). The mechanism of As uptake and localization of As in AMF is not yet known. By employing radioactive 73 As(V), the fungal potential for transport and uptake of As could be known (Smith et al. 2010a).

Zhang et al. (2014) examined the influence of AMF inoculation on accumulation of As and its speciation in *M. truncatula*. They reported that while specific Pi uptake was augmented by AMF colonization, As uptake was decreased. AMF mediated decreased influx of As species is due to reduced expression of phosphate transporters such as *OsPHT2* and silicon transporters, *Lsi1* and *Lsi2*, thus resulting in reduced uptake of As(V) and As(III), respectively (Ma et al. 2008). Lsi1 is a member of nodulin 26-like intrinsic membrane proteins (NIPs). They help in the transport of silicon (Si) from external medium into rice roots (Ma et al. 2008). They also help in the transfer of As(III) from soil into the rice roots (Li et al. 2009). Silicic acid and As(III) compete for transportation in the rice roots. AMF may suppress the expression of *Lsi1* to limit the uptake of As (Chen et al. 2015).

### *7.9.4 Sequestration of As*

Following the uptake of metal from the soil, AMF sequester it in various fungal structures such as vesicles and intra- and extraradical hyphae (Christie et al. 2004; Wang et al. 2007). In order to prevent the entry of metal in the cytoplasm, As is stored in the vesicular structures of AMF (Gohre and Paszkowski 2006). It could also be the case wherein the toxic compound is changed into less toxic forms and transported to plants or effluxed into the surrounding medium (Meharg 2003). For instance, conversion of As(V) to As(III). Also metal chelators such as amino acids, phytochelatins, and metallothioneins may have a role in transport and storage of toxic metal ions (Meharg 2003). AMF due to the presence of chitin in their cell walls have the ability to bind and immobilize As (Gaur and Adholeya 2004). The fungal cell walls also possess various free hydroxyl, amine, and imidazole carboxyl groups which offer active sites for binding As and prevent their movement in plants (Joiner et al. 2000). Due to intracellular precipitation of metallic cations with phosphate, there is decreased translocation of metals to the plants (Turnau et al. 1993).

#### 7.9.4.1 Production of Glomalin and Organic Acids

Glomalin is a glycoprotein produced by AMF that trigger soil formation (Rillig and Mummey 2006). During enhanced fungal growth, glomalin accumulates outside extraradical mycelium (Wright and Upadhyaya 1996). By forming complexes with various heavy metals including As, production of glomalin reduces As uptake in plants (Gaur and Adholeya 2004). This glycoprotein is also rich in iron; therefore, it may be involved in detoxification by forming As(III)-Fe(III) oxide compounds (Chen et al. 2005). Iron oxides when present have greater affinity for As(V) and As(III) (Meng et al. 2002). Role of glomalin in As tolerance has been known in various wetland plants (Meng et al. 2002).

AMF by producing organic acids help to mobilize elements essential for plant growth and development. Organic acids such as oxalic, malic, and citric acids are produced by the fungus (Jones 1998). For instance, in pine AMF colonize roots and result in greater production of oxalic acid as compared to that of non-colonized roots (Meharg 2003). Organic acids released by mycorrhiza have the ability to act as methyl donors resulting in bio-methylation and production of substrates that enhance microbial activity (Mukhopadhyay et al. 2002). These organic acids help to mobilize P ions from insoluble and complexed iron and aluminium phosphates in the rhizosphere (Ahonen-Jonnarth et al. 2000). Whereas, toxic metals present in the soil are immobilized by precipitation with organic acids (Meharg 2003). However, cycling and ability of organic acids to immobilize metals is influenced by their affinity for a particular metal (Meharg 2003).

#### 7.9.4.2 Change in Soil pH

Effect of soil pH on community composition of AMF is significant (Meharg 2003; Xiang et al. 2014). Soil pH of mycorrhizosphere influences spore density of the AMF species (Tchabi et al. 2008; Sun et al. 2016). Extent of AMF colonization and the community composition is influenced by hyphal growth, formation, and spore germination (Robson and Abbott 1989; Coughlan et al. 2000) which in turn is controlled by the pH of the soil. Some genera of AMF prefer acidic soils, while some others are present in a broader range of pH (Maia and Trufem 1990). For instance, AMF genus *Glomus* prefers neutral or alkaline pH (Schenck and Siqueira 1987) whereas *Gigaspora*, *Entrophospora*, and *Sclerocystis* prefer acidic soils (da Silva et al. 2005). At low pH, build up of positive charge occurs that repel cations resulting in metal cation insensitivity (Green et al. 1976). Inoculation with mycorhizza results in reduction of pH in the vicinity of rhizosphere due to selective uptake of nutrients (Smith and Read 1997; Sun et al. 2016).

#### 7.9.4.3 Higher Production of Phytochelatins and Metallothioneins

Arsenic chelation in the cytosol by high affinity ligands is potentially a very important mechanism of As detoxification and tolerance in plants under As stress. Two types of peptide metal binding ligands are synthesized by plants: phytochelatins (PCs) and metallothioneins (MTs). Phytochelatins are a family of cysteine-rich polypeptides, which play important role in detoxification of many HMs. Phytochelatin synthase (PCS) catalyzes the formation of phytochelatins from glutathione. Glutathione is known to play a central role in antioxidant defense system by upregulating cysteine synthase (CS), GST, and GR. Arsenate [As(V)] can be readily reduced to arsenite [As(III)] via arsenate reductase (ACR) enzyme (Mukhopadhyay et al. 2000; Smith et al. 2010a), which then subsequently complexed with thiols, particularly PCs. It has been proposed that PC-As complexes can be sequestered into the vacuoles by yef1p (ABC-type transporter) transporters and confers As(III) resistance in yeast (Ghosh et al. 1999; Zhu and Rosen 2009). Christophersen et al. (2012) confirmed that both *R. intraradices* and *Funneliformis mosseae* inoculation resulted in significantly higher expressions of *MtPCS* (*M. truncatula*) compared with non-mycorrhizal plants under As stress. They also reported increased expression of arsenate reductase (*MtACR*) gene that codes proteins thought to be involved in arsenate detoxification. Cicatelli et al. (2010) showed higher MTs gene expression in AMF inoculated plants of *Populus alba* clone AL35, suggesting that these polypeptides may provide protection from HM-induced toxicity. Rivera-Becerril et al. (2005) also reported similar results in *Pisum sativum*. Metallothioneins probably exert an antioxidant function (Akashi et al. 2004). Although role and production of PCs and MTs have been studied in many metals, not much is known about the effect of these protein ligands in response to AMF inoculation under As stress.

## 7.10 Amelioration of As Toxicity on Plant Physiology

The process of photosynthesis is stimulated due to improved nutrition in mycorrhizal plants (Dong et al. 2008). Total chlorophyll content was increased in *Solanum melongena* plants on inoculation with *G. mosseae* (Aziz et al. 2011). In maize leaves, AMF resulted in enhanced photochemical, non-photochemical efficiencies, and gas exchange, resulting in increased photosynthesis of plants (Sheng et al. 2008). Plants inoculated with AMF show increased nitrogen content as compared to that of non-colonized plants (Andrade et al. 2015). Colonization by AMF induces calvin–melvin cycle in plants (Sheng et al. 2008). This is done so as to increase the transport of triose phosphates to the roots, in order to reduce its limitation on photosynthesis resulting in increased carbon dioxide fixation in leaves (Kaschuk et al. 2009).

Comparative analysis of proteins induced in the presence of As contamination and mycorrhizal inoculation in *P. vittata* plants have shown upregulation of glycolytic enzymes (Bona et al. 2011). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) catalyzes the formation of 1,3-bisphosphoglycerate from glyceraldehyde-3-phosphate and phosphate in plants. GADPH can use As(V) instead of phosphate converting glyceraldehyde 3-phosphate into 1-arseno-3-phosphoglycerate (Gregus et al. 2009). One isoform of GADPH has been shown to increase in the presence of AMF (Bona et al. 2010). All enzymes that catalyze phosphorolytic–arsenolytic processes readily convert arsenylated metabolites to As(III)in plants (Bona et al. 2011). In plant roots colonized by *G. mosseae*, there is increase in phenylalanine-tRNA ligase or phenylalanyl-tRNA synthetase indicating the induction of protein synthesis in the presence of As (Zhou et al. 2010).

## 7.11 Protection from Oxidative Damage

In plants inoculated with AMF, increase in the concentration of glutathione (GSH) has been observed, suggesting glutathionylation as an approach for As detoxification in plants (Bona et al. 2011). High GSH pool in plants increases the tolerance of plants towards As toxicity. High GSH level was found in fronds of *P. vittata* where it could participate in the reduction of As(V) to As(III) in vitro (Singh et al. 2006). Increase in GSH levels have been reported on inoculation of plants with AMF (Garg and Kaur 2013). In AMF inoculated plants, increase in gene expression of GR has been observed that provides protection from antioxidants by recycling glutathione from oxidized to reduced form (Fuentes et al. 2016). In order to sustain increased ratio of GSH/GSSG, production of GSH is essential as it is involved in the synthesis of PCs and other enzymes of ROS scavenging pathway (Garg and Kaur 2013). Garg and Singla (2012) reported low levels of $H_2O_2$ and MDA in pea plants inoculated with AMF and grown on 30, 60, and 90 mg/kg As contaminated soil. Lipid peroxidation in plants can be ascertained by estimating the amount of thiobarbituric

acid (TBA) formed (Gonzalez-Chavez et al. 2002). On inoculation of soybean plants with *R. intraradices* at a dose of 25 or 50 mg/kg As, there was observed decrease in the content of TBA in both leaves and roots as compared to non-inoculated soybean plants (Spagnoletti et al. 2016). This decrease in lipid peroxidation on inoculation with AMF is attributed to reduced production of ROS resulting in reduced oxidative damage in plants (Garg and Kaur 2013; Fuentes et al. 2016).

Mycorrhizal plants in the presence of As show increase in the production of SOD, CAT, and APX (Wu et al. 2010; Garg and Singla 2012). Increase in concentration of ascorbate, glutathione, non-protein thiols, and cysteine has also been observed in plants treated with As (Mishra et al. 2008). Reduction in ROS production and lipid peroxidation occurs due to inoculation with AMF (Rahmaty and Khara 2008). In AMF colonized plants, high level of peroxidase (POD) is indicative of lower ROS production as compared to non-colonized plants (Santana et al. 2015). There is increase in the production of proline, glycine betaine, total proteins, and soluble sugars in pea (*P. sativum*) plants inoculated with *G. mosseae* when grown under As(V) stress (Garg and Singla 2012). Buildup of proline rich proteins provides protection against As stress (Matysik et al. 2002). Proline reduces As toxicity in plants by chelation of this HM in the cytoplasm (Schat et al. 1997), reducing hydroxyl radical production (Smirnoff and Cumbes 1989) and decreasing uptake of metal.

## 7.12 Use of Hyperaccumulators in Conjunction with AMF

Several species of fern from the genus *Pteris* are able to accumulate extremely high concentrations of arsenic (As) in the fronds. *P. vittata, P. cretica*, and *P. biaurita* are the well-known hyperaccumulators of As and are able to accumulate huge quantities of As from the soil (Ma et al. 2001). These hyperaccumulators show increased tolerance when grown in As contaminated soils in the presence of mycorrhiza (Leung et al. 2006). When supplied with AMF in the presence of As, *P. vittata* exhibited enhanced frond surface area and improved leaf area (Trotta et al. 2006). These parameters contribute to increase in As accumulating capacity of the fern as *P. vittata* accumulates As in the pinnae epidermis. Higher As translocation factor leading to high As storing ability was seen in the fern.

In the frond epidermal cells, majority of As is sequestered in un-complexed form in the vacuole. When As uptake exceeds the vacuolar sequestration capacity, PCs help in detoxification process in the fern (Zhao et al. 2003; Trotta et al. 2006). It has been found that under As stress, glutathione concentration is increased in fern roots and fronds which is a substrate for phytochelatin synthesis (Bona et al. 2011). Proteomic study of *P. vittata* suggested that As modulates the levels of numerous proteins related to glycolysis (Bona et al. 2011). Another study revealed that under As stress, a member of ABC transporter family, PDR-like protein is increased in *P. vittata* and helps in the detoxification of As (Shen et al. 2014). Mycorrhiza also

improves P nutrition lowering As toxicity by favoring the Pi uptake via AM pathway. Higher activity of arsenate reductase has been observed in *P. vittata* inoculated with mycorrhiza (Leung et al. 2013). In *P. vittata* inoculated with *G. mosseae* and *Gigaspora margarita*, there was upregulation of PgPOR29, porins that facilitate passive transport of small sized molecules in the presence of As (Bona et al. 2010). PgPOR29 may be involved in conferring As resistance in fern by either increasing As uptake or by causing As efflux and sequestration in the vacuoles of the frond (Bona et al. 2010).

## 7.13 Conclusion

Biological methods including the use of soil microbes and plants are amongst the most suitable methods, environmentally and economically. Use of AMF in alleviation of As stress is a reliable and efficient approach. Inoculation with AMF has shown to increase the yield of plants without increasing the concentration of As. AM fungi are known to absorb slight amounts of As as well. The absorbed As is retained in the fungal compartment preventing its translocation to roots and subsequently to the shoots. Ability of AMF to take up As strongly depends on the fungal isolate being used. It has been validated by various studies that best adapted fungal isolates for alleviating toxicity of As contaminated soils are the ones indigenously growing in the polluted soils. Therefore, for desired outcomes, AMF strains should be carefully selected, especially when being used in agriculture or in remediation of contaminated lands. It is also known that a variety of plants have the inbuilt mechanisms and the genetic potential to clean sites contaminated with As. Molecular understanding of As hyperaccumulators when used in conjunction with AMF is limited. Therefore, identification of As hyperaccumulator plants and their usage in conjunction with AMF can prove to be a beneficial technology. Understanding the physical, chemical, and biological mechanistic basis of the tripartite interaction between As, AMF, and plants will enable us to strategize efficiently phytoremediation of As contaminated land and rescuing of crop plants.

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# Chapter 8
# Co-cultivation of *Piriformospora indica* with *Azotobacter* sp.

**Prasun Bandyopadhyay and Ajit Varma**

**Abstract** The ever growing human population and depletion of resources have enforced progress in sustainable agriculture. Plant–rhizosphere microbe association has been known for some time now. To uphold sustainability, one would need to show better reliance upon the beneficial traits possessed by the root microbiome. To harness these traits, one would need to understand the process of recruitment and maintenance of microbiota as stable interactome. In this chapter, we highlight the process of recruitment and establishment of microbiota within rhizosphere. Further, we discuss the molecular basis of interspecies synergistic interaction where we have taken *Piriformospora indica* and *Azotobacter chroococcum* as model interacting partners. Lastly, we laid emphasis on the possibility of exploring the knowledge gained from such synergistic interaction to tailor the rhizosphere microbiome for better productivity and maintenance of agroecosystem. This chapter provides new insights into the broad principles of stable plant–microbe interactions which could be useful for sustaining agriculture and food security.

## 8.1 Introduction

Since the time humans have emerged, population has increased at a slow and steady pace. However, the past 100 years have witnessed tremendous increase than ever before, and it is expected to reach 9.6 billion by the end of 2050. If the population keeps on increasing in such an alarming rate, it may challenge the food security. As majority of the population depends on the agricultural sector, improving the quality and productivity will be advantageous. Therefore, considering the importance of the agricultural sector, it has become vital to ensure that genetic diversity is maintained for higher productivity and further avoidance of vulnerability to abiotic and biotic stress. Widespread research in plant–microbe association has highlighted the importance of microorganisms in determining the plant fitness (Gill et al. 2016).

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Thus, understanding the genesis of plant–microbe beneficial interaction will be advantageous in context to improve productivity.

Plants are usually found to be densely inhabited by diverse range of microorganisms both above and below the ground drawing mutual benefits. Depending on the niches of the plant colonized by microorganisms, they may be designated as epiphyte (present on the surface), endophyte (found inside the tissue), phyllospheric (growing on leaf surface), and rhizospheric (inhabiting soil closely associated with roots). Out of these diverse niches, rhizosphere is the most dynamic owing to its massive influence on plant nutrition and growth (Kowalchuk et al. 2010; Lakshmanan et al. 2014, Berendsen et al. 2012; Bakker et al. 2013; Mendes et al. 2013; Prasad et al. 2015). Given the enormous species diversity, staggering number of interactions, and complex community structure within the rhizosphere, understanding the biology of the root system and its microbiota as an interactome is still at its infancy. Plant hosts and their microbiota are often intertwined and are thought to have coevolved and function as meta-organism or holobiont having inseparable ecology (Bosch and McFall-Ngai 2011; Vandenkoornhuyse et al. 2015). To appreciate such a holobiont system, one needs to understand the interdependence of microbiota at various strata of their growth and development and may even view a plant's biology as the additive functions of the surrounding microbiota. With the advent of genomics and proteomics, recent scientific literature has explored the mechanisms coordinating the formation of plant–microbe mutualistic associations having the potential to improve plant productivity (Mabood et al. 2008; Pieterse et al. 2009; Berendsen et al. 2012; Bakker et al. 2013). However, the basis of microbe–microbe interaction is still at its early stage. So, if the factors that contribute to the formation of stable rhizosphere community are deciphered, they can be harnessed for sustainable agriculture to address the ever-increasing demand of food.

This chapter provides an outline of the root microbiome dynamics with an emphasis on the recruitment of the microbiota in the rhizosphere, considering bulk soil as a microbial sink. Further, we also address the formation of stable interspecies interactomes. Here, we have discussed upon molecular basis of trophic interaction between *Piriformospora indica* and *Azotobacter chroococcum*. Finally, we laid emphasis on the possibility of exploring the knowledge gained from such trophic interaction to tailor the rhizosphere microbiome for better productivity and maintenance of agroecosystem.

## 8.2 Rhizosphere as Microbial Incubator: Recruiting Microorganisms to Root Niche

Recruitment of root microbiota has gained much attention as it influences plant health and productivity. Ever since the origin of first terrestrial plants, a new set of biological factors have been introduced for the soil microorganism to deal along

with (Selosse and Strullu-Derrien 2015) the physiochemical factors. To understand the genesis behind the recruitment of microorganisms in the rhizosphere, one has to decipher their species and functional diversity. Though considerable progress has been made, it remains a huge challenge as currently only 1% of the entire soil dwelling microorganisms are cultivable (Walsh and Duffy 2013). With the development of omics and culture-independent techniques, scientists have been able to gain insight into rhizosphere microbiota. High throughput techniques have yielded estimates of up to $10^{11}$ microbial cells per gram of root which include at least 30,000 prokaryotes. These estimates may vary depending upon the plant species, genotype, and age. We begin this section by summarizing the role of root exudates as potential drivers, attracting microorganisms to the desired rhizosphere niche from the bulk soil.

Lorenz Hiltner coined the term "rhizosphere" to describe influence of root exudates on the soil microorganisms proliferating around and inside the roots (Hirsch and Mauchline 2012). Ever since rhizosphere has been defined, much has been learned on the effect of soil microorganisms on the plant host. In natural ecosystem, plants have been the driving force for assembling the rhizosphere microbiota constituting the diverse functional gene pool, including bacteria, fungi, and nematodes associated with various habitats like rhizosphere, rhizoplane, and endosphere. Plants tend to release 10–20% of their photosynthates as exudates including low molecular weight metabolites like sugar, organic acids, amino acids, and the dead border cells as mucilage (Dennis et al. 2010; Kaiser et al. 2015). These exudates alter the chemical and physical properties of the soil and cater the niche for microbial proliferation (Bais et al. 2006). Studies conducted on potato, sugarcane, and certain model plants like *Arabidopsis* and Barley have suggested genotype-dependent variation in the microbial community within rhizosphere. Hence, higher microbial count is obtained in rhizosphere in comparison to the bulk soil (Bulgarelli et al. 2012, 2015). These findings suggest a plant system can orchestrate its rhizobiota to optimize its own benefits and improve its fitness. Depending on the type of exudates, they may attract or repel certain groups of microbiota. Badri et al. (2013) demonstrated that exudates, particularly phenolics, can either stimulate or repel certain group of microorganisms highlighting the potential of the exudates in modulation of the microbial communities in the rhizosphere.

The association of microbes with plants may vary from obligate (endo) symbionts to transitory associates. Though ancestrally, prokaryotes and lower eukaryotes persisted as free-living microorgansims, the past millions of years have witnessed a transformation in their ability to socialize and create a niche where they can coordinate interactions. The diverse microbe–microbe and plant–microbe associations within the rhizosphere have received growing attention with regard to abundance, diversity, and complexity and hence considered as plant's secondary genome. They are often witnessed having major impacts on plant growth (Broeckling et al. 2008; Bulgarelli et al. 2012).

## 8.3 Formation of Stable Microbial Interaction May Influence Plant Growth and Fitness

Stable cooperation among microbial partners are comparatively unexplored as their mutual responses are difficult to observe. Once functionally diverse microorganisms have been assembled in the rhizosphere, it is important to identify the factors involved in the formation of stable yet dynamic communities. Keeping this in mind, here we have taken *Piriformospora indica* and *Azotobacter chroococcum* as model systems to study the molecular basis of stable interaction.

The mutualistic associations established by mycorrhizal fungi with plant and bacterial cells can range from seemingly disordered polymicrobial communities to highly specific symbiotic associations. Such association has been vastly seen in agricultural as well as forest ecosystems. Many bacteria and fungi either in combination or in isolation have been shown to produce beneficial effects on plants. Interaction between *Pseudomonas putida* and *Glomus* sp. has been shown to promote plant growth by enhancing phosphate solubilization (Villegas and Fortin 2002, 2011). Certain signaling metabolites of *Streptomyces* sp. AcH505 have also been shown to stimulate the hyphal growth of *Amanita muscaria*. Similarly, volatile substances produced by some bark beetle-associated bacteria stimulate the growth of their symbiotic fungi. Few PGPRs like *P. putida* IsoF promoted the growth of *P. indica* whereas *Pseudomonas fluorescens* WS5 and *Gluconacetobacter* sp. Comb19 inhibited fungal growth (Varma et al. 2012). Though this kind of interactions is known to occur in the rhizospheric region, the exact nature of molecular interaction is yet to be elucidated.

To address this, we conducted interaction between *P. indica* and strains of *Azotobacter chroococcum* in axenic culture. Initial in vitro screening revealed different patterns of the growth modulating interactions of strains of *A. chroococcum* with *P. indica*. We identified two strains—WR5 and M4 which have the tendency to modulate the fungal growth. WR5 has maximal growth promoting effect, and M4 strain has maximal growth inhibiting effect as seen in plate assay, dry cell weight content, and spore yield (Fig. 8.1). Electron microscopic (SEM and TEM) observation of *P. indica* did elucidate marked differences in the surface morphology and internal compartmentalization of cytoplasm and membranous organelles in interaction with WR5 and M4. Presence of healthy, thick hyphae in interaction indicates that WR5 supports fungal growth. Contrasting observations have been made in the presence of M4, where the hyphal architecture has been highly deformed. The cytoplasm has been disorganized in comparison to control. This suggests that in presence of M4, the fungus is metabolically less active (Figs. 8.2 and 8.3).

Further, to explore the mechanism of growth modulation of *P. indica* by both the strains, we demonstrated that WR5 and M4 lead to a specific modulation of protein expression in *P. indica*. In particular, *P. indica* cocultured with WR5 showed an increase in the level of expression of some major metabolic proteins. The latter were downregulated in the presence of M4. Based on a comparative analysis of

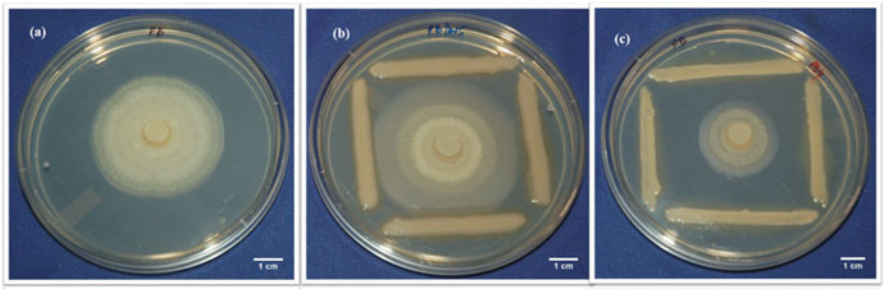


**Fig. 8.1** Visualization of the *A. chroococcum*—*P. indica* interaction in Hill and Kaefer agar plates in the presence and absence of *A. chroococcum* strains. (**a**) Control plate. (**b**) Interaction of *P. indica* with WR5. (**c**) Interaction of *P. indica* with M4

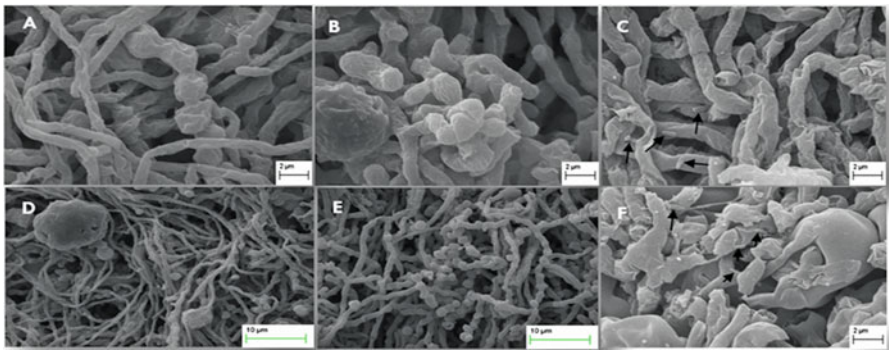


**Fig. 8.2** Scanning electron micrographs of *P. indica* morphology in isolation and coculture with *A. chroococcum* (WR5 and M4) specific elicit fungal growth at 28 ± 1 °C. (**A** and **D**) Control fungal mycelia appear to have normal hyphae, septa, and conidia. Hyphae showed uniform tubular shape in all parts. (**B** and **E**) Micrographs show a tendency of hyphal growth promotion induced by WR5. The main improvements are healthy fungal hyphae and more conidiation. (**C** and **F**) Hyphal growth affected by M4 showing damaged fungal hyphae with surface adhered rod-shaped bacteria (*arrow* in Fig. 8.2c) and lack of conidiation

major differentially expressed proteins, we present a hypothetical model in Fig. 8.4 suggesting the possible role of WR5 in stimulating the growth of *P. indica*. Upregulation of both ENO1 and Ure D in the presence of WR5 suggests that WR5 could trigger efficient uptake of hexose sugar by the activation of several glucose transporters. ENO1 is one of the key regulatory enzymes of glycolytic pathway for generating reducing power for ATP synthesis. Ure D is one of the accessory proteins of the apoprotein UreABC which is a nickel-dependent regulatory enzyme involved in recycling of urea. Ammonia generated by urease reaction is used as a source of nitrogen by the plant for its growth. This carbon supply generates the metabolic energy *via* glucose metabolism thus prompting the growth of *P. indica*. This has been well supported by increase in number of mitochondria as seen in cocultures with WR5. As the model reflects, the energy generated and the

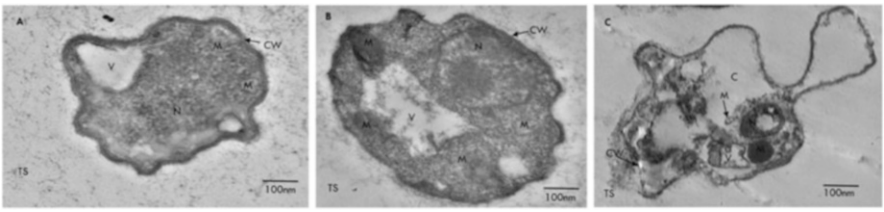


**Fig. 8.3** Transmission electron micrographs of *P. indica* in isolation and in coculture with *A. chroococcum* (WR5 and M4) grown at 28 ± 1°C. (**A**) Transverse section of control hypha showing cell wall (CW) mitochondria (M), vesicles (V), and nucleus (N). (**B**) Transverse section of hypha from coculture with WR5 well-organized hyphal cytoplasm, organelles, and number of mitochondria. (**C**) Transverse section of hypha treated with M4 showing disorganization of hypha and cytoplasmic organelles and formation of membrane-bound vesicles

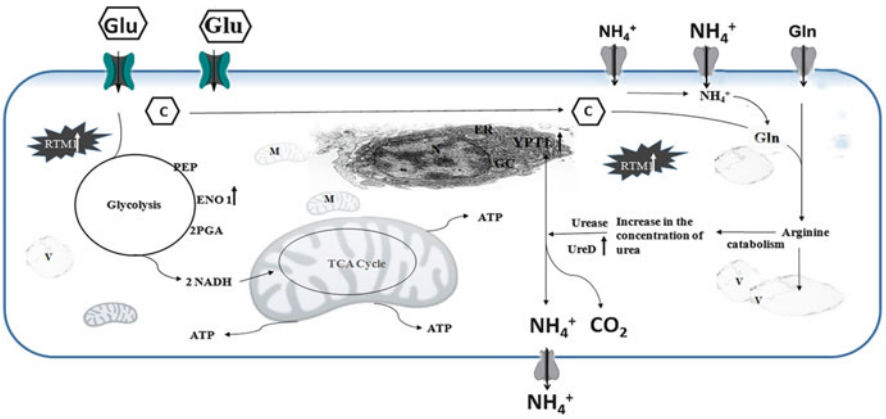


**Fig. 8.4** A hypothetical model showing changes in the carbon and nitrogen flux of *P. indica* in response to its interaction with WR5. Presence of WR5 enhances carbon pool in the mycelium triggering inorganic/organic nitrogen influx. This nitrogen is assimilated via glutamine synthetase/glutamate synthase cycle into arginine. Arginine is fed in the urea cycle; urea is further broken down to ammonia and carbon dioxide which is released. Stimulation in the expression of ENO1 and Ure D during carbon and in-/organic nitrogen influx has been indicated by up-arrows

carbon source may be further utilized in the active uptake processes of inorganic and organic nitrogen source by the mycelium further assimilating it into amino acid—Arginine, which is loaded into the vacuoles and transported along the hypha. As a regulatory process, arginine is loaded into the anabolic arm of the urea cycle in order to be degraded, leading to an increased concentration of urea. In the presence of active urease, urea can be converted into ammonia and carbon dioxide.

In conjunction with the activity of ENO1 and Ure D, YPT-1 and RTM1 proteins have unique importance in the growth of the fungus. YPT-1 does play a functional role in sporulation and the organization of the cytoskeleton during the vegetative state as it has been well documented in *S. cerevisiae*. In addition, it has also been identified as global GTP-binding protein associated with trafficking of secretory

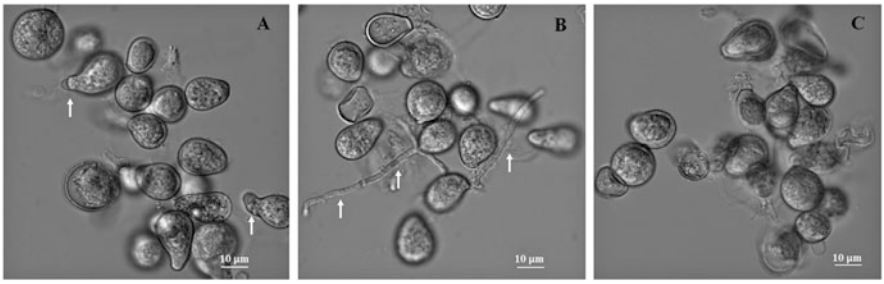


**Fig. 8.5** Chlamydospore germination pattern of *P. indica* after 12 h growth in Hill and Kaefer minimal medium in the absence (**a**) and presence of WR5 (**b**) and M4 (**c**) cell-free supernatant. Chlamydospores, grown in axenic culture, are at the initial stage of germ tube formation, whereas in the presence of WR5 cell-free supernatant, clear and distinct germinating tube is observed. In the presence M4 cell-free supernatant, growth is suppressed prior to germination. Images were taken at 400-fold magnification

vesicles between endoplasmic reticulum and Golgi complex. *RTM1* is a membrane-bound protein known to provide immunity and resistance to the fungus from the environmental toxic compounds. Similar pattern of response was observed in interaction of *L. bicolor* with soil bacteria though different sets of genes/proteins were found to be involved in the interaction (Deveau et al. 2015).

The specific growth response of the fungus could be caused by specific bacterial metabolites released into the environment during co-cultivation. To test whether diffusible low molecular weight active signaling molecules elicit the fungal growth response, we performed fungus growth assessment with 20-fold concentrates of culture supernatants. The results obtained from these experiments suggest that the cell-free culture supernatant might contain active metabolites for specific growth response of the fungus. The metabolites produced by WR5 contributed to early and better spore germination of *P. indica* with much elongated germ tube rising from the spores whereas in presence of M4 the spores did not show any visible mark of spore germination. In fact the spores were round suggesting that metabolites released from M4 suppressed the spore germination of *P. indica* and hence the overall growth (Fig. 8.5) (Bandyopadhyay et al. 2016a). This is in good agreement with our previous study, where WR5 was shown to improve fungal growth in terms of biomass and sporulation (Bhuyan et al. 2015). This also matches with a study conducted by Lumini et al. (2007), where endobacteria harbored by the arbuscular mycorrhizal fungus *Gigaspora margarita* were found to play a pivotal role in spore germination during the pre-symbiotic stage of infection. This observation reflects the potential strategy employed by free-living bacteria to attract mutualistic fungi and to repel competing bacteria from the same niche by manipulating their physiology with secreted factors.

## 8.4 Secreted Factors Involved in *P. indica–A. chroococum* Perception

For the identification of secretory factors involved in mutual perception, fungi and bacteria were cocultured, and proteins in the supernatant were extracted and analyzed by SDS-gel electrophoresis. Gel analysis of secreted proteins resolved numerous bands, ten protein bands of which were identified as differentially expressed. They are annotated as band A to J with molecular masses of approximately 37 kDa (A), 37 kDa (B), 40 kDa (C), 40 kDa (D), 73 kDa (E), 70 kDa (F), 73 kDa (G), 70 kDa (H), 73 kDa (I), and 70 kDa (J) (Figs. 8.2 and 8.3). Two distinct bands, A and B, were found to be upregulated by WR5 supernatant alone as well as in coculture with *P. indica*. Interestingly, two bands (E and F) of control *P. indica* cultures were found to be downregulated in coculture with WR5 (I and J). A similar result was obtained with M4 supernatants for bands G and H. Bands C and D (40 kDa) show ample expression with both M4-fungal coculture and M4 alone (Fig. 8.6).

To identify and elucidate the role of these secreted proteins involved in the *P. indica* growth modulation, they were subjected to LC-ESI-MS/MS analysis. For identification, proteins having individual ion score values >100 were selected. Band A of WR5 was found to be a flagellar domain protein. Band B was identified as glutamate dehydrogenase belonging to *P. indica*. Bands C and D were identified as probable flagellar biosynthesis protein FliC from *A. chroococum* M4 with a score of 368 and the mass 40.05 kDa and flagellin with a score of 314 and the mass 40.42 kDa. Proteins E and F correspond to α-glucosidase-b like secreted protein of *P. indica* with a score of 163 and the mass 98.83 kDa.

WR5 was shown to stimulate fungal growth, similar to observations reported earlier (Bhuyan et al. 2015). In this communication, we propose that the flagellar domain protein of WR5 induces the expression of fungal glutamate dehydrogenase. Glutamate dehydrogenase is usually involved in assimilation of inorganic nitrogen,

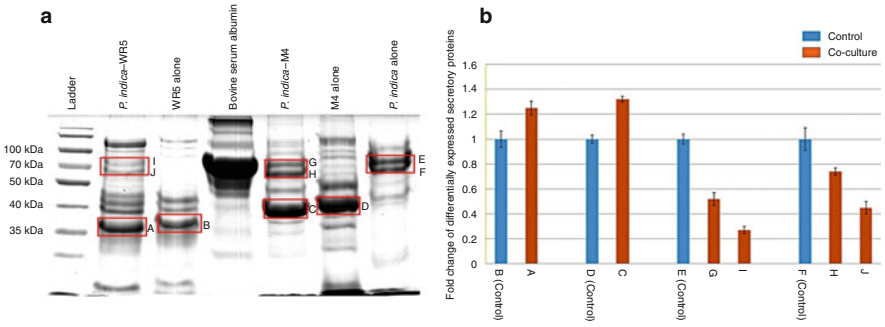


**Fig. 8.6** SDS-PAGE analysis of secreted proteins of axenically grown *P. indica* or in coculture with WR5 and M4 (**a**). Changes in differentially expressed secretory proteins of *P. indica* and *A. chroococcum* grown axenically or in coculture (**b**). Data were obtained by integrated densitometry values of the bands analyzed. Notations correspond to those in **a**

synthesizing glutamate from ammonium and α-ketoglutarate. Being a reversible enzyme, it can also degrade glutamate, yielding ammonium and α-ketoglutarate depending upon the concentration of ammonium. However, ammonium ions are toxic to the cell. Glutamate dehydrogenase converts it into glutamate, feeding the urea cycle for assimilating ammonium ion in the organic form (Bandyopadhyay et al. 2016a). This matches with our previous study, where glucose was shown to trigger nitrogen uptake in *P. indica* in the presence of WR5. Remarkably, α-glucosidase b of *P. indica* was found to be downregulated in the presence of WR5. α-Glucosidase b is a cell wall degrading enzyme, possessed by saprophytes and involved in invading the host by the pathogen (Favre et al. 2014). Downregulation of this protein suggests that WR5 enhances the beneficial relationship between *P. indica* and the plant by minimizing saprophytic traits of *P. indica*.

In interaction with M4, no such demarking proteins were identified that could elucidate the role of M4 in inhibiting fungal growth. However, the flagellar biosynthesis protein FliC was found to be highly expressed in both, M4 alone and in coculture with *P. indica*. An earlier report with *Pseudomonas aeruginosa* suggested that FliC could be involved as Type III secretion protein antagonizing the host by its toxicity. It may be assumed that the FliC protein secreted by M4 could play a similar role in antagonizing fungal growth. However, this study needs further data mining and additional validation to identify factors involved in inhibition of fungal growth. The interaction between the bacterium and the fungus may also involve secretion of volatiles and small diffusible secondary metabolites. Saponins may be seen as a reasonable assumption in this respect. Results obtained with observations on growth and motility of the bacteria *Collimonas pratensis* Ter291 and *Serratia plymuthica* Pri-2C in response to terpenes, secreted by *Fusarium culmorum*, point into a comparable direction. Several studies showed that fungi react with high sensitivity to volatiles emitted by bacteria, leading to reduction and inhibition of spore germination and growth (Schmidt et al. 2016). Similarly, *Streptomyces* AcH 505 has been shown to stimulate ectomyccorhizal growth by secreting auxofuran, whereas AcM11 inhibited it by secreting cycloheximide. This is also in close agreement with the studies by Bhuyan et al. (2015), where concentrated preparations of the cell-free *A. chroococcum* supernatant were shown to contain active fractions influencing fungal growth. This suggests that bacterial–fungal interactions may not simply depend on a single factor but instead on a blend of modulators, working together in modulating the phenotypic response that depends in addition on growth stage and nutrient conditions of the interacting partners.

The interaction of microorganisms with plant rhizosphere is not an autecological issue and has serious secondary impact on the performance of the plant vis-à-vis other organisms in its surroundings. Repeated selection for single traits in cultivars must have narrowed their interaction with microbiota in the rhizosphere. It has been argued that studies defining factors that determine the nature of microbiome would have to integrate results of systems-based approach in rhizospheric soil ecosystem. Plant species, soil type, agricultural practices, climatic factors, plant community structure, and nature of biotic interaction of microbes with plants would together

determine the course of plant–microbe co-evolution. The below-ground biological interactions influence biochemical constitution and behavior of aboveground multitropic organisms including pests and pathogens. This illustrates the probable relevance of the present observations on differentially expressed proteins of *P. indica* and *Azotobacter* for developing cultivars for sustainable agriculture by rhizosphere engineering.

## 8.5 Rhizosphere Microbiome Engineering: Novel Approach to Improve Plant Fitness and Restore Agro-Ecosystem

Deterioration of the biosphere and the global issue related to growing human population has raised the importance of alternate strategy to improve soil fertility and plant productivity. Global community has been intensively depending on the external inputs of chemical fertilizers to enhance production. However, such practices may deplete natural resources and weaken the ability of the agro-ecosystems to sustain production in the future. Development of transgenic plants could improve the fitness of the plant but has not been gaining importance because of the global ethical issues. Though many such approaches have been devised, engineering the microbial community has been found to be promising and recently gained importance. Microbiome engineering approach incorporates microbiota having ecologically distinct functions into niche to improve ecological balance and reduce anthropogenic inputs (Bandyopadhyay et al. 2016b) Thus, inputs of beneficial microorganism have been promising. These features make the *P indica–A. chroococcum* co-inoculation of non-legume crops most promising for optimal plant production. Along with *P. indica*, strains like WR5 can be a better choice as nitrogen biofertilizer in cold region or the region like Thar Desert in India where plants experience a variety of stresses like drought, heat (summer), and cold (winter). A field trial of *A. chroococcum* strain M4 inoculation with maize demonstrated a significant increase in yields and saving of nitrogen fertilizer when applied in combination with farmyard manure. The M4 strain can be further explored for its application as biocontrol for fungal pathogens associated with crops along with biofertilizer. The growth-promoting and inhibiting effects of *A. chroococcum* strains and their secondary metabolites on beneficial fungi have enormous potential for agronomical applications. Identification and validation of the potent molecular modulators (secondary metabolites) present in the culture supernatants and studying its effect on cellular genes of the fungus by undertaking a genome-wide profiling of transcripts of *P. indica* will help establish the mechanism of such interactions.

However, it raises question if such beneficial relationship can be maintained and sustained even in disease prone zones. The existence of beneficial plant–microbe relationship in disease suppressive soil encourages external inputs of the beneficial

microbiota to enhance productivity. Plants grown in such soil are either less challenged or are free of pathogens. The microorganisms can be incorporated as biofertilizers or may be as potential inoculants tailoring the native microbiome. Of late root endophytic fungus *Piriformospora indica* has gained importance in the quest of agronomical functional traits for sustainable agriculture. *P. indica* has been found to mimic AMF with respect to its ability to enhance phosphate uptake and protect the host plant from abiotic and biotic stress (Lahrmann et al. 2013). This raises the importance of adding beneficial microbiota as inoculants in maintaining the ecological functions and sustaining agriculture for improved food security. Some plants possess the ability to restrict nitrification process (transformation of ammonium to nitrate) by affecting nitrifying microorganisms. Thus increasing the available nitrogen and preventing leaching (Bender et al. 2016). Recent study done on interaction of *Azotobacter* with *Brassica napus* demonstrated enhanced root branching, plant biomass, seed yield, and oil content posttreatment (Namvar and Khandan 2015). Secretion of hormones like indole acetic acid, chelators, and various important metabolites like amino acids could play an important role in enhancing plant growth and productivity (Vacheron et al. 2013). These findings have provided valuable model system that could enforce microbiome approach to improve agricultural productivity.

Considerable effort has been made to understand the ecological significance of such bacterial fungal interactions in the rhizosphere. It has been found that even certain environmental changes could trigger free-living organisms to be mutualistic without necessitating adaptive co-evolution. This is in good agreement with the experimental evidences for mutualistic association between free-living organisms being induced by environmental conditions. However, the degree of progression in the lines of mutualism seems to be depending on species-specific traits. To address this issue, further environmental studies supported by biochemical and molecular profile of such interactions can be undertaken.

## 8.6 Conclusion and Future Remarks

In this chapter, we have tried to develop an understanding on formation of stable microbial assemblage in the rhizosphere system and its influence on plant fitness and productivity. Significant progress made in this area of research has opened up many unsolved puzzles. At community level, the plant system behaves as a holobiont and is never in isolation. Though genomes of the individual organisms are being sequenced, still it may not provide the entire information about the existence of plant within the ecosystem. Hence, there is need of holistic approach for integrative studies on plant–microbial community. With the development of molecular tools, one may even find the genes that are involved in acquiring reciprocal benefits from the plant–microbial community and harness them to sustain the agriculture. By implementing stable isotope and metagenomics approaches, one will also be able to understand the underlying food web connecting

the microbial communities of different rhizosphere niche. Increase in the population has been demanding new crops and implementation of alternate cropping system. With the application of beneficial microbial partners, methods need to be strategized for adequate plant productivity at lower environmental costs. In the recent past, advances made in understanding root microbiome have provided important cues related to formation and maintenance of the root microbial assembly. The next important step is to mine the beneficial plant attributes that are programmed by the rhizospheric microbial assembly. Based on this approach, microbiome can be manipulated/ engineered to test their efficacy towards host plant fitness. For example, artificial interaction of gnotobiotic hosts with certain microbial assemblies may give us an idea to elucidate the underlying roles of microbiomes in influencing host performance. Thus, from these insights it is clear that emergence of rhizosphere microbiota as stable interactome holds a great promise for the production of sustainable crops and open up new avenues for directed ecological intensification.

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# Chapter 9
# Arbuscular Mycorrhizal Symbiosis: Genetic and Functional Diversity

**Rekha Pandey and Neera Garg**

**Abstract** Arbuscular mycorrhiza (AM) is the most widespread plant symbiosis that improves plant productivity and resistance to nutrient stress. Numerous studies have demonstrated a high variability in the symbiotic outcome of different combinations of host plant and AM fungi. This reflects functional diversity in AM fungi in terms of variation in underlying physiological processes. The variability exists not only between isolates of different species but also within different isolates of the same species. This can be correlated to the high genetic variability observed within this group of fungi. However, little is known about the genetic diversity of AM fungi due to the strict biotrophy of these fungi, difficulties in obtaining sufficient fungal material, and to the lack of knowledge of the reproductive system and the mutation rate. Studies have shown that within the same cytoplasm, AM fungi contain thousands of nuclei and show extremely high levels of genetic variation for some loci. However, knowledge about the arrangement of this variation between, or within, nuclei remains controversial. It has been proposed that AM fungi could either be homokaryotic or heterokaryotic. In addition to genetic diversity, variability in life strategy patterns of different species could account for the functional diversity in AM symbiosis, for example, variation in the hyphal growth, rate of phosphate uptake and transfer and even in expression of specific genes. This review thus attempts to discuss the reported findings on the genetic and functional diversity within this mutualistic symbiotic association.

## 9.1 Introduction

Arbuscular mycorrhizal (AM) symbiosis is an association between plant roots and a specific fungal group, the glomeromycetes. This group of fungi-arbuscular mycorrhizal fungi (AM fungi; phylum Glomeromycota; Schüßler et al. 2001) are unique due to their age, lifestyle and genetic make-up. AM fungi may have evolved over 1000 million years ago and can be seen as living fossils because they have

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co-existed relatively morphologically unaltered with plants for more than 400 million years (Parniske 2008). The symbiosis is frequent in all early diverging lineages of the major plant clades. Non-mycorrhizal species or other mycorrhizal types developed in plant lineages of more recent origin. This suggests that this symbiosis is the ancestral form of mycorrhizal interactions and that it played a critical role in the evolution of land plants (Smith and Read 2008). They are keystone organisms that form an interface between soils and plant roots; and they are also sensitive to changes in soil and plant conditions (Giri et al. 2005). The main physiological basis for this symbiosis is usually considered to be bidirectional transfer of nutrients: the extraradical mycelium (ERM) of the fungus acts as an extension of the root system and takes up phosphate (P), nitrogen (N), sulfur and trace elements from the soil and delivers these nutrients *via* the intraradical mycelium (IRM) to the plant (Allen and Shachar-Hill 2009; Smith and Smith 2011). AM fungi may supply up to 90% of the host plant's nitrogen and phosphorus requirements (Smith and Read 2008). In exchange, they receive up to 30% of the host's photosynthate (Drigo et al. 2010; Kivlin et al. 2011). These fungi are obligate symbionts and cannot survive without this C supply (Bücking et al. 2012).

AM fungi comprise of intra- and extraradical structures. The intraradical hyphae can penetrate the outer cell wall of root and grow between or inside of the root cell wall and plasma membrane where they develop the intraradical structures, arbuscules and vesicles. The extraradical structures are hyphae and spores that develop outside of the roots in the soil. Root colonization by AM fungi is preceded by mutual recognition *via* diffusible molecules released by both symbionts, culminating in differentiation of the fungal tip of the growing hypha into a lense-shaped hyphopodium for docking at the root surface (Bonfante and Requena 2011; Nadal and Paszkowski 2013). The fungus then penetrates the outer root cell layers, spreads longitudinally within the root cortex and forms branched, tree-shaped hyphal structures called arbuscules, in cortical cells (Gutjahr and Parniske 2013). The formation of intracellular fungal structures and the extent of root colonization are dynamically regulated by the plant likely to optimize symbiotic benefit according to the plants physiological and developmental status that results from environmental conditions (Carbonnel and Gutjahr 2014). It has been reported that under high Pi supply, AM development is repressed (Breuillin et al. 2010; Balzergue et al. 2013). This suppressive effect of high Pi on root colonization by AM fungi is partially overruled by nitrogen (N) starvation and to a lesser extent by potassium, calcium or iron starvation (Nouri et al. 2014), suggesting that plants control the symbiosis in function of their nutrient requirements according to Liebig's law of the minimum (Carbonnel and Gutjahr 2014).

## 9.2 Diversity of AM Fungi: More Complex Than It Seems?

AM fungi are among the world's most common soil microorganisms and associate with more than 80% of vascular plant species ranging from bryophytes to tracheophytes, including many agricultural important crop species (Smith and Read 2008). It has been suggested that AM fungi play a key role in determining the distribution and abundance of plant species (van der Heijden et al. 1998, 2015). In order to understand the role of AM fungi in shaping the structure and composition of plant communities, information about their diversity and distribution is needed (Opik et al. 2013). Till date approximately 244 species have been described within the fungal phylum, Glomeromycota (Schüßler et al. 2001; Schüßler 2014; Lee et al. 2015; van der Heijden et al. 2015; Prasad et al. 2017). Molecular studies have suggested that diversity of these fungi may be much greater (Kivlin et al. 2011). In fact, high genetic variation has been reported even within different isolates of a species (Vandenkoornhuyse and Leyval 1998; Clapp et al. 2001); it has been shown that the genetic diversity in even one initial spore can be sufficient for the development of phenotypically different variants of one fungus (Ehinger et al. 2012) affecting various important functions such as colonization rates, growth of extraradical hyphae and phosphorus uptake of AM fungi giving rise to functional diversity (Munkvold et al. 2004; Avio et al. 2006; Croll et al. 2008; Angelard et al. 2010; Torrecillas et al. 2012). Genetic diversity within species or among isolates originates from the typical genetic structure of AM fungi. Hundreds or thousands of nuclei exist together within a single spore or hypha of AM fungi, meaning that the genetic structure of AM fungi is 'multigenomic' (Kuhn et al. 2001). Estimates of global AM fungal richness, based on environmental ribosomal DNA sequences, range from 341 (Opik et al. 2013) to 1600 operational taxonomic units (OTUs) (Koljalg et al. 2013) or even higher (Kivlin et al. 2011). These 300–1600 AM fungal taxa associate with approximately 200,000 plant species (Brundrett 2009), showing that host specificity must be very low (van der Heijden et al. 2015). Although AM fungi have not been found to be strictly host specific, many recent studies have indicated high functional diversity of AM fungi with different combinations of host plant and AM fungi having different effects on various aspects of symbiosis (Jansa et al. 2008; Wagg et al. 2011; Maherali and Klironomos 2012; Tian et al. 2013; Garg and Pandey 2015). There is still a lack of understanding about why a particular AM fungal isolate is much more beneficial than others although it has recently been demonstrated that both host and fungus can discriminate among their partners, reciprocally rewarding those partners that provide more mutualistic benefit (Kiers et al. 2011; Fellbaum et al. 2012; Bucking et al. 2016). Trade-offs in phylogenetically conserved traits have occurred during the evolution of the Glomeromycota lineage, affecting the growth of the fungus and plant host (Hart and Reader 2002; Powell et al. 2009). Hart and Reader (2002) demonstrated that members of Gigasporaceae family preferentially produce extraradical hyphal biomass in the soil, while members of Glomeraceae family extensively colonize roots. These trade-offs in hyphal traits contribute to higher nutrient acquisition and biomass of

plants in symbiosis with Gigasporaceae species, and greater pathogen protection of plants that associate with Glomeraceae species (Powell et al. 2009; Kivlin et al. 2011). These long-existing trade-offs may also lead to interactions between AM fungal taxa that could affect community assembly. If traits are phylogenetically conserved, competitive exclusion between closely related AM fungi could lead to a community in which species are less related than expected by chance (i.e. phylogenetic overdispersion). Alternatively, if environmental filtering or dispersal limitation selects for these traits, species within AM fungal communities could be more closely related than expected by chance (i.e. phylogenetic clustering) (Kivlin et al. 2011). The following sections therefore, explore the diversity in AM symbiosis at two levels: genetic and functional.

## 9.3 Genetic Diversity

While the ecological importance and evolutionary novelty of AM fungi have become clear, the basic genetics of these fungi remain enigmatic. Recent use of molecular-based methods in AM fungi studies has enabled direct identification of AM fungal species in plant roots or in soils, and it has been revealed that actual AM fungi diversity in ecosystems could be higher than expected (Gollotte et al. 2004; Kivlin et al. 2011). In addition, DNA polymorphism within AM fungal isolates by different geographic origin, even within a single spore, was identified by the use of molecular techniques (Clapp et al. 2001; Börstler et al. 2008). Stukenbrock and Rosendahl (2005) used a hierarchical design to study multilocus genotypes of three *Glomus* species. Significant genetic structure was found at a small scale, among plots separated by a few metres, whereas among neighbouring field sites, with differing agricultural treatments, no differentiation was detected. Vandenkoornhuyse et al. (2001) used inter-simple sequence repeat (ISSR) fingerprints and ribosomal gene polymorphisms to study differentiation among AM fungi from different sewage treatments in a field. A high degree of diversity was found for two *Glomus* species, and the observed diversity was structured among field plots of different treatments. Using in vitro propagated *G. intraradices* from a field population, Koch et al. (2004) found high genetic diversity and differentiation among field plots. In the same population, Corradi et al. (2007) found polymorphism in copy numbers of ribosomal genes. It has been shown that even the genetic diversity among isolates of the same phenetic species reflects diversity in development, function and symbiotic performance, thus reflecting diversity on the phenotypic level (Koch et al. 2004; Munkvold et al. 2004). Most of the knowledge on AM fungi genomes comes from *Glomus intraradices* (*Rhizophagus irregularis*) because this species can easily be cultivated in vitro. *G. intraradices* DNA harbours a low content of Guanine and Cystein (30–35% of the whole genome) (Hosny et al. 1997) against 50% for the majority of other organisms and particularly plants. This difference allows detection of plant or microorganism contaminations in the extracted DNA (Corradi et al. 2004). The size of the *G. intraradices* genome was

estimated around 16.54 megabase pairs (Mbp) by reassociation kinetics (Hijri and Sanders 2004). The DNA content per nucleus was estimated at between 0.14 picogram (pg) for AM fungi *Scutellospora pellucida* and 1.15 pg for *Scutellospora gregaria* by flow cytometry (Hosny et al. 1998). The ploidy level is unknown for most AM fungi species, although *G. intraradices* and *Scutellospora castanea* appear to be haploid, based on reassociation kinetics (Hijri and Sanders 2004).

The genomic structure of AM fungi is unusual in at least two respects. First, AM fungi are multinucleate at all stages of their life history. Individual cells may contain as many as a few hundreds to tens of thousands of nuclei depending on the fungal species and the method of analysis employed (Cooke et al. 1987; Becard and Pfeffer 1993; Hosny et al. 1998). As the AM fungal hyphae lack regular septa and the fungi do not appear to go through a uninucleate or sexual stage, the vegetative structures can be thought of as free-flowing populations of nuclei (coenocytic). The second unusual aspect of their genetics is that individual cells can have very large amounts of genetic variation, with repetitive regions such as ribosomal RNA genes (rDNA) having several genetically different copies derived from single spores (Hijri et al. 1999; Clapp et al. 1999; Pringle et al. 2000; Pawlowska and Taylor 2004). While a component of this variation has been found to be due to non-mycorrhizal fungi that cohabit with, and contaminate, AM fungi (Hijri et al. 2002), these contaminants do not negate the high diversity of rDNA of AM fungal origin (Pringle et al. 2003). Moreover, a similar level of variation within spores has been observed within single-copy regions of the genome (Kuhn et al. 2001; Pawlowska and Taylor 2004), with, for example, 13 different variants of putatively single-copy gene, DNA polymerase 1 (*PLS1*), being found within individual spores of *Glomus etunicatum*. To explain this, two basic organizational structures have been proposed (Fig. 9.1). Firstly, it is possible that all intracellular variation is present within individual nuclei and all of the nuclei within a cell are identical, i.e. *homokaryotic* (Pawlowska and Taylor 2004; Pawlowska 2005; Fig. 9.1a). Alternatively, it is possible that much of the genetic variation may be distributed between nuclei, with each cell containing multiple genomes, i.e. *heterokaryotic* (Kuhn et al. 2001; Hijri and Sanders 2005; Bever et al. 2008; Boon et al. 2015; Fig. 9.1b).Thus, the entire intrasporal rRNA variation could be either contained in every single nucleus (homokaryosis) or distributed among different nuclei (heterokarysis) (Pawlowska 2005) (Fig. 9.1). The homokaryotic organization implies that the nuclear polymorphism reported in these organisms is the result of orthologous allelic variants partitioned between chromosomes (i.e. polyploidy) or paralogous copies within a chromosome, while in the heterokaryotic state, different allelic variants could be evenly partitioned among distinct nuclei or be present in a group of complementary nuclei. There are supporters of both the theories (Pawlowska and Taylor 2004; Hijri and Sanders 2005; Pawlowska 2005; Bever et al. 2008; Boon et al. 2015) providing evidences in their support. However, it is possible that these scenarios might not be mutually exclusive, and the genetic variation among and within AM fungi isolates is likely to be a continuum between these two states, being shaped by modest rates of hyphal fusion and segregation (Bever and Wang 2005).

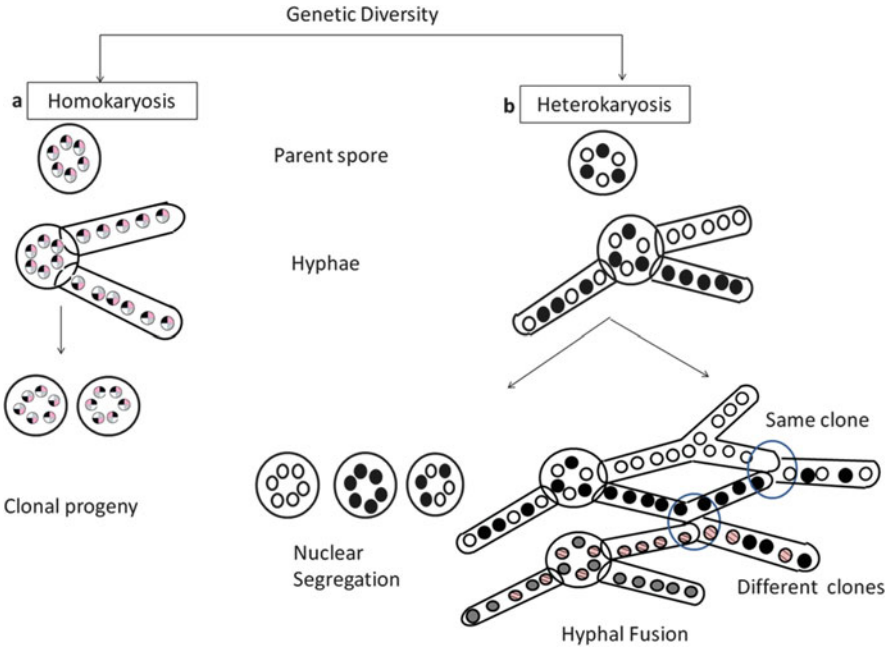


**Fig. 9.1** Two alternative hypotheses on genomic organization in AM fungi: (**a**) Homokaryotic, where entire intrasporal rRNA variation is contained in every spore; clonal progenies in this case contain the genetic composition of parental spore. (**b**) Heterokaryotic, where the variation is distributed between different nuclei with each cell containing multiple genomes—under heterokaryotic nuclear organization, process of nuclear segregation and hyphal fusion could segregate and remix variation. Hyphal fusion between same hyphal clones restores genetic diversity, while between different clones creates new genetic diversity

Pawlowska and Taylor (2004) found that high levels of intracellular molecular variation within isolates of *Glomus etunicatum* were not lost due to segregation. They observed that each of 20 single progeny spores had all 13 variants of PLS1, a putatively single-copy gene. They argued that this was inconsistent with heterokaryotic organization of the genome, with their statistical confidence in this conclusion coming from simulations of the segregation process that assumed haploidy, no hyphal fusion and no selection. Instead, they proposed that all 13 variants of the *PLS1* gene were present within each nucleus (and all nuclei in the hyphae were identical), with the persistence of the large number of variants within individual spores resulting from very high ploidy in these fungi (i.e. at least 13 ploids) and the suspension of gene conversion (Fig. 9.1a). This explanation came into conflict with the subsequent observation that *G. etunicatum* is actually haploid (Hijri and Sanders 2005). Bever and Wang (2005) presented a resolution to this apparent conflict by using a simulation similar to that of Pawlowska and Taylor (2004) to demonstrate that modest levels of hyphal fusion would allow remixing of the nuclei and reduce the effective rate of segregation to a level consistent with the Pawlowska and Taylor's laboratory observations. With sufficient rates of hyphal fusion, high levels

of variation can be maintained within spores over long periods of time, which is consistent with Pawlowska and Taylor field observations (Bever et al. 2008). According to Pawlowska (2005, 2007) coexistence of polymorphic rRNA gene sequences observed in individual nuclei indicate that the process of concerted evolution may have been impaired in this group of fungi during evolution. [Concerted evolution is a universal recombination-driven process responsible for rRNA gene sequence homogeneity within an individual and among individuals of a recombining population (Arnheim et al. 1980; Dover 1982). Over time, these processes can reduce variation within multicopy gene families, accounting for the low variation within rDNA gene families in other organisms (Hamby and Zimmer 1992; Avise 2004)]. A study by Stukenbrock and Rosendahl (2005) used three codominant genetic markers of confirmed AM fungi origin to estimate the genetic structure of two AM fungal populations from agricultural fields, and they did not find evidence of significant recombination. However, Bever and Wang (2005) noted that the presence of the three types within a nucleus is not a definitive test for homokaryosis as the nuclei could still vary in the numbers for each of the three ITS types as well as in other regions of the genome. Pawlowska and Taylor (2005) suggested that the changes in copy number of rDNA are not relevant, citing evidence that copy number can be dynamic within a cell cycle.

As opposed to this, proponents of the theory of heterokarysis postulate that although AM fungi like *G. intraradices* and *G. etunicatum* are haploid as other fungi, but rRNA variation occurs between different genomes that are present in genetically different nuclei in the coenocytic mycelium (Kuhn et al. 2001; Hijri and Sanders 2005; Bever et al. 2008) (Fig. 9.1b). Heterokarysis has been proposed to have arisen by hyphal anastomosis and accumulation of mutations (Bever and Wang 2005; Croll et al. 2009). Pawlowska and Taylor (2005) argued that there is no evidence of the level of hyphal fusion assumed in Bever and Wang's simulation, citing evidence of barriers to hyphal fusion between geographically isolated populations of *G. mosseae* (Giovannetti et al. 2003). However, this same body of work shows very high rates of hyphal fusion within isolates of many species of *Glomus* (Giovannetti et al. 2001, 2004). The simulation of Bever and Wang simply assumed that offspring from a single spore could fuse, which is exactly what had been demonstrated by the work of Giovannetti et al. (2003). Using a stochastic simulation of nuclear segregation, Bever and Wang (2005) demonstrated that modest rates of hyphal fusion can maintain high levels of nuclear variation within spores at equilibrium. While species within *Glomus* bearing small spores may have relatively small numbers of nuclei, which makes them very vulnerable to drift, *Glomus* spp. generally have high rates of hyphal fusion which will reduce the rate of genetic drift (de la Providencia et al. 2005; Voets et al. 2006). In contrast, *Scutellospora* and *Gigaspora* generally appear to have lower rates of hyphal fusion (de la Providencia et al. 2005; Voets et al. 2006), but these fungi consistently have larger numbers of nuclei with estimates ranging from a thousand to tens of thousands (Hosny et al. 1998). Croll et al. (2009) confirmed that genetically distinct AM fungi, from the same field, could anastomose, resulting in viable cytoplasmic connections through which genetic exchange can occur. These results suggest that

hyphal fusion rates are sufficient to offset the force of drift in AM fungi, potentially providing an explanation for persistence of high levels of variation in AM fungal nuclei (Bever et al. 2008). The variation that exists between nuclei would segregate as hyphae grow and divide in a process analogous to assortment during meiosis. Fusion of genetically different hyphae could remix and recombine variation in an analogous manner as fusion of gametes in sexual organisms (Fig. 9.1b). Assuming that the phenotype is a function of the nuclear composition of the hyphae, this process could mimic the creative process of sexual reproduction by bringing together novel genetic variants into the same functional organism (Bever et al. 2008). Recently, Boon et al. (2015) have cited evidences in favour of heterokarysis in AM fungi. First, they cite the evidence for within-isolate sequence polymorphism in *Rhizophagus irregularis* DAOM 197198 (synonym *Glomus irregulare*) and *Glomus etunicatum* (synonym *Claroideoglomus etunicatum*) transcripts (Boon et al. 2010; Tisserant et al. 2012). Second, segregation of genetic variation between parent and offs-pring has been demonstrated for *R. irregularis* (Angelard et al. 2010) and *G. etunicatum* (Boon et al. 2013). Patterns of genetic segregation between parent and clonal offspring indicate that different fractions of genetic variation are passed on to different spores. Moreover, this variation appears to make a difference to the phenotype of the offspring isolate (Angelard and Sanders 2011). Third, within-isolate heterokarysis has been demonstrated for several loci (Boon et al. 2010). Fourth, several AM fungi taxa seem at no part of their life cycle, reduced to a single nucleus (Jany and Pawlowska 2010; Marleau et al. 2011; Ehinger et al. 2012). In 2010, Jany and Pawlowska examined the dynamics of spore nuclei in *Glomus etunicatum* using live three-dimensional imaging and mathematical models. They observed that spores of *Glomus etunicatum* are formed not by false sporogenesis (where serial divisions of a single founder nucleus occurs), but the spores are populated by an influx of a stream of nuclei from the surrounding mycelium which might account for the heterogeneity. Marleau et al. (2011) found that spores used for dispersal of AM fungi contained nuclei with two origins—those that migrate into spore from the coenocytic mycelium and those that arise by mitosis in spore which led them to postulate that probably AM fungi lack the genetic bottleneck of a single-nucleus stage at any point in the AM fungi life cycle, which sets AM fungi apart from filamentous fungi (that are heterokaryotic only in a part of their reproductive cycle) (Boon et al. 2015).

Although recent publications of the *Rhizophagus irregularis* genome (Tisserant et al. 2013) and single-nucleus sequencing (Lin et al. 2014) have reported evidence in favour of homokaryosis, it is unclear whether the approach adopted in these studies is sufficient to provide a definite answer to the debate. In a latest report, Boon et al. (2015) have addressed the question of the extent of genome differentiation and its physical partitioning in *Rhizophagus*. They found evidence for genome differentiation within the *Rhizophagus* cytoplasm, both genome-wide and on the scale of a single locus which led them to propose that this population of partly heterogeneous genomes in *Rhizophagus* is analogous to a pan-genome, since there may not be one typical genome within an isolate representative of all the other but rather a population of partly differentiated genomes.They cite four observations

to support this interpretation. First, for several AM fungi, it has been shown that they are at no point in their life cycle reduced to a single genome as stated earlier (Jany and Pawlowska 2010; Marleau et al. 2011; Boon et al. 2013). Second, *Rhizophagus* spores do not germinate below a certain number of nuclei per spore, which is roughly 65 nuclei for *R. irregularis* (Marleau et al. 2011). Third, for *R. irregularis* and *G. etunicatum*, it has been shown that genetic polymorphism is expressed in the transcriptome (Boon et al. 2010; Tisserant et al. 2012), which indicates that differentiation at the genome level could play a role in the functioning of *Rhizophagus* isolates. Finally, the high amounts of genetic variation in AM fungi isolates have been proposed to play a role in the ability of AM fungi to adapt to a wide range of host plants (Angelard et al. 2010). However, basic parameters can differ substantially between different members of the Glomeromycota (Gianinazzi-Pearson et al. 2012)—their genome sizes vary, not much is known about the genome structure except the model species—*G. intraradices* and retrotransposons have been suggested to play an important role in the genome of atleast one species (*Scutellospora castanea*; Gollotte et al. 2006). Not only that, anastomosis has not been observed in Gigasporaceae and other lineages, suggesting a completely different of the mycelium in such cases (Purin and Morton 2011). Also in contrast to *G. intraradices*, *G. mosseae* has been shown to have a rather uniform worldwide population structure suggesting a different genetic disposition (Gianinazzi-Pearson et al. 2012).

Despite recognition of the importance of genetic diversity of AM fungi, little is known about its role in ecosystems. It has been demonstrated that genetically different AM fungal isolates, even from the same species, have different effects on their host plants (Avio et al. 2006; Koch et al. 2006). Recently, Colard et al. (2011) observed that genetically different AM fungal isolates could differ in their ability for survival or functionality in their host plants. This supports the view that genetic variation could lead to functional diversity of AM fungi in ecosystems.

## 9.4 Functional Diversity

The relationship between arbuscular mycorrhizal (AM) fungi and their plant hosts is considered as a classic example of a reciprocally beneficial mutualism; both partners benefit from the symbiosis, with plants providing carbohydrates to their fungal partners and fungi providing mineral nutrients, such as nitrogen (N) and phosphorus (P) to their host plants. The presence of AM in virtually all terrestrial habitats (Smith and Read 1997; Brundrett 2004) together with the hitherto, comparatively small number of identified AM fungi taxa (244, Schüßler 2014; van der Heijden et al. 2015), could indicate a high promiscuity among the fungal species, and it was long believed that most AM fungi species are able to form a successful symbiosis with most plant hosts and are not host specific. However, recent studies have clearly brought out the host preference in AM fungi, thus emphasising the need for selecting efficient AM fungi for inoculating a particular host. This

variation has been observed among AM fungi isolates belonging to different species, as well as among isolates of the same species (van der Heijden et al. 1998; Klironomos 2003; Munkvold et al. 2004; Smith et al. 2004; Burleigh et al. 2002; Lerat et al. 2003a, b; Avio et al. 2006; Jansa et al. 2008; Wagg et al. 2011; Maherali and Klironomos 2012; Tian et al. 2013; Pellegrino and Bedini 2014). Antunes et al. (2011) even found evidence for functional diversity in AM fungi originating from contrasting climates. Many reports have stated the higher efficiency of *G. intraradices* (= *R. irregualris*) over other AM fungi (Avio et al. 2006; Peng et al. 2011; Tufenkci et al. 2012). As opposed to this, many reports have shown *Funneliformis mosseae* to be more beneficial (Stancheva et al. 2006; Song et al. 2012; Huang et al. 2013). The AM fungi species commonly used in functional studies, e.g. *Glomus intraradices* and *Glomus mosseae* from the Glomeraceae or *Gigaspora margarita* from the Gigasporaceae, dominate almost all biodiversity studies even across drastically different environments. Functional studies therefore often contain a certain bias towards fungal species that either confer a clear benefit on their host plant or are highly abundant and therefore easily detected in culture systems (Feddermann et al. 2010; Opik et al. 2013). However, there are numerous examples of negative effects of AM fungi on host-plant growth (Johnson et al. 1997; Klironomos 2003; Li et al. 2008). In a recent study, de Novais et al. (2014) evaluated the functional variability of 41 isolates of 20 species and eight genera of AM fungi for root colonization, growth promotion and P uptake of corn and observed the relationship of this functional variability with the isolates genetic variability revealed by PCR-RFLP analysis. All the isolates abundantly colonized the corn roots but only 23 promoted higher shoot dry mass and P leaf content. A functional variability was observed between isolates of distinct species and isolates of the same species showing no correlation between the ability to promote growth with the genus, species or the origin of the isolates. Cluster analysis based on functional variability data separated the isolates *Acaulospora morrowiae* (Am2), *Acaulospora* sp. (Aca), *A. colombiana* (Ac3, Ac4 and Ac5), *Gigaspora albida* (Gia1), *Gi. margarita* (Gim4 and Gim5), *Gi. rosea* (Gir), *Rhizophagus clarus* (Rc2, Rc3, Rc4, Rc5 andRc6), *Claroideoglomus etunicatum* (Ce4), *R. manihotis* (Rm), *Scutellospora calospora* (Sc), *S. heterogama* (Sh2, Sh3, Sh4 and Sh5) and *S. pellucida* (Sp3) from the others at the distance of 80% functional similarity. These were considered efficient in promoting functional symbiosis in corn, while the other isolates were considered inefficient. The functional diversity of the AM fungi could be manifested at various levels from colonization strategies to the induction of specific genes in the host (Fig. 9.2) each of which is discussed in detail.

### *9.4.1 Colonization Strategy*

AM fungi form a number of different infective propagules that are used to form new mycorrhizal associations. These are spores, extraradical hyphae and infected roots.

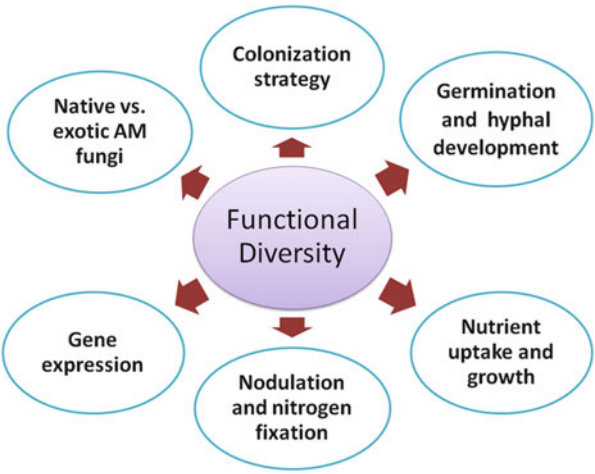


**Fig. 9.2** Different levels at which functional diversity in AM symbioses are displayed by different AM fungi

However, not all fungi are equally capable of colonizing roots with all of the above-mentioned propagules, and there is conflicting evidence of major differences in colonization strategy between members of the Glomineae and Gigasporineae. Abbott et al. (1994) showed that mycorrhizal root pieces were effective propagules for *Glomus* and *Acaulospora* isolates but not for *Scutellospora* isolates. Biermann and Linderman (1983) reported a similar result. They examined the role of root fragments as propagules and found high infectivity from those containing *Glomus* and *Acaulospora* species, but none from root fragments containing *Gigaspora* species. They attributed this difference to the presence of vesicles. Later, Klironomos and Hart (2002) tested the abilities of eight fungal species from four different genera to colonize roots using three different types of inoculum. *Glomus* and *Acaulospora* isolates colonized from all inoculum types, whereas *Gigaspora* and *Scutellospora* isolates colonized mainly from spores and to a limited degree from root fragments. Extraradical hyphae were not suitable propagules for the species of *Gigaspora* and *Scutellospora* tested. This clearly highlighted that AM fungi differ on the basis of different colonization strategies.

### *9.4.2 Germination of Spores and Hyphal Development*

The development of AM fungi prior to root colonization, known as presymbiosis, consists of three stages: spore germination and hyphal growth, host recognition and appressorium formation. Spores of the AM fungi are thick-walled multinucleate resting structures. The germination of the spore does not depend on the plant, as spores have been germinated under experimental conditions in the absence of plants both in vitro and in soil. However, the rate of germination can be increased by host root exudates (Douds and Nagahashi 2000). AM fungal spores germinate given

suitable conditions of the soil matrix, temperature, carbon dioxide concentration, pH and phosphorus concentration. AM fungi recognize and respond to their potential hosts, whereas the presence of non-host plants has no stimulatory effect on hyphal growth or is even inhibitory (Requena et al. 2007). AM fungal colonization of host roots involves a series of events that are tightly regulated by both partners. Recognition and the subsequent initiation of the symbiotic program in the AM fungi and potential host plant could be described as compatibility and is genetically predetermined. According to Giovannetti et al. (2003), spores and/or sporocarps of the six *G. mosseae* isolates from different geographical areas, showed different germination abilities, depending on the experimental system. Surface-sterilized spores grown in vitro exhibited the lowest germination percentages in all isolates, and some of these (BEG25, IN101C and SY710) did not germinate at all in axenic culture. In contrast, spore germination was higher in all isolates when the in vivo culture system was used. Hyphal growth per germinated spore, assessed in vivo, varied with the different isolates and ranged from $34.5 \pm 3.5$ mm and $35.9 \pm 2.9$ in BEG69 and SY710, respectively, to $119.5 \pm 14.4$ mm in IMA1. Within the root, fungal hyphae grow intercellularly until they reach the inner cortex where they penetrate cortical cell walls and form characteristic intracellular hyphal structures (Genre et al. 2008). A varied range of structures is formed by AM fungi in the roots of plants, as first highlighted by Gallaud (1905) such as hyphal coils, arbuscules and intermediate structures involved in nutrient transfer (Smith and Smith 2012). Within the mycorrhizal roots, different types of hyphal morphology can be identified. Depending on the type of mycorrhiza, characteristic, highly branched arbuscules (Arum-type) or heavily curled "coils" (Paris-type) (Smith and Read 1997) are developed, although there is a continuum of intermediate structures (Dickson 2004). The colonization morphology depends on the combination of the plant and fungal species (Feddermann et al. 2010). In general, AM fungi of the Glomeraceae usually form the Arum-type of mycorrhiza (Cavagnaro et al. 2001; Burleigh et al. 2002; Dickson 2004; Feddermann et al. 2008), while other genera, e.g. Gigasporaceae, form Arum-type AM or intermediate types of AM with Paris-type hyphal coils (Cavagnaro et al. 2001; Dickson 2004; Karandashov et al. 2004; Smith et al. 2004; Dickson et al. 2007). The extraradical mycelium (ERM) plays critical roles in uptake and rapid translocation of nutrients to the intraradical structures and in foraging to locate new roots on the same or different plants, which are new sources of organic C (Smith and Smith 2012). The ability to develop extensive and highly interconnected extraradical mycorrhizal networks could represent an important feature of efficient AM fungi. Recently, the genetic diversity, i.e. the genetic composition, of the coenocytic ERM has also been discussed as being an important factor in the recognition process (Koch et al. 2006; Croll et al. 2009). Some studies have provided data on the large diversity among different AM fungal isolates in the extension, viability, structure and anastomosis formation ability of ERM originating from mycorrhizal roots (Abbott and Robson 1985; Hamel et al. 1990; Friese and Allen 1991; Giovannetti et al. 2001). Avio et al. (2006) investigated the functional significance of extraradical mycorrhizal networks produced by geographically different isolates of the AM fungal species

*Glomus mosseae* and *Glomus intraradices* and detected a large functional diversity between the two, since *G. intraradices* isolates were generally more effective than *G. mosseae* isolates. They observed not only interspecific but also intraspecific functional diversity, both in *G. mosseae* and in *G. intraradices*. In particular, AM fungal isolates producing higher total hyphal lengths and densities yielded larger increases in total shoot biomass, confirming that the growth ability and developmental pattern of ERM are important factors of fungal efficiency (Jakobsen et al. 1992). Plant P content also correlated positively with hyphal length, which could be responsible for overall functional diversity (Avio et al. 2006). Mycelia produced by different fungi have quite varied characteristics, in terms of hyphal diameters (usually in the range of 2–20 μm), extent of growth away from the root and ability to absorb nutrients at a distance [up to 25 cm (Jansa et al. 2003)] and translocate them to the root (Jakobsen et al. 1992; Smith et al. 2000; Drew et al. 2003; Munkvold et al. 2004). Many AM fungi produce runner hyphae of relatively large diameter that can subtend tufts of finely branched hyphae; the latter turn over rapidly and are probably involved in nutrient uptake (Bago et al. 1998). Hyphal length densities in soil associated with plants in pot experiments are variable and usually in the range of 1–40 m $g^{-1}$ depending at least in part on the identity of the AM fungus (Jakobsen 1999; Munkvold et al. 2004). Smith et al. (2004) investigated structural and functional diversity in arbuscular mycorrhizal (AM) symbioses involving three plant species and three AM fungi and measured contributions of the fungi to P uptake using compartmented pots and $^{33}P$. They observed that flax (*Linum usitatissimum*) responded positively to all fungi, and medic (*Medicago truncatula*) to *Glomus caledonium* and *G. intraradices*, but not *Gigaspora rosea*. Tomato (*Lycopersicon esculentum*) showed no positive responses. Not only host genotype but AM fungal identity also influenced the outcome of the association. Hyphal growth in soil was very low for *Gi. rosea* and high for both *Glomus* spp. Hypha lengths in root + hyphal compartment (RHC) and hyphal compartment (HC) were similar for *G. intraradices* but much higher in HC for *G. caledonium*. In order to evaluate host plant performance relative to different soil arbuscular mycorrhizal fungal (AM fungi) communities, *Andropogon gerardii* seedlings were grown with different AM fungi communities (Gustafson and Casper 2006). The communities consisted of spores of *Glomus etunicatum* and *Glomus intraradices*. There was no difference in root AM fungi colonization rates between single species communities of either *G. etunicatum* or *G. intraradices*, but *G. intraradices* enhanced plant growth and *G. etunicatum* did not.

### *9.4.3 Nutrient Uptake and Growth Response*

In the context of nutrient uptake in AM symbiosis, the soil-root interface provides the direct pathway (DP), in contrast to the mycorrhizal pathway (MP). The latter involves uptake by the ERM and rapid translocation to the IRM. Delivery is followed by nutrient export from the fungus across the interfacial apoplast to the

plant. Positive mycorrhizal growth responses arise largely from increased P uptake via the MP, alleviating P deficiency, but can also come from increased uptake of other growth-limiting nutrients (Smith and Smith 2012). However, the contribution of the plant or mycorrhizal pathway to total P uptake also depends on the plant and fungal species. Important functional differences in terms of P acquisition strategies have been recognized among AM fungi species and also among AM fungi isolates belonging to the same species. These are mainly expressed as (1) morphological traits such as the ability (rate and extent) of the AM fungi to colonize the root and the soil and (2) physiological traits that mainly include the efficiency of the mycorrhizal pathway to take up the P from the soil solution, transport and deliver it to the roots, along with the carbon requirement from the plant host (van der Heijden and Scheublin 2007). There is a consensus (Avio et al. 2006; van der Heijden and Scheublin 2007) that the differential increases in P supply to host plants are mainly attributed to morphological and physiological properties of the mycorrhizal extraradical mycelium (ERM). However, recent work by Mensah et al. (2015) demonstrated that the greater effect of some AM fungal isolates on plant P and N nutrition was more likely the result of more efficient P and N uptake systems and/or higher nutrient transport rates to the host than the length of ERM. This is consistent with other studies in which no correlation between the dimensions of the ERM and P uptake and/or MGR (mycorrhizal growth responsiveness) was found (Hart and Reader 2002; Smith et al. 2000). A meta-analysis recently revealed that the mycorrhizal colonization is only in part responsible for the high diversity in MGR that can be observed but that AM fungal taxa also differ in their mycorrhizal benefit per unit root length colonized (Treseder 2013). Thonar et al. (2011) quantified differences in P acquisition and use efficiency of medic (*Medicago truncatula*), when colonized by three different AM fungi species (*Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita*) using dual radioisotope labeling ($^{32}$P and $^{33}$P): $^{33}$P labeling determined hyphal P uptake from different distances from roots over the entire growth period, whereas $^{32}$P labeling investigated hyphal P uptake close to the roots over the 48 hours immediately prior to harvest. *G.intraradices*, *G. claroideum* and *Gi. margarita* were able to take up and deliver P to the plants from maximal distances of 10, 6 and 1 cm from the roots, respectively. *G. intraradices* most rapidly colonized the available substrate and transported significant amounts of P towards the roots but provided the same growth benefit as compared to *G. claroideum*, whose mycelium was less efficient in soil exploration and in P uptake and delivery to the roots. *Gi. margarita* provided low P benefits to the plants and formed dense mycelium networks close to the roots where P was probably transiently immobilized. Based on numerical modelling, they concluded that high external hyphal production at the root-fungus interface together with rapid hyphal turnover as important factors governing hyphal network development by *Gigaspora*, whereas nonlinearity in apical branching and hyphal anastomoses was key features for *G. intraradices* and *G. claroideum*, respectively. Similarly, Veresoglou et al. (2011) also observed that *G. intraradices*-inoculated *Plantago lanceolata* plants had 27.8% and 40.8% more total N and 55.8% and 23.3% more total K when compared to *Gigaspora margarita*-inoculated

counterparts in a native, nutrient limited, coastal dune soil. *G. intraradices* inoculated and non-mycorrhizal plants generally exhibited N:P:K ratios indicative of P limitation, whereas for *Gi. margarita* mycorrhizal plants, corresponding ratios strongly implied either N or K limitation. Plant P transporters that are involved in the uptake *via* the plant pathway are downregulated in response to the AM symbiosis (Chiou et al. 2001; Grunwald et al. 2009), while mycorrhiza-specific transporters that are involved in the P uptake from the mycorrhizal interface are induced (Harrison et al. 2002; Xu et al. 2007; Paszkowski et al. 2002). However, this effect can be largely species specific. *Glomus intraradices* has been shown to suppress the expression of plant P transporters of the plant pathway the most, whereas *G. mosseae* had the least effect (Grunwald et al. 2009). In tomato, almost 100% of the plant's P was taken up by *G. intraradices* via the mycorrhizal pathway, but the contribution of *Gigaspora rosea* to total P uptake was much lower (Smith et al. 2003). Wu et al. (2011) found that the benefits of AM fungi on nutrient uptake in peach were better in *G. mosseae* treatment when compared to *G. versiforme* and *Paraglomus occultum* treatments. A high functional diversity in nutritional benefit, not only among different fungal morphospecies but also among isolates within one morphospecies, has been observed (Pellegrino et al. 2011; Tian et al. 2013; Pellegrino and Bedini 2014). Recently, the effect of 31 different isolates from 10 AM fungal morphospecies on the P and nitrogen (N) nutrition of *Medicago sativa* and the P allocation among different P pools was examined by Mensah et al. (2015). The results of these investigations revealed that plant growth benefit was positively correlated to the mycorrhizal effect on P and N nutrition. A high variability in the mycorrhizal growth response (MGR) across AM fungal isolates was detected. The per cent increase in total plant biomass ranged from 7.3 ± 10.8% in plants colonized with *R. irregulare* (not significantly higher than the controls) to 207.4 ± 36.4% in plants colonized with *Acaulospora colombiana*. They divided the different isolates into high, medium and low performance isolates based on increase in total plant biomass relative to controls. The high performance isolates increased plant biomass by as much as 170% and contributed substantially to both P and N nutrition, whereas the effect of medium performance isolates particularly on the N nutrition of the host was significantly lower (18–170%). Of all the AM fungal species tested, the four *Rhizophagus* isolates led to the lowest MGR (average of 20.2 ± 9.3%) and the plants did not differ in their biomass from the non-mycorrhizal controls. The high performance isolates belonged to different morphospecies and genera, indicating that the ability to contribute to P and N nutrition is not conserved and is widespread within the Glomeromycota, and differences in symbiotic performance and P metabolism are not specific for individual fungal morphospecies. Garg and Pandey (2015) demonstrated that higher beneficial effects of *R. irregularis* over *F. mossease* in pigeon pea-AM associations were related to its higher P and N acquisition traits as well as more favourable $K^+/Na^+$ ratios. According to a meta-analysis study by Augé et al. (2014), AM-induced increases in root $K^+/Na^+$ ratio ranged from 12 to 107 percent based on AM taxa, and this heterogeneity was significant—*R. intraradices* had the largest effect on root $K^+/Na^+$ (average increase of 107%), followed by *F. mosseae* (45%) and *R. clarus*

(17%). More recently, Kohl and van der Heijden (2016) demonstrated that the effects of different AM fungi on nitrogen leaching varied depending on host plant species and the identity of the AM fungal species present in soil, using experimental microcosms with two different host plants (the grass *Lolium multiflorum* or the legume *Trifolium pratense*) and three different AM fungal species (*Claroideoglomus claroideum*, *Rhizoglomus irregulare* and *Funneliformis mosseae*). Their results show that the differential effects were, at least in part, explained by species-specific differences in root colonization. The AM fungus with the highest levels of root colonization (Ri) had the strongest effects on plant biomass [resulting in the greatest growth stimulation (1170%) for the mycotrophic plant species (*Trifolium*) and the greatest growth suppression (18%) for the grass species (*Lolium*)]. This was in confirmation with an earlier study by Verbruggen et al. (2012) who demonstrated that the abundance of specific AM fungal taxa, as determined by terminal-RFLP, correlated well with plant productivity and $PO_4^{3-}$ leaching from microcosms. Thus, together, these studies showed that the AM fungal composition can influence nutrient leaching in soil.

### *9.4.4 Nodulation and Nitrogen Fixation in Legumes*

It is also important to study the interaction of *Rhizobium* with different AM fungal species/isolates since such interactions may be relevant to $N_2$ fixation and to nutrient and water uptake by the legume plants. In a study using chickpea plants, the symbiotic efficiency was found to be dependent on the particular combination of the *Rhizobium* strain and *Glomus* species, indicating selective and specific compatibilities between the bacterial strain and fungal isolate (Ruiz-Lozano and Azcon 1993). Geneva et al. (2006) evaluated the response of pea (*Pisum sativum* cv. Avola) to AM species *Glomus mosseae* and *Glomus intraradices* and *Rhizobium leguminosarum* bv. *viceae*, strain D 293, regarding the growth, photosynthesis, nodulation and nitrogen fixation activity. Their results demonstrated that the dual inoculation of pea plants significantly increased the plant biomass, photosynthetic rate, nodulation and nitrogen fixation activity in comparison with single inoculation with *Rhizobium leguminosarum* bv. *viceae* strain D 293. The effectiveness of coinoculation with *Rhizobium leguminosarum* and *Glomus mosseae* was higher at the low phosphorus level, while the coinoculation with *Glomus intraradices* appeared to be the most effective at higher phosphorus level. Assessment of comparative effects of three AM fungi species, *Glomus intraradices*, *Acaulospora tuberculata* and *Gigaspora gigantea*, was combined with cultivar specific *Bradyrhizobium japonicum* (CSBJ) in soybean cultivars on nodulation, plant growth and seed yield by Meghvansi et al. (2008) revealed that amongst the single inoculations, *G. intraradices* produced the largest increases in the parameters studied followed by *A. tuberculata* and *G. gigantea* indicating that plant acted selectively on AM symbiosis. The dual inoculation with AM fungi and CSBJ further improved these parameters demonstrating synergism between the two

microsymbionts. Among all the dual treatments, *G. intraradices* + *B. japonicum* brought about the largest increases in the studied characteristics particularly in seed weight per plant that increased up to 115.19%, which suggested that a strong selective synergistic relationship existed between AM fungi and *B. japonicum*. More recently, tripartite symbiosis of common bean (*Phaseolus vulgaris* L.) recombinant inbred line (RIL) 147 with rhizobia and three AM fungal species—*Glomus intraradices*, *Gigaspora rosea* and *Acaulospora mellea*—was assessed in sand culture by comparing the effects on the mycorrhizal root colonization, rhizobial nodulation, plant growth and phosphorus use efficiency (PUE) for symbiotic $N_2$ fixation by Tajini and Drevon (2012). They found that although *Glomus intraradices* well-colonized the roots of RIL147 plants, *Gigaspora rosea* and *Acaulospora mellea* colonized the roots weakly. Significant differences among colonization and nodulation of the roots and growth were found between AM fungal species—significantly more nodules were encountered for plants inoculated by *Glomus* than other AM fungal species—even nodular dry mass was higher in these plants. In addition, the combined inoculation of *Glomus* and CIAT899 strains resulted in significantly higher N and P accumulation of common bean plants and improved PUE compared with their controls. Recently, Garg and Pandey (2016) observed higher nodulation and nitrogen fixation in pigeon pea plants inoculated with *R. irregularis* as compared to *F. mosseae* which has been correlated to higher AM colonization percentage as well as higher trehalose turnover in the nodules of these plants.

### *9.4.5 Gene Expression*

It has been suggested that differences in AM compatibility reflect differences in plant and fungal gene expression (Feddermann et al. 2010). Genetic variation caused by the composition of hyphal nuclei is important in mutual recognition of AM symbiosis. In addition, genetically different isolates of AM fungi could affect colonization strategy and mycorrhizal morphology of the plant (Koch et al. 2006; Lee et al. 2015). Koch et al. (2006) showed that genetically different *Glomus intraradices* isolates from one AM fungi population significantly alter plant growth in an axenic system and in greenhouse experiments. Isolates increased or reduced plant growth meaning that plants potentially receive benefits or are subject to costs by forming associations with different individuals in the AM fungi population. This suggested that genetic diversity of AM fungi plays a pivotal role in host-plant fitness. Croll et al. (2008) also reported a strong preference for AM fungal genotype by host plants in his experiment. Angelard et al. (2010) used genetically different AM fungal isolates of *G. intraradices* to promote the growth of rice and found that specific AM fungal genotypes could increase the biomass of rice up to five times compared with other isolates. Recent gene expression studies on plants interacting with AM fungi from different taxonomic groups have showed a partial overlap in the gene expression patterns after colonization of fungi of the Glomeraceae,

Diversisporacea and Gigasporaceae. In an early study using array techniques, Liu et al. (2003) showed that a high number of AM-specific genes are induced in their host plants. Transcriptional analysis dissecting the common symbiosis dependent and independent signalling in rice revealed that the symbiosis signalling pathway is conserved in angiosperms (Gutjahr et al. 2008). Despite the apparent similarities in plant transcriptional responses to AM fungi, a large number of genes found in recent studies show significant variation in gene expression levels. The symbiotic phenotype in the presumed *myc*$^{-1}$ tomato mutant *rmc* (Barker et al. 1998) differs depending on the AM fungi against which it is challenged. The Paris-type of AM formed by *S. calospora* in tomato induced high levels of a number of defence-related genes, whilst the Arum-type of AM formed by *G. intraradices* did not induce these defence reactions (Gao et al. 2004). Hohnjec et al. (2005) reported that the gene expression pattern was similar in infections by two AM fungi species but that some genes were expressed more in specific host plants, meaning that mycorrhizae-specific gene expression was affected by the combination of host plant and AM fungi species (Lee et al. 2015). Marulanda et al. (2003) have shown that *Glomus intraradices* is one of the most efficient fungi in improving plant-water uptake in lettuce plants, while *Glomus mosseae* showed a reduced ability for the same. These differences have been related to the different regulation of plant *PIP* aquaporin genes by the fungi. Up-regulation of the *PIP* gene expression induced by *G. intraradices* enhances the water uptake of root and the root water movement. AM-inducible plant Pi transporters are involved in the acquisition of Pi released by the AM fungus at the symbiotic interface and can be used as markers for the symbiotic Pi uptake pathway (Grace et al. 2009). It has been observed that different AM species or isolates have varying influence on the expression of Pi transporters in plant species such as *M. truncatula* and tomato (Burleigh et al. 2002; Poulsen et al. 2005; Tian et al. 2013). Tian et al. (2013) studied the functional diversity of different AM fungal species (*Glomus deserticola*, *Glomus intraradices*, *Glomus mosseae*, *Gigaspora gigantea*) in influencing the expression of Pi transporters in maize roots. The expression patterns of the two genes (*ZEAma:Pht1;3*, Pi starvation inducible, and *ZEAma:Pht1;6*, AM inducible) were quantified using real-time, reverse transcription polymerase chain reaction (real-time RT PCR). It was observed that expression of the two genes differed with inoculation treatment, and increasing the diversity of AM fungi in maize roots led to greater expression of *ZEAma: Pht;6* as well as Pi uptake in shoots. The percent root colonized by *Gigaspora gigantea* was significantly lower than the other four AM fungal inoculations. All AM fungal inoculations significantly increased the expression level of the AM-inducible Pi transporter (*ZEAma:Pht;6*) and decreased the Pi starvation-inducible Pi transporter (*ZEAma:Pht;3*) in maize roots. The expression of *ZEAma:Pht1;6* was higher in *G. mosseae* or *G. intraradices* compared to *G. deserticola* or *Gigaspora gigantea*, while the greatest repression of genes occurred in roots colonized by *G. mosseae*, *G. deserticola* and the AM mix. This also suggested that AM-inducible Pi transporter genes can be used as effective markers for a functional mycorrhizal Pi uptake pathway in plants (Poulsen et al. 2005; Javot et al. 2007). Recently, Estrada et al. (2013a, b) studied the expression of

different ion transporters and genes involved in water uptake in maize grown under saline conditions and demonstrated that the differential expression of *ZmAKT2*, *ZmSOS1* and *ZmSKOR* genes as well as chaperone and aquaporin genes (*GintBIP*, *Gint14-3-3* and *GintAQP1* genes) in maize plants led to the increased salt tolerance of the plants inoculated with *Glomus intraradices* collected from saline soil as compared to *G. intraradices* from the collection.

### *9.4.6 Native Vs. Exotic AM Fungi*

As opposed to the introduction of new AM fungal species in an ecosystem, the use of an inoculum based on locally sourced AM fungi may be a more suitable choice because of its better adaptation to the prevailing conditions (Lambert et al. 1980), and also it would avoid the ecological risks of the introduction of foreign species (Schwartz et al. 2006). Several studies have shown higher or similar plant growth and nutritional performances of locally sourced AM fungi as compared to foreign selected ones (Requena et al. 2001; Klironomos 2003; Caravaca et al. 2003; Tchabi et al. 2010; Pellegrino et al. 2011; Estrada et al. 2013a, b; Pellegrino and Bedini 2014). Klironomos (2003) tested the effect of multiple AM fungi isolates from native and non-native sources on the mycorrhizal plant-growth responses for a number of grassland species. He found that plant growth associated with AM fungi that naturally co-occurred with a species (native AM fungi treatment) ranged from highly parasitic to highly mutualistic, depending on the combination of plant and fungal species. Calvente et al. (2004) compared the effect of native strains of AM fungi (*G. intraradices* BEG 123, *G. mosseae* BEG 124, *G. clarum* BEG 125 and *G. viscosum* BEG 126) with two non-native AM fungi (*G. intraradices* and *G. mosseae*) on olive plants and observed that the native strains of *G. intrradices* and *G. viscosum* were most effective in improving the growth of two varieties of olives. Similarly, when Williams et al. (2013) treated rooted cuttings of an endemic New Zealand tree species (*Podocarpus cunninghamii*) and an exotic and invasive grass (*Agrostis capillaris*) with an indigenous, pot-cultured AM fungi (*Acaulospora laevis*) and an exotic commercial AM fungi product (*Glomus* spp.), they observed significant increases in plant growth rates and tissue concentrations of both nitrogen and phosphorus upon inoculation with indigenous AM fungi, while the commercial AM fungi had either no effect or a negative effect on host growth and nutrient levels. Sharma et al. (2009) studied the effect of *G. geosporum*, *G. microcarpum* and a native consortium of AM fungi on post-transplanting performance of 'in vitro' raised *Curculigo orchioides* plantlets and reported plantlets inoculated with the native consortium of AM fungi consistently performed better in terms of biomass accumulation and nutrient uptake. Pellegrino and Bedini (2014) evaluated the effectiveness of the inoculation of locally sourced and foreign/exotic AM fungi on chickpea (*Cicer arietinum* L.), cultivated under a rainfed low-input system. The foreign/exotic AM fungi *Funneliformis mosseae* and *Rhizophagus irregularis* were used as single and dual species inocula. Better overall yield performances of

chickpea inoculated with the local inoculum were recorded compared to the foreign/exotic AM fungi inocula.

In contrast, there are also reports where non-native AM fungi isolates have provided greater benefits to the hosts than the native ones (Requena et al. 2001; Tian et al. 2004; Schreiner 2007). Requena et al. (2001) found that inoculation with the exotic AM fungi *Glomus intraradices* promoted faster growth of *Anthyllis* than inoculation with a mixture of native AM fungi during the first year after seedling transplanting in a degraded semiarid area. Higher effectiveness of non-native isolate of *G. mosseae* over native AM fungi mix (*Glomus mosseae*, *Glomus intraradices* and *Scutellospora calospora* isolated from Jory soil) in promoting growth and nutrient uptake in 'Pinot noir' grapevine cuttings, growing in Jory soils, was also evidenced by Schreiner (2007). More recently, Garg and Pandey (2015, 2016) have found higher benefits with the exotic single isolates of *R. irregularis* and *F. mosseae* over the native saline mix isolated from saline soils in pigeon pea plants growing under salt-stressed conditions.

## 9.5 Different Strategies Driving Functional Diversity In AM Fungi

The functional diversity of AM fungi has been linked to different life history strategies employed by AM fungi species (Boddington and Dodd 1999; Thonar et al. 2011; Maherali and Klironomos 2012). For example, AM fungi species differ in the amount of carbon they extract from their host (Zhu and Miller 2003; Li et al. 2008; Olsson et al. 2010), their ability to acquire phosphorus (P) (Smith et al. 2000; Drew et al. 2003) and their nutrient-storage strategies (Kiers et al. 2011). These differences in life-history strategies likely dictate the nature of competition inside and outside the host. Specifically, AM fungi strains will compete intraradically for host-derived carbon (Herrera Medina et al. 2003) but also extraradically for available mineral nutrients (Johnson et al. 2003; Parniske 2008). The classification of AM fungi into functional groups or on the basis of their life-history strategy (LHS) has become a major field of interest in the last few years (Hart et al. 2001; van der Heijden and Scheublin 2007). The LHS concept aims to explain how organisms invest their resources under perturbed (i.e. unpredictable) or stable (i.e. predictable) environments. This positions organisms on an r-K continuum with the "r" endpoint representing a quantitative and the "K" endpoint representing a qualitative extreme (Pianka 1970; Begon et al. 1996). The K-strategists are organisms that evolved traits to enhance survival in stable and predictable environments where competition is high. K-strategists principally allocate resources to growth and enhanced survival (Pianka 1970; Begon et al. 1996). The r-strategists invest their energy mainly in the production of many offspring and evolved traits that are favoured in unstable environments. Life-history patterns have been determined by the amount of resources allocated to growth, survival and reproduction over time (Pianka 1970;

Begon et al. 1996). Based on these traits, it was suggested that AM fungi belonging to the Gigasporaceae resemble the LHS of K-strategists (de Souza et al. 2005). In contrast, Glomeraceae and in particular *Glomus* species show an opportunistic behaviour, similar to r-strategists (Sykorova et al. 2007). Ijdo et al. (2010) examined the effect of repeated defoliation of in vitro grown barrel medic (*Medicago truncatula*) on the spore and auxiliary cell (AC) production dynamics of a presumed r-strategist (*Glomus intraradices*) and a presumed K-strategist (*Dentiscutata reticulata*). Decreasing the host plant's photosynthetic ability (e.g. through defoliation, shading or reducing the number of hours of daylight) reduces AM fungal colonization of the root as well as spore production in the extraradical mycelium (Daft and El Giahmi 1978; Olsson et al. 2010). *G. intraradices* modulated the production of spores directly to C availability, showing direct investment in reproduction as expected for r-strategists. In contrast, spore production of *D. reticulata* was not affected after a single defoliation and thus showed higher resistance to fluctuating C levels, as expected for K-strategists (Ijdo et al. 2010). Recent work has shown that plants supply more carbohydrates to fungal partners that provide more phosphorus and *vice versa* (Hammer et al. 2011; Kiers et al. 2011; Fellbaum et al. 2012, 2014; Bucking et al. 2016) giving rise to the 'fair trade' in 'biological market theory' (Fig. 9.3a). Kiers et al. (2011) used the model plant *Medicago truncaluta* and three AM fungal species (*Glomus intraradices*, *G. custos* and *G. aggregatum*) to demonstrate this theory to explain the mutualism in AM symbiosis. These AM fungi exhibited either high or low levels of cooperation (symbiont quality), based on plant growth responses, costs of carbon per unit P transferred and resource-hoarding strategies. According to these traits, two species were classified as less-cooperative species directing more carbon resources either into storage vesicles—*G. aggregatum* or spores—*G. custos*, while *G. intraradices* was termed the cooperative species. Although colonization with all single species inoculation was above 80%, in two-species and three-species experiments, the cooperative fungus, *G. intraradices*, was significantly more enriched with host $^{13}C$ than both less-cooperative species of the same genus. The cooperative species also transferred more P to roots with greater access to C resources, confirming that fungi can discriminate among hosts differing in C supply. In contrast, the less-cooperative species, *G. aggregatum*, responded differently. Like the cooperative species, it transferred more P to the root compartment with access to more C, showing that it was able to assess and respond to the rate of C supply. However, this species predominantly stored the P resources in long-chained polyphosphates, a host-inaccessible form which potentially reduces P availability for competing fungi and P directly available for host uptake. The investigations thus illustrate key differences in fungal strategies, with *G. intraradices* being a 'reciprocator' and *G. aggregatum* a less cooperative 'hoarder'. Mensah et al. (2015) have also suggested that the high functional diversity within species of AM fungi is associated with differences in phosphate and nitrogen uptake and fungal phosphate metabolism. Recently, Walder and van der Heijden (2015) challenged the importance of reciprocally regulated exchange and thereby market dynamics, for resource exchange in the AM symbiosis, and suggested that such reciprocity is only found in

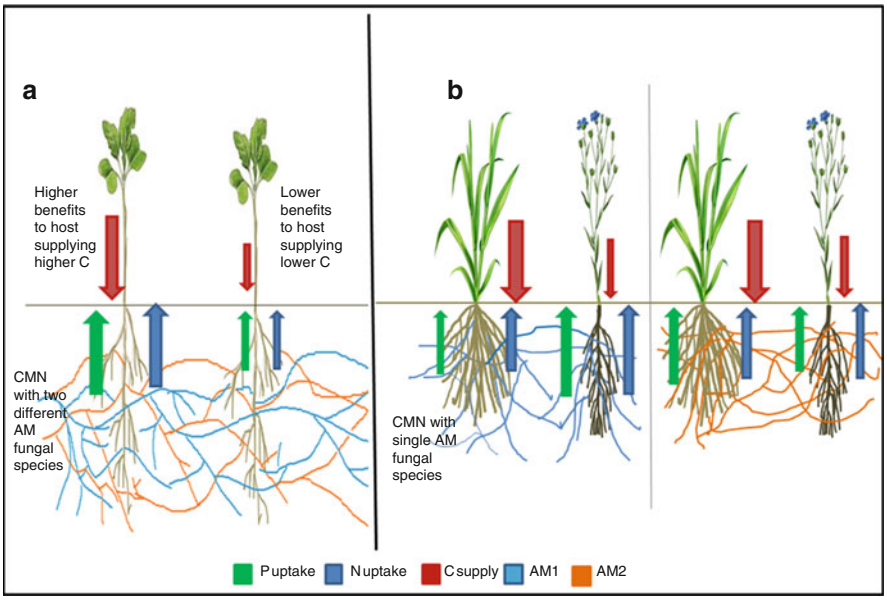


**Fig. 9.3** Two different approaches to explain the nutrient exchange and functional diversity in AM symbioses: (**a**) 'fair trade' in 'biological market theory' (Kiers et al. 2011) where different AM fungi discriminate among hosts and transfer more nutrients to hosts providing higher C supply and (**b**) 'unequal terms of trade' (Walder et al. 2012; Walder and van der Heijden 2015) wherein the symbiosis between plants and AM fungi is not so tightly controlled and instead is based on exchange of 'luxury goods'

a subset of symbionts, under specific conditions. Instead they proposed that it could be the exchange of luxury goods and sink strength that controls resource exchange in the plant—AM fungal symbiosis (Fig. 9.3). To study this, they set up microcosms containing sorghum and flax plants, interlinked by a common mycorrhizal network (CMN) of *Glomus intraradices* or *Glomus mosseae* and assessed the carbon investment of the two plants into the CMN through stable isotope tracing (Walder et al. 2012; Arguello et al. 2016). The plants' 'return of investment' (i.e. the acquisition of nutrients via CMN) using $^{15}N$ and $^{33}P$ as tracers was also calculated. They observed that nutritional benefit to the two host plants strongly depended on the fungus involved: in the case of *G. intraradices*, flax behaved as a 'cheater' on sorghum, acquiring 80% to 90% of the total labeled nitrogen and phosphorus provided by the CMN, whereas the acquisition of labelled nitrogen and phosphorus was more balanced in the case of *G. mosseae*. In mixed cultures containing both AM fungi, sorghum, in return for a similar expenditure of carbon, received much more phosphorus from *G. mosseae* than from *G. intraradices*, whereas for flax it was the inverse. This agrees with the theory that the symbiosis between plants and AM fungi is based on the exchange of 'luxury goods' (Kiers and van der Heijden 2006) with the symbionts offering luxury goods in exchange for more limited resources (Fig. 9.3b). Therefore, Walder et al. 2012 argue that the biological

trade is not simply reciprocal or 'fair exchange' as proposed by Kiers et al. (2011), rather it depends on transient sink strengths and the efficiency of exchanges at the various symbiotic interfaces which may differ for different plant—AM fungal combinations. However, the debate between the two groups regarding the nutrient exchange in the underground market continues with both groups citing evidences in their favour (Kiers et al. 2016; van der Heijden and Walder 2016).

## 9.6 Conclusion

AM fungi are one of the most abundant symbionts prevalent in the world ecosystems. However, this mycorrhizal association is not a homogeneous association; each association of plant and fungus species combination depends strongly on the particular partners involved. Each AM is essentially a phenotypic response to the different fungal and plant genotypes involved and the environment they inhabit. The AM fungi may vary in germination patterns, in hyphal traits, in nutrient uptake and transfer capacity as well as in symbiotic efficiency. This functional diversity is also often found to be reflected in the gene expression patterns. The functional diversity of AM likely results from the genetic structure of AM fungi, which is multi-genomic and composed of hundreds or thousands of nuclei with different genetic composition. The use of molecular studies has indicated high genetic diversity within a population and even within a single spore. This genetic variation of nuclei in a single spore affects genetic diversity at the population level and plays a major role in increasing the functional diversity of AM fungi in ecosystems. Thus, in order to better understand the functional diversity of AM, it is imperative to study the pattern of genetic variation in AM fungi. Not only genetic diversity, different life strategies employed by different AM fungi are also directly responsible for the functional diversity observed. In order to get a clear understanding on functional diversity and the factors controlling it, further studies on the variable responses of AM fungi under controlled conditions need to be studied along with genetic variation studies to correlate the two.

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# Chapter 10
# Mycorrhizal Symbiosis: Ways Underlying Plant–Fungus Interactions

**Shaily Javeria, Vivek Kumar, Pratibha Sharma, Lakshman Prasad, Manoj Kumar, and Ajit Varma**

**Abstract** Dissimilar and diverse symbiotic mycorrhizal interactions within plants and fungi occur, which is almost ubiquitous and universal, in the broad range of global ecosystems. The entire mycorrhizal communications achieve symbiotic functioning through development of an extensive contact surface area between plant and fungal cells, where exchange of nutrients and signals takes place. The swap of beneficial molecules within the plant and the fungal cytoplasm takes place both through their cell walls and the plasma membranes, having a purposeful chamber, known as symbiotic interface. Amongst all symbiotic interfaces, the arbuscular mycorrhizal (AM) relationship has intricate intracellular interface which gains major consideration since its first portrayal. It is dissimilar in ectomycorrhizae (ECM); here the fungus grows outside and inside the roots cell walls, which are constantly in direct contact and form interface within both the partners. The mycorrhizae are diverse fungi belonging to dissimilar fungal taxa and interact with roots around of 90% plant species and supply important nutrients for their growth. This also hypothesizes the flow of energy-rich composites required for nutrient mobilization and simultaneously transportation of mobilized products back to their host. Traditionally, these have chiefly been considered within pretty precise perspective of their effects on devouring dissolved mineral nutrients by plants. Enormous research work has been done which put emphasis on multifarious outlook of the mycorrhizal association with plant and also with associated microbial communities and ultimately on ecosystem processes. Consequently, the inputs of both partners in mycorrhizal association are starting to be decrypted to understand this knowledge for enhanced and progressive agricultural practices. The foremost aim of this chapter is to understand the prevailed information on mycorrhizal communications and interactions by integrating morphological observations with plants.

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## 10.1 Introduction

Bernhardt Frank in 1885 coined the term "mycorrhiza" by identifying some special structures in tree roots. "Mycorrhiza" obtained from two Greek words, viz., myco means fungus and rhiza means root. Frank described its morphology as well as its physiological role (Strack and Fester 2006).

Mycorrhizas are found in many environments, and their ecological success reflects a high degree of diversity in the genetic and physiological abilities of the fungal endophytes (Bonfante and Anca 2009). About 6000 species in the Glomeromycotina, Ascomycotina, and Basidiomycotina have been recorded as mycorrhizal, and the advent of molecular techniques is increasing this number (Bonfante and Anca 2009).

H. Anton de Bary in 1869 described "Symbiosis" as a long-term closed interaction between two or more biologically distinct species, which may range from mutualism to parasitism. Later this term was used only with mutualistic association organisms, viz., lichens (Smith and Read 2008). Root colonizing fungi are associated with more than 90% of terrestrial plants, establishing stable and closer mutualistic symbiosis known as mycorrhiza, which generate a huge hyphal network in the soil, which also associate with complete plant communities offering nutrients and energy flow within soil and plants (Cardon and Whitbeck 2007; Prasad et al. 2017), while the association and the relationships of roots and fungi are known as mycorrhizal associations, which are taking part in the nutrients absorption from the soil, and mostly found within fungal hyphae and plant's underground organs. This association is one of the most important associations in this planet (Mohammadi 2011). Mycorrhiza increases significantly in surface area of plant root by production of extensive hypha which enhances plant growth under relatively harsh conditions, viz., deficiency of nutrients and drought stress, etc. (Mohammadi 2011; Prasad et al. 2017).

### *10.1.1 Mycorrhiza: Plant–Fungus Communication*

Classically, a mycorrhiza is defined as an interaction from which both partners benefit. Generally, it is claimed that mycorrhizal fungi improve plant nutrient uptake thanks to fine exploration of the rhizosphere by the hyphae, which in return receive plant carbohydrates that are essential for completion of the fungal life cycle. This retains the concept of mutualism, i.e., an interaction of net benefit to both parties (Thompson and Cunningham 2002) and poses questions about the molecular mechanisms that allow nutritional exchange. A breakthrough on this front has come from some important findings: AM fungi possess active phosphate transporters that take up inorganic phosphate (Pi) from the soil, allowing its delivery to the plant (Harrison and Buuren 1995). Furthermore, plants also possess phosphate transporters that are mycorrhiza specific. Their role is to receive Pi from the fungus and

deliver it to plant cells. A *Medicago truncatula* Pi transporter exclusively expressed during AM symbiosis and located in the periarbuscular membrane (Harrison 2005) not only is essential for acquisition of Pi delivered by the AM fungus but is also required to maintain arbuscule vitality and sustain development of the fungus (Javot et al. 2007). Pi transport therefore seems to be a signal to sustain fungal growth inside the root and a determinant of arbuscule morphogenesis. Nitrogen is the other important element taken up by most mycorrhizal fungi. Genes involved in organic and inorganic uptake of N have been identified in AM and ECM fungi (Cappellazzo et al. 2008; Lucic et al. 2008; Smith and Read 2008). Many molecular and physiological data show that plant N transporters are activated during mycorrhization (Guether et al. 2009a, b; Smith and Read 2008), suggesting that mycorrhizal fungi release a substantial amount of N to their hosts. While these fungal and plant transporters may be used as clear markers of mycorrhizal function, the reverse nutrient flow is not so clearly characterized. Carbon transfer from plants to mycorrhizal fungi was demonstrated in the 1960s (Smith and Read 2008), but the molecular mechanisms are still unclear. With the exception of the gene described in the glomeromycotan *Geosiphon pyriforme* (Schüßler et al. 2006), which forms symbiosis with a cyanobacterium, and of the *AmMst1* gene from the ECM fungus *Amanita muscaria* (Nehls et al. 1998), no other hexose transporter responsible for the uptake of C released by host cells has so far been characterized in mycorrhizal fungi. In addition, the transfer does not always go in the expected direction; for example, in orchid mycorrhizas or in other heterotrophic plants, C moves from the fungus toward the plant (Selosse and Roy 2009). In this case, the nature of the benefit for the fungus is not obvious, although it might gain advantages, for example, by living within a protected niche.

A crucial consequence of nutrient exchange is that the partners must be living and in physical contact through their cell surfaces (Bonfante 2001). The result is a specialized interface that is particularly complex during intracellular interactions. Here, the fungus is in all cases engulfed by a plant-derived membrane, one result of a developmental program leading to intracellular accommodation of microbes by plants (Parniske 2000). In AM, this new compartment is known as the interfacial compartment and consists of the invaginated host membrane, cell wall-like material, and the fungal wall and plasma membrane (Bonfante 2001). Cellular and molecular approaches have provided many insights into the structure, function, and biogenesis of this complex compartment (Guether et al. 2009a; Harrison 2005; Parniske 2000). Some fungi, such as *Piriformospora indica*, are sometimes defined as mycorrhizal because of their capacity to stimulate plant growth, even if an interface between living partners is not always present and the fungus may surprisingly cause the death of plant cells (Deshmukh et al. 2006).

### *10.1.2 Fundamental Assortment of Mycorrhizal Interactions*

Arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) associations differ in their structural characteristics and in the plant and fungal species that they involve. In AM roots, the fungus penetrates intercellularly and intracellularly into the root cortex, whereas in ECM roots the fungus only penetrates intercellularly into the root cortex. The main structural differences between AM and ECM associations of angiosperms or gymnosperms are discussed (Fig. 10.1) (Bücking et al. 2012).

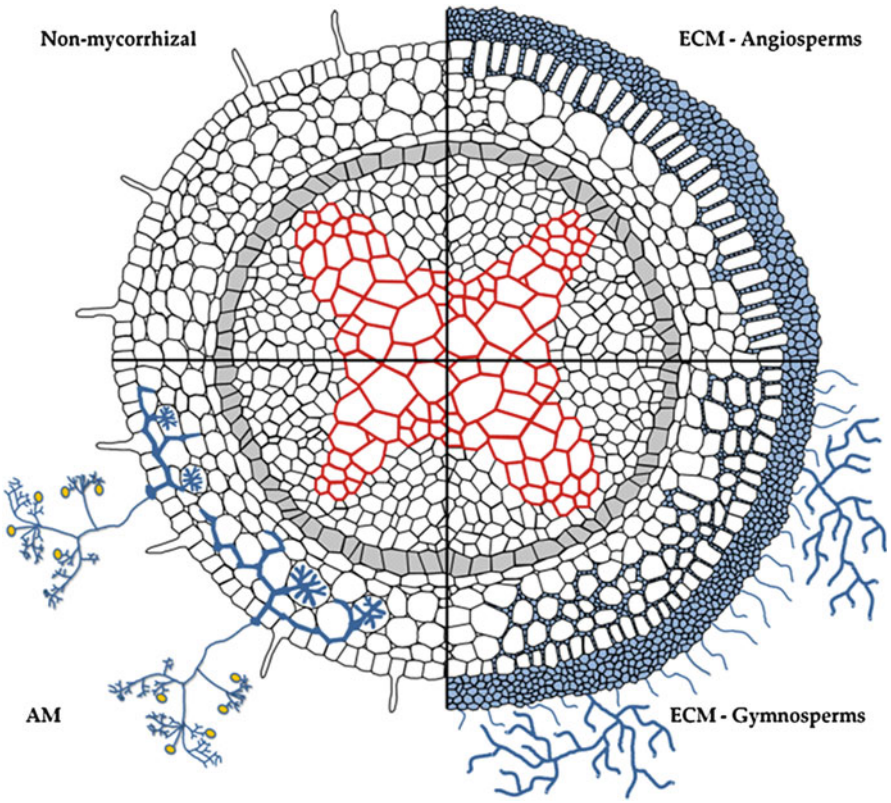


**Fig. 10.1** Arbuscular mycorrhizal (AM) structural characteristics or gymnosperms or angiosperms of ectomycorrhizal (ECM) root adapted from Bücking et al. (2012)

## 10.2 Arbuscular Mycorrhizal Fungi Symbiosis

Endomycorrhizas are further divided into orchid, ericoid, and arbuscular mycorrhizas (Smith and Read 2008). There are numerous types of mycorrhizas present in nature, but endotrophic arbuscular mycorrhiza (AM) is the most common type, present in large number of plant species. Nearly all herbaceous plants, shrubs, and trees of temperate and tropical habitats can form arbuscular mycorrhizas. "Vesicular-arbuscular mycorrhiza" (VAM) was the earlier term replaced by "Arbuscular mycorrhiza" (AM) because all endomycorrhizas didn't produce vesicles, but all produce arbuscules. The "arbuscular" as a name was derived from the distinct structure known as the arbuscules, which is present in the cortical cell of plants root (Smith and Read 2008). For the diagnostic purposes of AM symbioses, these structures are used.

The AM symbioses, formed between soil fungi and vascular plants, have a long history, with fossils providing evidence of AM fungi in the roots of the earliest land plants more than 400 m years (Remy et al. 1994). Sequence data and fossils of spores and hyphae point to the existence of AM fungi even earlier, more than 460 m years, and it is suggested that the AM fungi assisted plants in their colonization of land (Pirozynski and Malloch 1975; Redeker et al. 2000; Simon et al. 1993). Certainly, it is clear that the ability to form an AM symbiosis occurred early in the evolution of plants, and today the capacity to form these associations is distributed widely throughout the plant kingdom and includes angiosperms, gymnosperms, pteridophytes, and some bryophytes. More than 150 species of AM fungi belong to the Zygomycota which includes in the Glomales (Morton and Benny 1990).

Within the angiosperms, at least 80% of the species are able to form AM symbioses (Harley and Harley 1987; Newman and Reddell 1987; Smith and Read 1997) AM fungi are obligate symbionts that establish a symbiosis with the plant in order to obtain carbon, which enables them to grow and complete their life cycle. Their main contribution is to assist the plant with the acquisition of mineral nutrients, particularly phosphorus, and recently it was suggested that in an AM symbiosis, plants receive all of their phosphorus via their fungal symbiont (Smith et al. 2003). Phosphorus is an essential mineral nutrient that constitutes up to 0.2% (dry weight) of each plant cell and is thus required in significant quantities (Schachtman et al. 1998). In many soils, the concentration of phosphorus available to plants is limiting for growth (Holford 1997). Consequently, improvements in phosphorus acquisition have a significant impact on plant growth, health, and subsequently on plant biodiversity and ecosystem productivity (Smith and Read 1997). While enhanced plant mineral nutrition is of immense significance, other aspects of the AM symbiosis have far-reaching effects (Newsham et al. 1995). The extra radical phase of the arbuscular mycorrhiza includes meters of AM fungal hyphae that impact soil aggregate stability (Bearden and Petersen 2000; Requena et al. 2001; Rillig et al. 2003). Furthermore, AM fungi receive 100% of their carbon from the plant and this increase in carbon flow to the roots, estimated at up to 20%

of the plants' photosynthate, translates to a huge amount of carbon worldwide. Thus, the AM symbiosis also plays a significant role in carbon cycling between the atmosphere and biosphere (Bago et al. 2000; Zhu and Miller 2003). The perfect example of obligate symbionts is an AM fungus which totally depends on plant roots for reducing carbon, and in return they provide numerous benefits such as uptake of nutrients, etc (Entry et al. 2002). AM fungi take about 20% of photosynthetic product which allocate by plants and roughly equal to 5 billion tonnes of carbon provide to AM fungi by plants (Dahlgren et al. 2004; Ganry et al. 1985). In contrast to this important phenomenon, some mycoheterotrophic plants consume their source from mycorrhizal fungi. The mycorrhizal fungi actively play role in nutrient cycling; they also help in absorbing the nutrients from the soil as nitrogen and phosphate uptake and reduce the biotic (pathogens of root) and abiotic (heavy metals, salinity, and drought) stress of the host plant (Mohammadi 2011). In endomycorrhizas, no sheath is formed, and the fungi colonize the root cortex both intercellularly and intracellularly.

Ectotrophic and arbuscular mycorrhizal interactions are highly beneficial economically, and their applications and ecological significances are also very high. AM fungi colonize the roots of many agriculturally important plants (food and bioenergy crops). They may serve as biofertilizers and bioprotectors against pathogens and toxic stresses in environmentally sustainable agriculture (Bücking et al. 2002). Ectomycorrhizal fungi on the other hand colonize a smaller number of plant species, but play as symbiotic partners of tree and shrub species as a key role in forest ecosystems (Finlay 2008), and could be a critical component in phytoremediation or revegetation applications (Bücking 2011; Giri et al. 2007). In many cases, individual plants may found to be infected by multiple strains of mycorrhizal fungi (Gherbi et al. 2008; Akiyama et al. 2010; Maillet et al. 2011; Bonfante and Requena 2011) which increase host–plant growth.

## 10.3 Ecological Aspects

In accordance with the evolutionary history, AM symbioses can be found in almost all ecosystems. They have been described from deserts (Corkidi and Rincón 1997; Dalpé et al. 2000; Titus et al. 2002), tropical rainforests (Brundrett et al. 1999; Guadarrama and Alvarez-Sanchez 1999; Siqueira and Saggin-Júnior 2001; Zhao et al. 2001; Gaur and Adholeya 2002), aquatic environments (Khan 1993), as well as from ecosystems with strong saline (Carvalho et al. 2001; Sengupta and Chaudhuri 2002a, b), sodic, or gypsum soils (Landwehr et al. 2002). The relatively low number of plants colonized by AM fungi in some arctic and antarctic habitats seems to be due to a lack of suitable vectors for fungal spores rather than to other causes (Allen 1996). In addition to the global distribution of AM symbioses, there is large functional diversity as well. Whereas most AM symbioses are mutualistic, a growing number of non-photosynthetic plants are described, which are receiving a large portion of their nutrients from AM fungi (Imhof 1999; Yamato 2001),

resembling the functioning of orchid mycorrhizas (Rasmussen 2002). In some cases, these mycotrophic plants are living epiparasitically on other plants using the hyphae of their fungal partner for the transfer of nutrients (Bidartondo et al. 2002). On the other hand, AM fungi may become parasitic themselves in relation to their host plant under special circumstances (Allen 1996).

Fossil record suggests that the mycorrhizal symbiosis originated from the Ordovician, 450–500 million years ago (Redeker et al. 2000), and they also performed an important role in filling land with plants. The journey of mycorrhizal fungi starts with the germination of spore and growth of the fungal hyphae toward a host root (Martin and Nehls 2009). The cell of the plant prepares its intracellular environment (Handelsman 2004). For the exchange of nutrients, the parenchyma cortex of plant is primarily attacked by fungus.

There are two groups of Mycorrhizal fungi known till this era, viz., aseptate endophytes such as Glomeromycota and septate such as Basidio and Ascomycota (Smith and Read 2008). Anatomically, mycorrhizae are broadly of three major types: ectomycorrhizas, ectendomycorrhizas, and endomycorrhizas, which depend upon the colonization of mycorrhizal fungi on the root intercellular spaces or develop inside the cell (Bonfante and Genre 2008).

## 10.4 AM Fungi

The AM fungi are obligate biotrophs and depend entirely on the plant to provide them with carbon. It's considered to be asexual, although the hyphae of genetically distinct strains can anastomose and exchange genetic material (Hijri and Sanders 2005; Croll et al. 2009). Our inability to grow AM fungi in the absence of the plant has impeded the study of these organisms, and in comparison with other groups of fungi, relatively little is known about them. When not in association with a plant, AM fungi exist in the soil as resting spores, which in some species are large enough to be visible with the naked eye (Schüßler et al. 2001). Currently, little is known about their genetics or the organization of their genomes. Their resting spores are multinucleate, and analyses of the ribosomal DNA sequences of many species indicated unusually high levels of polymorphism at these loci (Clapp et al. 2001; Kuhn et al. 2001). Initially, AM fungi were classified as zygomycetes, and the morphological characteristics of their spores were used as taxonomic markers (Morton and Benny 1990). Recently, analyses of the small subunit rRNA sequences led to a reclassification and the creation of a new phylum, the Glomeromycota, a sister clade to the Ascomycota and Basidiomycota (Schüßler et al. 2001; Kramadibrata et al. 2000). Some analyses suggested that they are heterokaryotic, whereas other studies predicted that they are homokaryotic (Kuhn et al. 2001; Pawlowska and Taylor 2004). Estimation of genome sizes for these fungi varies greatly and most indicated large genomes (Hosny et al. 1998). In contrast, a recent study found that *Glomus intraradices*, a species that has been maintained in coculture with excised roots for many years (Bécard and Fortin 1988), has a haploid

genome of 15 Mb (Hijiri and Sanders 2004). The genome of this species is now being sequenced, which will provide significant insights into this ancient, obligate symbiont.

## 10.5 Organizational Features of AM Plant Roots and Fungal Life Cycle

AM fungi are obligate biotrophs and rely on their autotrophic host to complete their life cycle and to produce spores which are able to germinate without the presence of a host, but the spores respond with an increase in hyphal branching and metabolic activity to root exudates (Bücking et al. 2008; Gachomo et al. 2009; Tamasloukht et al. 2003). Plant roots release strigolactones like substances that are able to induce pre-symbiotic growth of AM fungal spores (Akiyama et al. 2010).

On the host root surface, AM fungi form a specific appressorium (hyphopodium) from this hyphopodium; fungal Hyphae penetrate into the root through the pre-penetration apparatus, which guides the fungal hyphae from root cells up to the cortex. In the cortex, the hyphae enter the apoplast, and grow laterally along the root axis, and penetrate into inner root cortical cells (Fig. 10.2). In "typical" AM associations, the fungus enters the cell by small hyphal branches that continuously branched and develops into highly branched arbuscules. By contrast, in some cases, the mycorrhizas spread the fungus primarily from cell to cell and develops extensive intracellular hyphal coils that sometimes show an arbuscular like branching (Smith and Read 2008). The fungus does not enter the plant symplast and is excluded from the host cytoplasm by the enlarged periarbuscular membrane (PAM) of the host. Some fungi also form vesicles, fungal storage organs in the root apoplast (Bücking 2011).

Despite its coenocytic nature, the mycelium that is formed within the root, the intraradical mycelium (IRM) differs morphologically and functionally from the extraradical mycelium (ERM), the mycelium that grows into the soil (Bücking et al. 2012). The ERM absorbs nutrients from the soil and transfers these nutrients to the host root. The IRM on the other hand releases nutrients into the interfacial apoplast and exchanges them against carbon from the host. The fungus uses these carbon resources to maintain and enlarge the ERM, for cell metabolism such as active uptake processes, nitrogen assimilation, etc. and for the development of spores, which are able to initiate the colonization of a next generation of host plants (Bücking et al. 2012).

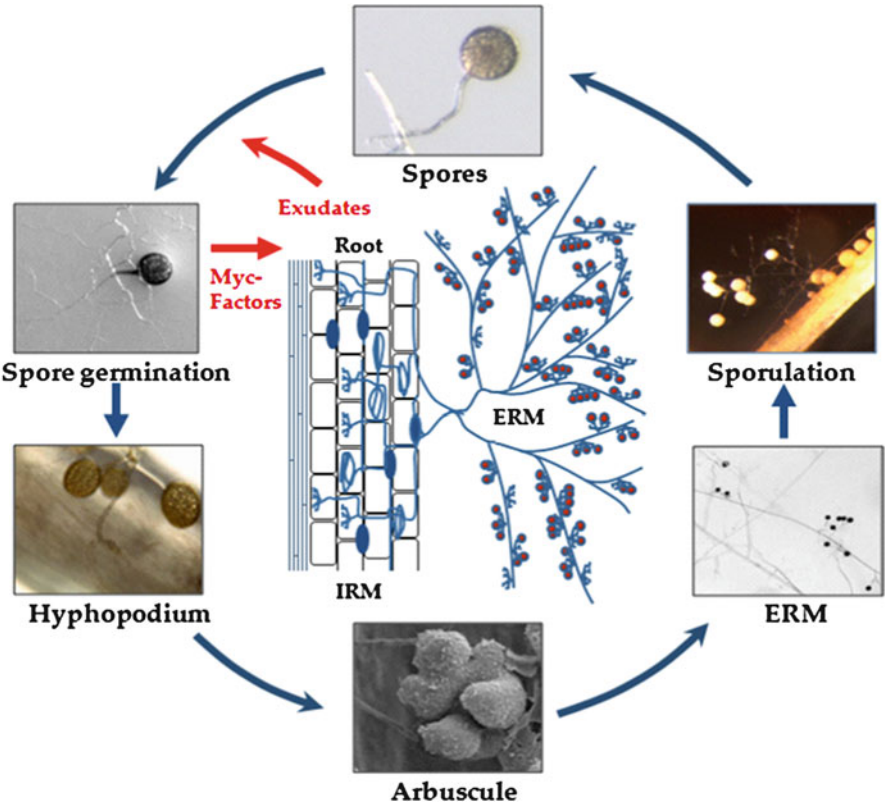


**Fig. 10.2** Life cycle of an AM fungus and the different steps during AM development (Bücking et al. 2012)

## 10.6 Root Colonization with AM Fungi

Similar to Nod factors that play an important role in root nodulation, AM fungi release Myc factors that lead to an expression of plant symbiosis related genes and prepare the root for AM symbiosis. One active Myc factor has been identified as lipochitooligosaccharide (Maillet et al. 2011). Nod factors are also lipochitooligosacccharides and have a similar composition. It has been suggested that Nod factors developed from Myc factors, and that the functions of Myc and Nod factors overlap (Bonfante and Requena 2011). This is also supported by the fact that AM and rhizobial symbiosis share parts of the same signal transduction pathway—the so-called common symbiosis pathway. So far seven genes (SYM genes) of the common symbiosis pathway have been identified that are required for both root symbioses.

### *10.6.1 Plant Root Colonization and Morphological Changes*

The process starts with germination (hyphal growth) of fungal spores, followed by poorly understood events. Subsequently, appressoria are formed from which the fungus penetrates the root surface and colonizes the intercellular space of the root cortex. On the fungal side, nonaggressive cell wall-lytic enzymes become active, and both the plant root cells and the fungus change their gene expression pattern and morphology. The hyphae penetrate the cell walls and develop within the cortex cells tree-like structures, called arbuscules, by repeated dichotomous branching. In some cases, intercellular storage organs, lipid-rich vesicles, and finally extraradical spores are formed, which may enter another colonization process. Fungal root colonization is under control of the plant aiming at a morphological and functional compatibility of the two partners (Bonfante and Perotto 1995). The key feature of AMs is the arbuscule, a highly branched haustorium-like structure within root cortex cells, responsible for nutrient exchange. However, the arbuscules represent a dead-end in the growth of AM fungi (Bonfante and Perotto 1995), and they finally senesce and collapse after 4–10 days of symbiosis (Sanders et al. 1977), possibly caused by the continuously stressful environment of the host cortex cell (Harley and Smith 1983).

Formation of arbuscules is accompanied by alterations in morphology of the host cell: the central vacuole is fragmented; the volume of cytoplasm and number of cell organelles increase significantly, and the nucleus moves into a central position and undergoes hypertrophy (Balestrini et al. 1994). The host cytoplasm and cell organelles proliferate around the branching hyphae. The number of plastids in colonized cortex cells increases (Bonfante and Perotto 1995) and networks are formed covering the arbuscules (Fester et al. 2001; Hans 2003). The plastids in these networks are connected to each other by so-called "stromules" (stroma-filled tubules) (Köhler and Hanson 2000). It has been shown that microtubules are involved in changes of host cell morphology and cytoplasmic architecture.

Four types of microtubule patterns were observed in arbusculated cells: (1) long bundles of microtubules crossing the cytoplasm among the arbuscule branches and passing through the arbuscule; (2) short microtubules connecting fine arbuscule branches or connecting arbuscule branches either to the cortical region of the cell or to the cell nucleus; (3) bundles of microtubules in the periphery (cortical region) of the host cell and along the hyphal trunk; and (4) perinuclear bundles of microtubules.

## 10.7 Mycorrhizal Interface in AM Interactions

The interface compartment that develops between the plant and the fungus is continuous with the peripheral plant cell wall (Bonfante and Perotto 1995). Although the fibrillar interface differs from the peripheral plant cell wall in

structure, its components reflect the composition of the wall of the host cell that is being invaded. This mixture of primary plant cell wall components indicates that the arbusculated plant cells have maintained their abilities to synthesize and secrete cell wall material. That this material does not assemble further to build up a secondary wall might be the result of lytic activities of the fungus (Peretto et al. 1995). When the arbuscule begins to senesce, the fibrillar material encapsulates the collapsed fungal structures that are then degraded completely by the plant cell. Subsequently, the cells regain their original morphology (Jacquelinet-Jeanmougin et al. 1987) and are able to allow another arbuscule formation.

Some processes of AM establishment are known to be mediated by phytohormones on the plant side, as suggested by application experiments (Barker and Tagu 2000). The levels of cytokinins are higher in shoots and roots of mycorrhizal plants compared to non-mycorrhizal ones (Allen et al. 1980). A possible role of abscisic acid was suggested from the fact that its level increases in AM roots (Danneberg et al. 1992; Bothe et al. 1994). Jasmonic acid applied exogenously promotes colonization and development of mycorrhizal structures (Regvar et al. 1996). The observed endogenous rise of jasmonates in barley roots correlating with myorrhization, however, is more indicative for a role in AM (Hause et al. 2002).

Critical for the mutualism in the AM symbiosis is the bidirectional exchange of nutrients across the mycorrhizal interface. The interface between the fungus and the host includes the PAM and the fungal plasma membrane, the fungal cell wall, and the periarbuscular space between the fungal cell wall and the PAM. The PAM differs in its protein composition from the plant plasma membrane of non-arbusculated cells and is characterized by mycorrhiza-inducible transporters that facilitate the uptake of nutrients from the mycorrhizal interface. One of these transporters is Pt4, a high affinity phosphate (P) transporter that is only expressed in mycorrhizal roots and that is involved in the acquisition of P delivered by the fungus (Dewbre et al. 2002). A high-affinity ammonium ($NH_4^+$) transporter (AMT2;2) is also localized in the PAM. This transporter is exclusively expressed in arbusculated cells of mycorrhizal roots, but not in root nodules (Guether et al. 2009b). In contrast to other high affinity $NH_4^+$ transporters of plants, AMT2;2 of *Lotus japonicus* (LjAMT2;2) transfers $NH_3$ instead of $NH_4^+$, and it has been suggested that the transporter takes up the positively charged $NH_4^+$ from the mycorrhizal interface and releases uncharged $NH_3$ into the plant cytoplasm. The detection of mycorrhiza-inducible sulfate transporters in AM roots suggests that also sulfate is transferred from the AM fungus to the host across the mycorrhizal interface (Casieri et al. 2012; Allen and Shachar-Hill 2009). The transport of carbon from the host to the fungus is driven by a monosaccharide transporter in the fungal arbuscular membrane (MST2) (Helber et al. 2011). This transporter takes up glucose but also other monosaccharides, such as xylose, what indicates that the fungus can also use cell wall sugars of the plant as alternative carbon source.

### 10.7.1 The Ect-endomycorrhiza

In ect-endomycorrhizas, the sheath may be reduced or absent; the Hartig net is usually well developed, but the hyphae penetrate into the cells of the plant. As already mentioned, the same species of fungus may form ectomycorrhizas on one species of plant and ect-endomycorrhizas on others. Arbutoid mycorrhizas possess sheath, external hyphae, and usually a well-developed Hartig net (Mohammadi 2011).

### 10.7.2 The Ectomycorrhizal Fungi

Ectomycorrhizas are very much important in numerous boreal and temperate forests which contributed approximately 30% of total microbial biomass of forest soils. Ectomycorrhizas are present in some families of woody gymnosperms (e.g., *Pinaceae*) and angiosperms (e.g., Betulaceae, Dipterocarpaceae). In ectomycorrhizas, the mycorrhizal fungi form a structure known as mantle (sheath) which encloses the rootlet (Mohammadi 2011).

Hyphae also penetrate inside the cells of the root to form a complex intercellular system, which appears as a network of hyphae in section known as the Hartig net, where a minute or no intracellular penetration takes place. In a few plants, the development of the Hartig net is negligible (Smith and Read 2008).

There are approximately 7000 to 10000 fungal species and 8000 plant species that form ectomycorrhizal (ECM) associations (Taylor and Peterson 2005). The number of plant species is relatively small (approximately 3%), but the group includes plants with high global and economic importance due to the disproportionate large terrestrial land surface that these plants cover and as main producers of timber. The plant species include wooden perennials, trees, or shrubs from cool, temperate boreal or montane forests, but also species from arctic alpine shrub communities (Smith and Read 2008; Tamasloukht et al. 2003). However, most of these plant species are not exclusively colonized by ECM fungi. Many species, such as *Populus*, *Salix*, *Betula* and *Fagus*, also form AM interactions, and there are indications that the AM symbiosis is the common mycorrhizal form of this taxon (Smith and Read 2008).

ECM fungi are relatively closely related to saprotrophic fungi and mainly belong to the Basidiomycota (e.g., *Amanita muscaria*, *Hebeloma cylindrosporum*, *Laccaria bicolor*, *Paxillus involutus*, *Pisolithus tinctorius*, *Suillus bovinus*, *Xerocomus badius*), but also include some Ascomycota (e.g., *Cenococcum geophilum*, *Tuber borchii*, *Scleroderma hypogaeum*) (Smith and Read 2008). The switch from the presumably ancestral saprotrophic to the symbiotic behavior developed convergently in several fungal families during evolution. In contrast to AM fungi, many ECM fungi can be grown in axenic culture without a host, and this has allowed screening of their ability to use different carbon or nutrient sources

(Salzer et al. 1997). ECM fungi have a dual life style and are considered to be facultative saprotophs. In the soil, they are highly competitive in nutrient acquisition and secrete a number of hydrolytic enzymes that allow them to degrade litter polymers and to use organic nutrient sources (Finlay 2008). At the same time they live within plant roots as symbionts, and this requires a set of adaptation mechanisms to avoid plant parasitism. ECM fungi have for example lost their ability to degrade plant cell wall polysaccharides (cellulose, pectins, and pectates), and this restricts their penetration into the root to the intercellular spaces (Martin and Martin 2010).

#### 10.7.2.1 Root Colonization with Ectomycorrhizal Fungus

Typical for ECM roots are changes in the root morphology, such as the dichotomous branching of lateral roots, e.g., in pines, the production of a large number of root meristems and as a result an extensive root branching, inhibition of root hair formation, and the enlargement of cortical cells. Many of these morphological effects can be observed prior to colonization and can be interpreted as a preparation of the plant to increase root symbiosis.

Prior to the establishment of a functional ECM root and similar to the processes during AM development, there is an exchange of signals and cross-talk between both partners. The fungal tryptophan betaine hypaphorine has been shown to trigger reduced root hair elongation and swelling of the root hair tip and a stimulation of short root formation (Tamasloukht et al. 2003). ECM fungi also produce phytohormones, including auxins, cytokinins, abscisic acid, and ethylene, and it has been shown that the changes in the root morphology are caused by an overproduction of auxin in ECM fungal hyphae and changes in the endogenous hormone levels in the roots. The effect of ECM fungi on lateral root formation is independent from the plant's ability to form ECM associations. The ECM fungus *Laccaria bicolor* can induce lateral root formation also in *Arabidopsis thaliana*, a non-mycorrhizal plant, and the effect is correlated to an accumulation of auxin in the root apices (Felten et al. 2009). The auxin accumulation in the root tips and/or other fungal signals could stimulate basipetal auxin transport and lateral root primordia formation by an induction of plant genes involved in auxin transport and signaling.

The fungal partner responds to root exudate components, such as rutin and zeatin, with stimulation in hyphal growth and branching and growth towards the root and an accumulation of hypaphorine (Martin and Martin 2010). In response to host signals, ECM fungi also release effector proteins into the rhizosphere, such as nucleus after its uptake, and alter plant gene expression (Plett et al. 2011). *MiSSP7* has been shown to be crucial for the establishment of the ECM symbiosis and resembles effector proteins of pathogenic fungi and bacteria with similar function. A transcriptional response of the host can be observed within hours after an initial contact between both partners has been established. Plant genes encoding proteins involved in stress and defense response, as well as genes involved in signal

transduction and communication, and water uptake are upregulated in response to the presence of an ECM fungus in the rhizosphere (Sebastiana et al. 2009).

#### 10.7.2.2 Organizational Characters of Ectomycorrhizal Roots

An established ECM symbiosis is characterized by three structural components: the hyphal sheath or mantle, the Hartig net (in later passages of this text sometimes also referred to as intraradical mycelium or IRM), and the extraradical mycelium. The hyphal sheath or mantleen closes the root completely. The structural composition of the mantle is very diverse and can range from relatively thin, loosely arranged assemblages of hyphae to very thick, multilayered and pseudoparenchymatous mantles. The surface of the mantle can be compact and smooth or rough with numerous emerging hyphae and hyphal strands or rhizomorphs. The fungal sheath is involved in nutrient storage and controls the nutrient transfer to the host. The fungal mantle can represent a significant apoplastic barrier (Bücking et al. 2002; Ashford et al. 1988) and thereby creates a closed interfacial apoplast, in which the conditions can be controlled by both partners.

The Hartig net plays the key role in the nutrient transfer between both partners. The Hartig net is formed by hyphae that penetrate into the root cortex intercellularly. The penetration depth of the Hartig net differs between angiosperms and gymnosperms. Most angiosperms develop an epidermal Hartig net and confine the penetration of the Hartig net to the outer epidermis, which is often radially elongated. By contrast, the Hartig net in gymnosperms normally encloses several layers of cortical cells and sometimes extends up to the endodermis (Smith and Read 2008).

The extraradical mycelium (ERM) of the fungus acts as an extension of the root system, and it has been estimated that the ERM of the fungus *Pisolithus tinctorius* can represent 99% of the nutrient-absorbing surface length of pine roots (Ashford et al. 1988). The ERM of ECM fungi can account for 32% of the total microbial biomass and 700–900 kg $ha^{-1}$ in forest soils (Högberg and Högberg 2002). The ERM can have a relatively simple organization with individual hyphae with similar structure that grow into the soil (mainly in ascomycetes) or can be differentiated into singular hyphae and rhizomorphs. Rhizomorphs are aggregates of hyphae which grow in parallel and whose organization level can range from simple assemblages of undifferentiated and loosely woven hyphae to complex aggregations of hyphae with structural and functional differentiations (Agerer 2001).

#### 10.7.2.3 Mycorrhizal Interface in Ectomycorrhizal Links

Transport studies suggest that in ECM associations, nutrients are exchanged simultaneously across the same interface (Bücking and Heyser 2001). The interface includes the plasma membranes and cell walls of both partners and the interfacial matrix between both partners. The plant transfers photosynthates as sucrose from

source to sink organs and ECM roots act as strong carbon sinks in mycorrhizal root systems. It is generally accepted that in contrast to phytopathogenic fungi or ericoid mycorrhizal fungi, AM and ECM fungi are not able to use sucrose as a carbon source, and that they take up simpler sugars, such as glucose or fructose, from the mycorrhizal interface. The presence of invertase genes in fungal genomes is correlated with the nutritional mode and in contrast to other plant-associated fungi, such as pathogens or endophytes, there are no indications that AM or ECM fungi possess invertase genes (Parrent et al. 2009) or have invertase activity (Salzer and Hager 1991). Consequently, mycorrhizal fungi rely on the invertase activity of the host in the interfacial apoplast for sucrose hydrolysis. Sucrose hydrolysis makes the hexoses glucose and fructose available for the fungus, and it has been suggested that glucose is mainly taken up by hyphae of the Hartig net and fructose mainly by hyphae of inner mantle layers (Nehls et al. 1998). Compared to the ERM, fungal hexose transporters are upregulated in ECM roots, indicating that the fungus in symbiosis takes up carbon primarily from the mycorrhizal interface (Lopez-Pedrosa et al. 2006).

The high affinity $NH_4^+$ importer *AmAMT2* of *Amanita muscaria* is upregulated in the ERM, but downregulated in Hartig net and the fungal sheath (Willmann et al. 2007). The high expression of this transporter in the ERM suggests a high capability of the ERM for $NH_4^+$ uptake. The low expression level in the Hartig net on the other hand indicates that $NH_4^+$ can serve as a potential nitrogen source that is delivered by the mycorrhizal fungus to the host. A low expression level of this $NH_4^+$ importer in the Hartig net would reduce the re-absorption of $NH_4^+$ by the fungus from the interfacial apoplast and increase the net transport of $NH_4^+$ to the host. The potential transport of $NH_4^+$ across the ECM interface is also supported by the presence and upregulation of plant high affinity $NH_4^+$ importers in ECM roots (Selle et al. 2005).

## 10.8 Phosphorus Uptake Improvement

The AM symbiosis is a highly compatible association, and in phosphate-limiting conditions, intraradical development of the fungus can occur in more than 80% of the root length. In addition to the intraradical growth phase, the fungus also maintains an extraradical mycelium that can extend several centimeters from the root (Mohammadi 2011). The fungal hyphae within the root are connected to the extraradical mycelium and form a single continuum. The extraradical hyphae acquire phosphate, initiate the colonization of other roots, and, in most species, are also the site of sporulation. Phosphate is delivered to the plant across the arbuscule cortical cell interface, and, recently, plant phosphate transporters involved in this process were identified (Harrison et al. 2002; Paszkowski et al. 2002; Rausch and Bucher 2002). Although not proven directly, it is anticipated that carbon is taken up by the arbuscule. The arbuscule cortical cell interface shares some structural and functional similarities with the endosymbiotic interfaces of other plant–microbe endosymbioses including the symbiosome, the symbiotic

interface of the rhizobium-legume symbiosis, and the haustorial-plant interface formed by the biotrophic fungal pathogens (Smith and Smith 1997; Parniske 2000).

Under conditions of low P availability, which occur in many soils, the AMF mediated transfer of nutrients has been reported from the host plant to another plant. Hyphae of mycorrhizas may spread from one infected plant and enter the roots of one or more other plants (Heap and Newman 1980). It has been shown that assimilates may be transported from one plant to another through AM hyphal connections. In a study, transfer of 14C photosynthate from one plant to another was found primarily through AM hyphae rather than leakage from the roots of the donor plants. Similar results were obtained in a 32P experiment, where hyphal linkage between plants was the dominant factor for transferring P (Chiariello et al. 1982). Ganry et al. (1985) conducted an experiment to investigate the effect of P fertilization on AM colonization and BNF, and based on the results of a preliminary pot study, field site is selected with a low colonization potential. In the absence of P fertilizer or in the presence of insoluble rock phosphate, there were no significant differences in AM colonization between -AM and +AM treatments, but when soluble superphosphate fertilizer was applied, AM colonization of inoculated roots at 26 days was greater than for the −AM treatment. These early differences disappeared by day 40 with AM frequencies 490% in all treatments. As mentioned, the most prominent effect of AMF is to improve P nutrition of the host plant in soils with low P levels due to the large surface area of their hyphae and their high affinity P uptake mechanisms. To substantiate this concept of plant growth promotion by AMF, several studies have shown that AM fungi contribute to up to 90% of plant P demand (Vander Heijden et al. 1998).

## 10.9 Mitigation of Environmental Stresses

Soils rarely provide ideal conditions for growth and survival of plants and soil microorganisms. Since soil conditions are constantly changing, the soil environment may favor development of arbuscular mycorrhizas at one point in time and inhibit them at another time. AM fungi have an important role in promotion of biological and chemical properties of plants under stressed environment (Mohammadi 2011). AM help plants to adapt to and resist a wide range of biotic and abiotic stresses they encounter in the environment. Adequate soil moisture and temperature may favor development of arbuscular mycorrhizas. However, when soil moisture or temperature becomes too high or low, mycorrhizal formation may be inhibited. AMF may alter the metal concentration in plants by metal immobilization in intra- or extra-radical hyphal cell wall components, metal chelation by fungal secreted compounds, such as glomalin, or by metal compartmentalization inside fungal cells. Thus, these AMF act as metal sinks, reducing local concentrations in soils and creating a more suitable environment for plants growing in soils with high metal contents. At molecular level, some reports show that the expression of plant genes related to metal tolerance was altered by mycorrhizal colonization

(Andrade et al. 2010). Arbuscular mycorrhizal fungi may affect host plant function and productivity under both high and low moisture conditions in greenhouse studies; drought-stressed maize infected with *Glomus mosseae* had higher concentrations of glucose, fructose, and total amino acids in leaves and roots than non-mycorrhizal plants (Schenck and Smith 1982). After applying periods of drought stress of varying length and severity, arbuscular mycorrhizal colonization increased leaf area, total plant and root biomass, number of tillers, and grain yield of wheat.

The arbuscular mycorrhizal symbiosis may alleviate plant responses to moderate moisture deficit by several mechanisms including increased water uptake from the soil by hyphae, altered hormonal levels, causing changes in stomatal conductance, increased turgor by lowering leaf osmotic potential, improved nutrition of the host, and improved plant recovery after drought by maintaining the soil-root continuum (Entry et al. 2002). AM fungi can enhance plant growth under salinity stress, especially in soils with low level of P and are able to enhance plant tolerance under salinity through altering plant physiology and increasing water and nutrient uptake. For example, mycorrhizal plants absorbed less amounts of $Na^+$ and $Cl^-$ or inhibit their transfer to the shoots resulting in the increased dry weight of cotton by 68% under the salinity of 3 g/kg (Tian et al. 2004).

## 10.10 Mitigation of Heavy Metals Stress

Heavy metal uptake and tolerance depend on both plants and soil factors including soil microbes; therefore, information on interactions between plant roots and their symbionts such as AM fungi is required in order to understand heavy metal effects. Only few plants (the metallophytes) can cope with the adverse conditions on heavy metal soils. Availability and toxicity of metals to plants and mycorrhizal fungi varies, depending on the actual concentrations and oxidation states of the metals; soil and rhizosphere pH; and soil cation exchange capacity, CEC, texture, organic matter content, and redox potential. In roots, metals such as aluminum can impair cell division, increase cell wall rigidity, alter root respiration, precipitate nucleic acids, and interfere with the uptake and transport of Ca, Mg, P, and Fe. Fungal hyphae sequester metals, which may serve to reduce movement into and toxicity to the host stress tolerance. Detoxification mechanisms enable the plant and fungus to avoid toxic effects (Entry et al. 2002). Most reports note a positive effect of mycorrhizal inoculation on growth of plants in metal-contaminated soils. This protective benefit may be related to the adsorptive or binding capability for metals of the relatively large fungal biomass associated with host plant roots, which may physically minimize or exclude the entry of metals into host plants.

Several biological and physical mechanisms have been proposed to explain the generally lower metal toxicity to plants colonized by arbuscular mycorrhizal fungi. These include adsorption onto plant or fungal cell walls present on and in plant tissues or onto or into extraradical mycelium in soil (Joner et al. 2000; Meharg and

Cairney 2000) chelation by such compounds as siderophores and metallothionens released by fungi or other rhizosphere microbes and sequestration by plant-derived compounds like phytochelatins or phytates. Other possible metal tolerance mechanisms include dilution by increased root or shoot growth, exclusion by precipitation onto polyphosphate granules, and compartmentalization into plastids or other membrane-rich organelles (Entry et al. 2002).

Metallophytes have developed various different physiological adaptations which enable them to compete successfully with the harsh conditions in heavy metal soils.

In addition, protection by AMF that colonize plant roots and considerably reduce the uptake of heavy metals into plant cells may be one of the means that allow metallophytes to thrive on heavy metal-polluted sites (Ouziad et al. 2005; Vogel-Mikus et al. 2006). For example, both zinc violets are strongly colonized by AMF, and leaves of *Viola lutea* ssp. *calaminaria* collected from a heavy metal site were earlier found to contain low amounts of heavy metals in ranges similar to those detected in non-metallophytes. This correlation is not likely to be coincidental, since mycorrhizal colonization of the roots increases with increasing heavy metal content of the soil. Since under adverse conditions, AM might be more important for plant metal resistance and under the optimized conditions of normal agricultural practice; however, AM colonization even could increase plant absorption from polluted soil and cleansed polluted sites by removing aboveground parts. It is suggested that metal-tolerant mycorrhizal inoculants might be considered for soil reclamation; thus, *G. caledonium* might be a promising mycorrhizal fungus for bioremediation of heavy metal-contaminated soil (Mohammadi 2011).

## 10.11 Conclusions

It should be apparent from the preceding discussion that different types of mycorrhizal symbioses show fundamental aspects in the frame of the terrestrial ecosystems and that the distinctive plant communities lead the major terrestrial biomes of the today's world because different kinds of symbiotic associations have been favored by selection that are adapted functionally to prevalently lodging of climatic and edaphic conditions characterizing different environments. The primary producer (plants) of an ecosystem is connected by mycorrhizal fungi which are its main significance to the distribution of required nutrients for their growth and also facilitate the flow of energy needed for nutrient mobilization and translocation of mobilized products backward to their hosts. This process enlightens the way of mycorrhizal fungi role in regulating the biogeochemical cycles. Old views of mycorrhizal symbiosis that are entirely based on the mineral nutrition of individual plants are hence giving way to new theories with extensive functional basis, making use of major ecologically relevant species and substrates. Comparative analysis of diverse systems will enhance our understanding of responses to environmental and climatic perturbations. New molecular tools have empowered identification of mycorrhizal fungal symbionts with more advance degree of resolution and have

contributed to the realization that the degree of functional specificity in mycorrhizal associations may be much greater than hitherto appreciated. This new knowledge is an imperative prerequisite for future, sustainable management of terrestrial ecosystems.

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# Chapter 11
# The Management of the Mycorrhizal Soil Infectivity: Ecological and Technical Approaches

**Adrien Lies, Yves Prin, Robin Duponnois, and Hicham Ferhout**

**Abstract** Arbuscular Mycorrhizal Fungi have a large potential to help increase global food security. They constitute the most important microbial symbiosis for the majority of terrestrial plant species. Their ecological functions in the productivity and stability of agroecosystems have been recognized for many years. Many studies have shown that these symbionts improved plant growth and plant resistance to biotic and abiotic stresses. Despite the proven potential of mycorrhizal symbiosis to sustainably improve the productivity of agroecosystems, this biotechnology is still under exploited. This failure mainly results from technical difficulties to mass-produced fungal inoculum of high quality and a lack of knowledge about the biological factors regulating the soil receptivity of arbuscular mycorrhizal inoculation. In order to promote mycorrhizal soil infectivity, two main approaches could be considered: (1) the "reductionist" approach that consists to add into the soil, a large quantity of fungal propagules of a specialized AMF and (2) the "holistic" approach that aims to conserve and restore native AMF diversity and abundance. In this chapter, we will examine the environmental factors that affect the mycorrhizal diversity and abundance and limit both approaches as they can both be of interest, trying to explain to what environmental solution they would be more adapted.

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## 11.1 Introduction

Producing enough food to feed a global human exceeding 7 billion, and estimates to reach 9 billion by 2050, has become the main challenge leading to a global crop yield increases of up to 100% (Godfray et al. 2010). The current global productivity of agrosystems is largely not efficient enough to reach these objectives of such yield increases. In addition, increasing crop resource use efficiency (yield per unit of resource input) has to be achieved by following the recommendations of the "doubly green revolution" that combines the objectives of the Green Revolution and the maintenance of biological diversity and ecosystem resilience but without decreases of actual yields. Hence, it becomes urgent to identify new technologies and better apply long-known agricultural practices (Bennett et al. 2013).

The main environmental factors that limit increasing crop yields are the poor soil fertility and particularly the availability of nitrogen and phosphorus (Tilman et al. 2002). Among the soil microbial components, it is well known that the arbuscular mycorrhizal fungi represent a potential low-input solution to increasing the overall yield of important stable crops resulting from their positive impacts on phosphate acquisition, water stress, or disease resistance (Rodriguez and Sanders 2015).

About 80% of all plant species, including most agricultural crops, can form arbuscular mycorrhizal (AM) symbiosis in all major terrestrial ecosystems (Opik et al. 2006). AM symbiosis (Fig. 11.1) plays a major role in soil fertility and plant nutrition and in the maintenance of stability and biodiversity within plan communities (Smith and Read 2008).

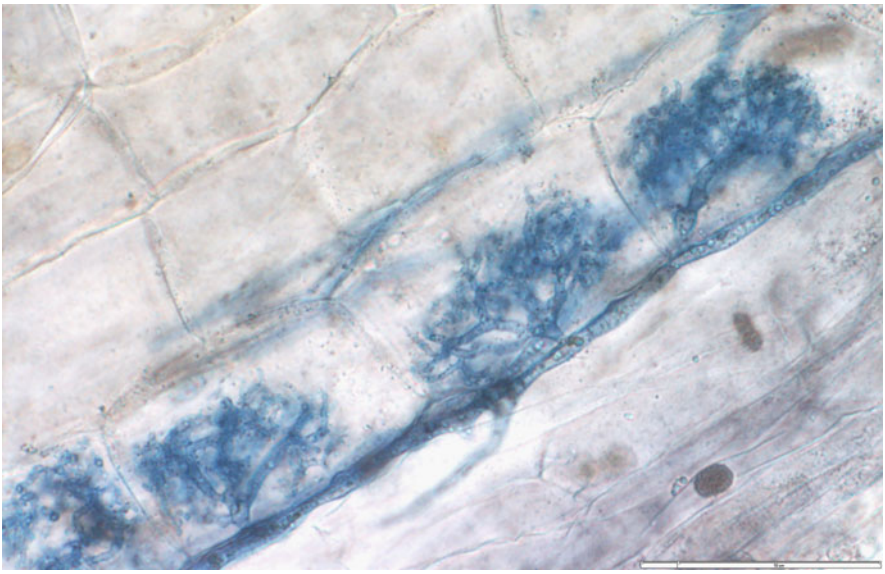

**Fig. 11.1** Typical intracellularly formed arbuscules of an AMF within the cortical cells of *Tagetes* roots. Arbuscules are the main site of exchange between the plant and the fungus

AM fungi facilitate plant nutrient uptake and transport of less mobile soil nutrients (i.e., phosphorus) (Jakobsen et al. 2001), promote drought tolerance (Kaya et al. 2003), and limit pathogenic infections (Del Fabbro and Prati 2014). Although AM fungi (AMF) have been traditionally considered as non-host specific in their ability to infect plants, benefits resulting from AM symbiosis establishment for each partner could be highly dependent on the particular species involved (Burrows and Pfleger 2002). It has been reported that AMF taxa can differ significantly in their growth strategies (Hart and Reader 2002) and in their impact on plant growth and development (Klironomos 2003). In particular, AMF species or AMF assemblages mediate plant interspecific competition and plant community structure and diversity (van der Heijden et al. 1998; Klironomos et al. 2011; Koorem et al. 2012). In parallel, it has been shown that host species and species mixture impacted individual fungal species or fungal assemblages (Eom et al. 2000; Johnson et al. 2004).

Obviously, in the current context of development of an environment friendly agriculture, it has been suggested that the integration of key natural processes (i.e., facilitation, plant soil feedback) in agricultural practices could be an efficient strategy for agricultural management. These natural processes with significant potential for plant stress resistance and plant mineral nutrition are mainly subjected to the arbuscular mycorrhizal establishment (Fester and Sawers 2011). Hence, the management of these symbioses is of prime interest in practices of controlled fallows or crop successions, mixed crop cultures (e.g., cereals/legume associations), or agroforestry.

Although the potential of AMF to contribute to improved crop yields has been demonstrated for decades and despite the current knowledge on AMF establishment and loss, there are a lot of limitations that reduce the efficiency of this biotechnological approach. For instance, one of the main obstacles to AMF large-scale uses is their non-cultivability and availability as a pure microbial inoculant, as easy to use and apply as chemical fertilizers. In fact, if a number of commercial inoculants can be found worldwide, they represent, at the best, a very low taxonomic diversity limited to a few strains or species with more or less presumed wide plant spectrum compatibility. However, the recent emergence of massive soil microbiota sequencing has evidenced an extremely high taxonomic diversity among Glomeromycetes, with a lot of undescribed clusters, and a range of putative associated functions to be exploited in a smart agriculture.

Two main approaches could be considered: (1) the "*reductionist*" approach that consists to add into the soil, a large quantity of fungal propagules of a specialized AMF like in commercial AMF inoculants and (2) the "*holistic*" approach that aims to conserve and restore native AMF diversity and abundance (Fester and Sawers 2011). In the holistic approach, it is proposed to take benefit of the ability of some plant species like legumes or some aromatic plant species to associate and multiply a wide range of AMF partners. Introducing such plants in agricultural practices may considerably diversify the mycorrhizal soil potential and benefits to the associated crops.

Although nearly all soils are inhabited by indigenous AMF (Abbott and Robson 1982), their distribution and their abundance (i.e., the indigenous inoculum potential) show large variations within regions, soil types, and crop production systems (Gianinazzi-Pearson et al. 1985). In this chapter, we will consider the environmental factors that affect the mycorrhizal establishment and limit both approaches as they can both be of interest, trying to explain to what environmental solution they would be more adapted.

## 11.2 Environmental Factors that Affect AM Establishment and Efficiency

All cultivated soils contain diverse communities of AMF and, globally, all the important food crops are naturally colonized by AMF independently from mycorrhizal inoculation. Since the expected impact of AMF introduction is to increase plant productivity, a qualitative and quantitative assessment of the AMF community composition has to be performed before the application of fungal inoculum. Hence and in order to optimize the expected impact of the mycorrhizal symbiosis, an important starting point is to determine at what level AMF are limiting to the crop yield. These limitations have to be considered at least at two different parameters: abundance and diversity. It has been reported that abundance of AMF can be negatively affected by tillage, high levels of nutrients, and frequent fallow periods in the intensive agricultural production resulting in an insufficient root colonization and, consequently, a lower mycorrhizal effect on plant growth (Karasawa and Takebe 2011).

The other form of limitation concerns the meaning levels of AMF diversity. The main differences between natural ecosystems and agroecosystems are recorded in nutrient cycling and biological diversity. It has been reported that AMF diversity was higher in natural systems than in agricultural systems (Verbruggen et al. 2010) and that agricultural soils harbored a few select taxa within the AMF order Glomerales (Oehl et al. 2010). The poor biological diversity of current agroecosystems and its consequences on crop yield could be alleviated by using fungal inocula with low sensitivity for such agricultural practices.

## 11.3 The "*Reductionist*" or Microbe-targeted Approach: *AM Fungi Isolation, Purification, Multiplication, and Application*

In the soil, AMF are found as hyphae and spores. Spores can be separated from a soil sample by sieving and gradient centrifugation (Fig. 11.2) and separated in sublots according to their size (generally ranging from 10 to 1200 μm) and color.

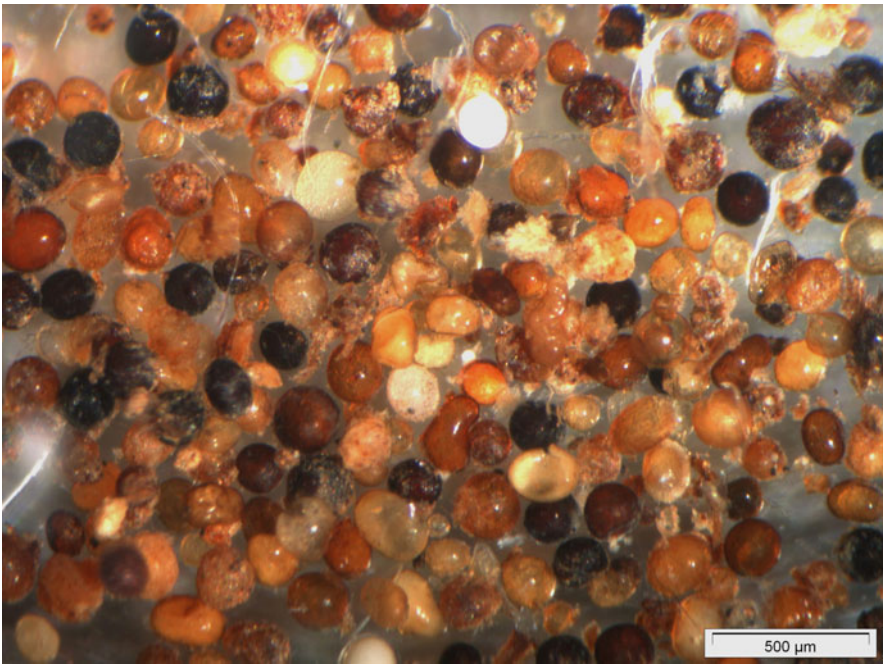


**Fig. 11.2** A crude mix of AMF spores after wet sieving and sucrose gradient separation from a soil sampled in Morocco. Note the diversity of shape, size, and color

After this first separation, spores have to be manually and individually ranked under a stereomicroscope. A first taxonomic assignment may then be done from spore characteristics: color size, morphology cell wall organization, etc.

In a second step, spores can be multiplied on an easy-to-cultivate mycotrophic plant species (Tagetes, sorghum, leek, onion, maize, Bahia grass, etc.) in controlled conditions, and new mycorrhizal roots can then be used to multiply the fungi by re-inoculation of these sheared root systems on new plants. This inoculum allows to evaluate the mycorrhizal potential of the new AMF strain and can be molecularly identified by PCR/sequencing. Various cultural substrates could be used for propagation and large-scale production of AMF such as disinfected sandy soil, peat, vermiculite, perlite, and calcinated clay (Ijdo et al. 2011)

Alternatively, this multiplication step can be made on in vitro cultured *Agrobacterium*-transformed roots of carrot or alfalfa, according to Declerck et al. (1996), following surface-sterilization of spores. With this technical practice, it is possible to obtain fungal DNA free of DNA of other organisms (Koch et al. 2004).

Unfortunately, this last step may not be equally feasible with any AMF taxa, and fungal subculturing may be intrinsically blocked at any moment for an undetermined reason. Slow growth is generally observed with AMF and may constitute an open door to a wide range of fungal or bacterial contaminants stopping the multiplication step. Aside these difficulties, some strains like MUCL46238

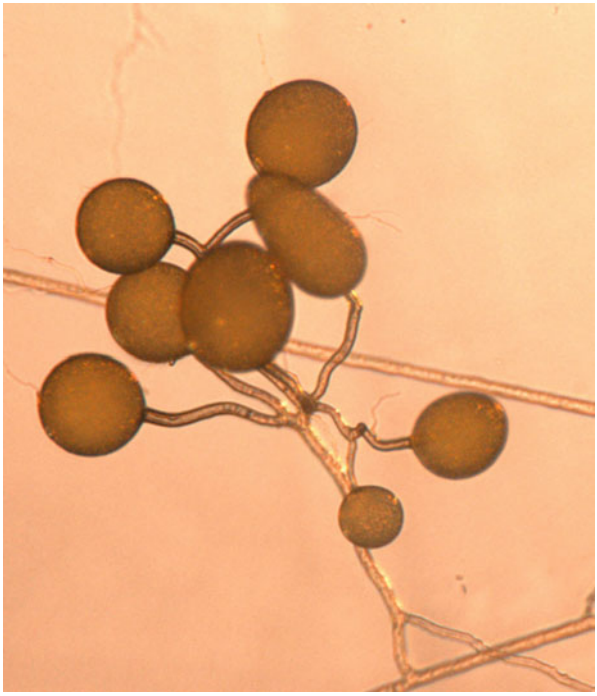

**Fig. 11.3** Monoxenic sporulation of the AMF strain MUCL46238 of *Rhizophagus clarus,* associated to transformed roots of carrot

(*Rhizophagus clarus*) (Fig. 11.3) are quite easy to multiply in microbiologically controlled conditions. They constitute the basis of most biologically active commercial AMF inoculants. Another AMF, *Rhizophagus irregularis*, is easily cultured in axenic conditions (Bécard and Fortin 1988), and its genome has recently been sequenced (Tisserand et al. 2013). The importance of this mycorrhizal fungus for the future applications of this fungal symbiont to improve food security is recognized principally because of: (1) its worldwide distribution and (2) its ability to be easily and efficiently cultured in axenic conditions (Rodriguez and Sanders 2015). Many field studies have reported its beneficial effect on yields of a globally important crop (Table 11.1).

To be compatible with field uses on large-scale applications, AMF strains' spores or sheared roots have to be embedded in or mixed with different types of substrate (peat, perlite,. . .) or polymer (alginate, gums,..) (Fig. 11.4) allowing their manipulation, survival, and field application to soil and plants. Such inocula are being used with success in different countries with various crops.

A number of studies and patents exist that compare the type and quality of different inoculant technologies, including seed coating or embedding (Malusá et al. 2012).

Diversifying the microbial offer of inoculants, i.e., mixing several AMF strains and possibly associating bacteria may be an efficient strategy to enhance the positive impact on plant growth. Some studies have reported that close interactions

**Table 11.1** Effect of *R. irregularis* inoculation on the growth of different crop species in field conditions

| Plant species | Biomass yield | Fruit yield | References |
|---|---|---|---|
| Wheat cv. Tetra | +22.1% | +22.4% | Babana and Antoun (2006) |
| Wheat | +23% | +7.7% | Wahbi et al. (2015) |
| Wheat | +15.4% | +13.4% | Suri et al. (2011) |
| *Trifolium alexandrium* | +51.7% | – | Pellegrino et al. (2011) |
| Maize (Pioneer "3025W") | +68.1% | +22.4% | Franco et al. (2013) |
| Maize | +53.6% | – | Celebi et al. (2010) |
| Maize | – | +44.6% | Hagh et al. (2016) |
| *Solanum lycopersicum* cv. Ercole | +19.6% | +29% | Conversa et al. (2013) |
| Maize (Zheng Dan 958) | +5% | +12.6% | Li et al. (2013) |

(1)nd: not determined

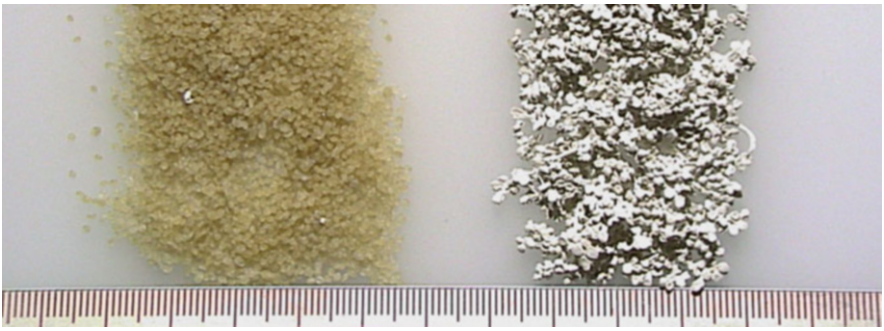

**Fig. 11.4** Two formulations of an alginate-embedded inoculant with either pure (*left*) or 5% kaolin-supplemented (*right*) alginate. Kaolin is intended to stabilize the relative humidity level and improve survival rate of the microbial strain

occurred between AMF and rhizobacteria underlying the existence of a trophic complex where multitrophic interactions take place between AMF, mycorrhizosphere microbiota, and host plants (Duponnois et al. 2011). The beneficial traits of root-colonizing bacteria and mycorrhizal fungi have been frequently studied separately. However, it is now well known that synergistic effects of bacteria and mycorrhizal fungi occurred with respect to their combined beneficial impacts on plant growth (Vessey 2003). For instance, recent studies have shown that inoculation of PGPR with AMF is more beneficial in promoting plant growth compared to inoculation with either one of them (Ratti et al. 2001; Gamalero et al. 2004). In addition, it has been reported that rhizobacteria can improve the mycorrhizal establishment by stimulating the growth of fungal hyphae through the production of compost like vitamin or enzymes increasing the permeability of the cell wall of the root epidermis (Jeffries et al. 2003), but also indirectly at the "molecular dialogue" preceding the mycorrhizal symbiosis. These bacteria have been named Mycorrhization Helper Bacteria, MHB (Duponnois and Garbaye 1991). Some companies are commercializing such composite inoculants; however,

the technical elaboration of mixed inoculants associating AMF and other plant growth promoting rhizobacteria may be a brake in terms of quality, constancy, and cost.

## 11.4 The Holistic Approach: Taking Advantage of the Symbiotic Dependency of Some Plant Species

At this stage, an efficient alternative could be the selection and use of plants with particularly high level of mycotrophy, *i.e.*, that will recruit a diversified range of AM fungi and their associated bacteria directly in soils. Combining the direct use of plants and their associated microbial communities (also called "nurse plants" (Duponnois et al. 2013) or "holobionts" (Vandenkoornhuyse et al. 2015) as planted fallows or directly in association with the targeted crops may be an efficient way of introducing a diversified microbial inoculant. Among candidate mycotrophic plants are species of the Crotalariae, within the nitrogen-fixing legume (Fabaceae) family. Among these tropical plants, some species (*Crotalaria juncea* (Fig. 11.5), *C. grantiana*, *C. spectabilis*,...) produce alkaloids with a nematicidal activity, altogether these properties making them to be highly appreciated in the tropics as fallows producing green manure and nematode control.

Regarding AMF, Germani and Plenchette (2004) demonstrated that all *Crotalaria* species they tested significantly responded to AM inoculation with *Glomus intraradices* (now *Rhizophagus irregularis*), resulting in increased plant P content and plant growth. Moreover, as reported for other legumes (Medina-Gonzales et al. 1987), *Crotalaria* species showed their high mycorrhizal dependency, up to 90%, and their potential to increase the level of beneficial mycorrhizal fungi in soil (Germani and Plenchette 2004).

Other candidate plants include aromatic like *Lavandula* or *Thymus* that can be co-planted in Morocco with trees like *Cupressus* to improve tree growth and survival after field plantation (Duponnois et al. 2011).

Considering the symbiotic characteristics of plant species and varieties in the pluriannual and rehabilitation design of crops is also a way of maintaining the biological quality of soils. For example, plants of the brassicaceae (rapeseed, mustard), chenopodiaceae (spinach), or amaranthaceae (beet) are non-mycorrhizal (i.e., non symbiotic); cereals like wheat or corn are mycorrhizal; and legumes like bean, faba, or alfalfa are both mycorrhizal and nitrogen-fixing species. Such characteristics were more or less empirically considered in traditional agriculture and are regaining interest in agro-ecological practices.

Additionally, a number of studies have shown that enhancing aboveground (plant) diversity has a positive impact on belowground (microbial) diversities and this is true for AMF. In this context, multispecies cropping systems may often be considered as a practical application of key ecological processes depending on biodiversity, plant interactions, and diverse natural regulation mechanisms that

**Fig. 11.5** *Crotalaria juncea* used as a fallow in a vegetable production farm during a greenhouse trial in Noves (France)

ensure the productivity, resistance to disruption, and ecological sustainability of the agro-systems, especially in Mediterranean areas (Vandermeer 1989). For instance, the association of trees with crops (agro-forestry) allowed nutrient recycling by coexisting plant species exploring different soil depths increasing nutrient and water-use efficiency by the crops (van Noordwijk et al. 1996) (Fig. 11.6) Moreover and in these cropping systems, tree species could be considered as beneficial microbe inoculum sources for inter-row crop species (e.g., mycorrhizal fungi, PGPR, etc.) (Haselwandter and Bowen 1996).

**Fig. 11.6** The association of *Argania spinosa* and peas in a vegetable farm near Agadir (Morocco). This agro-forestry system associating perennial oleaginous trees and short-term crops optimize land use, soil depth exploration, soil microbial activity, and diversify sources of cash incomes

## 11.5 Conclusion

This chapter has reviewed the large potential of AMF to help increase global security resulting from their positive impact on the development of all globally important food crops. However, despite numerous studies focused on this symbiosis, few data are available on large-scale inoculation experiments performed on important crops. According to the scientific knowledge that shows the complexity of the interactions between the plant, the physico-chemical characteristics of the soil and the soil microbiota, the traditional focus on nutrient exchange and plant growth response in order to evaluate the "symbiotic efficiency" in field conditions seems too simplistic to expect a large-scale application of AMF ensuring significant increases in food production. To enhance chances of successful inoculation, research efforts should have to be directed towards the impact of the introduction of nonnative AMF on the composition of resident AMF communities (i.e., distribution of inocula in large geographical areas, risk of "outbreeding depression" resulting from genetic exchange between the introduced exotic AMF strain and the native AMF strains, etc). Better knowledge is also required on the indirect effects on plant growth resulting from AMF inoculation that are not related to mycorrhizal root colonization. Hence and in order to make commercial application of AMF, a sum of scientific results have to be acquired especially to predict under which environmental conditions AMF inoculation (the reductionist approach) will

promote yield and agricultural sustainability compared to the "holistic approach" that will manage the mycorrhizal soil infectivity through an adequate cultural practice (i.e., agroforestry, intercropping, rotation).

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# Chapter 12
# Reactive Oxygen Species (ROS) Metabolism and Signaling in Plant-Mycorrhizal Association Under Biotic and Abiotic Stress Conditions

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**Abstract** A stringent regulation between reactive oxygen species (ROS) generation and scavenging is an essential process that helps a plant to adaptively utilize ROS as a primary defense molecule against biotic and abiotic stress condition. ROS at lower level primarily acts as a signaling molecule regulating plant cellular processes that include plant-microbe interaction. However, ROS generated at higher levels often leads to the inhibition of cellular processes, thus consequently leading a detrimental effect in plant growth and homeostasis. Rhizosphere being the "chemical space" around the roots which proves to be biologically active zone for plant-microbe interactions forms a link responsible for mutual signaling in each of the partners. Moreover plant fitness is said to be enhanced by these symbiotic mycorrhizal associations which are known to alleviate detrimental effects caused by environmental stresses thereby enhancing overall plant growth and development. This present chapter summarizes a precise interlink between biotic-abiotic stressed plants and its mycorrhizal association linking ROS modulation with plant signaling thereby establishing a link between stress tolerance and ROS metabolism. The literature reviewed herein will help to delineate the basic mechanism of ROS signaling, by ascertaining the physiological responses via altering the ROS metabolism, in mycorrhizal-associated stressed plants. This will ultimately help in designing innovative strategies to improve the overall plant productivity under stressful regimes.

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## 12.1 Introduction

Reactive oxygen species (ROS) are generated in response to stress which includes both abiotic and biotic stress conditions as well as in normal metabolic processes, e.g., in chloroplast and mitochondrial electron transport chains. Enzymatic components are known to serve as one of the major ROS scavenging systems in plants, e.g., monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which are predominantly distinguished as one of the major players of the detoxification pathway. On the other hand, glutathione (GSH) and ascorbic acid (AsA) are often demarcated as components of nonenzymatic system (Gill and Tuteja 2010; Rasool et al. 2013). Additionally, respiratory burst oxidase homologues (RBOHs) and NADPH oxidases are also known to be major components of ROS production system in plants (Suzuki et al. 2013; Kadota et al. 2015).

However, in response to stress, ROS generation acts as a signaling agent that aware the plant for stress adaptations (Mittler et al. 2011; Sewelam et al. 2016). Contrarily an accurate balance, between ROS scavenging and ROS generation system, is indispensable for the utilization of ROS as signaling molecule under stress (Baxter et al. 2014). However, long duration of stress results in an increased ROS level that further leads to oxidative stress, thus inhibiting the crucial cellular activities and cell viability (Gill and Tuteja 2010; Barna et al. 2012). Therefore, antioxidant signaling, redox homeostasis, and continuous generation/scavenging of ROS are designated as key components of stress signals (Bose et al. 2014; Jajic et al. 2015). ROS generation is also known to occur during early mycorrhiza-plant symbiotic interactions (Fester and Hause 2005; Tanaka et al. 2006; Puppo et al. 2013; Espinosa et al. 2014; Kiirika et al. 2014).

Plants often interact with several microbes in rhizosphere; however, among these interactions, some beneficial interactions are known to enhance plant growth and fitness. However, in present scenario, a relatively small number of beneficial plant-microbe interactions are well characterized and utilized (Farrar et al. 2014). Microbes are capable to alleviate the effect of environmental stress on plants via decreasing the stress impacts thus ultimately increasing the plant fitness (Schouteden et al. 2015; Doty 2016). On the other hand, a huge number of microbes interact with the plant root, in the rhizosphere, thus affecting plant growth and fitness (Mine et al. 2014). The major active region identified for plant root-microbe interactions is the edge between soil and roots, i.e., through mutual signaling that occurs during plant-microbial association (Evangelisti et al. 2014).

Several fungal species are reported which are capable in colonizing plant roots. Additionally research on plant-microbe interaction has majorly spotlighted the areas of plant-arbuscular mycorrhizal fungi (AMF) symbiosis and legume root-rhizobium interaction for nitrogen fixation and pathogenesis (Smith and Smith 2011; Oldroyd et al. 2011; Kachroo and Robin 2013; Farrar et al. 2014). Mycorrhizal fungi are also well recognized to facilitate nutrient transfer from soil, e.g.,

transfer of phosphorus and nitrogen in plants (Behie and Bidochka 2014). Furthermore, the symbiotic beneficial fungal counterpart is also known to be capable of increasing the plant fitness via modifying the chemical plasticity such as modulation in response to stress (Goh et al. 2013).

Regardless of the facts, research work related to link ROS metabolism and plant-mycorrhizal association under stress is still scanty. Therefore, an attempt has been made here to briefly summarize ROS metabolism including generation/scavenging, signaling, and homeostasis in connection with plant-microbe interactions. Interestingly, research on AMF-like fungi, *Piriformospora indica*, has recently depicted their role in plant growth promotion under stressful conditions which has consequently led to an increase in plant yield (Sherameti et al. 2008; Vadassery et al. 2009a, b; Varma et al. 2012; Cruz et al. 2013; Jogawat et al. 2013; Bakshi et al. 2014; Trivedi et al. 2016; Gill et al. 2016). Therefore, ROS metabolism linked with *P. indica*-plant root association under stress will also be briefly summarized.

## 12.2 ROS Metabolism and Plant-Mycorrhizal Association Under Stress Conditions

Plants continuously interact with microbes, e.g., mutualists and pathogens, that affect plant growth (Mine et al. 2014). Beneficial mutualists such as AMF are well reported in providing plant growth sustainability under different stress conditions (Muthukumar and Udaiyan 2010; Porcel et al. 2012; Tahat and Sijam 2012). ROS generated in both radical and non-radical forms includes superoxide radicals ($O_2^{\bullet-}$), perhydroxyl radical ($HO_2^{\bullet}$), and alkoxy radicals ($RO^{\bullet}$) which constitute radical form, while hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$) constitute non-radical forms. However, radicals prove to be highly toxic in nature when compared to non-radicals (Gill and Tuteja 2010; Sewelam et al. 2016). The role of mycorrhiza in ROS scavenging is established from the ROS metabolism study of AM-colonized roots of *Zea mays*, *Medicago truncatula*, and *Nicotiana tabacum* (Fester and Hause 2005). Likewise, AMF-colonized plants accumulated less ROS ($H_2O_2$) and malondialdehyde (MDA) than non-colonized olive plants which ultimately helped in alleviating oxidative stress, thus increasing drought tolerance (Fouad et al. 2014). Similarly, AMF were also reported to enhance the antioxidant system of host plant and decrease the impact of drought stress condition in date palm (Benhiba et al. 2015) and *Citrus reticulata* (Sarkar et al. 2016).

AMF mediate the control of ROS metabolism and antioxidants and further diminish the effect of oxidative stress in host plants in response to stress conditions (Vos et al. 2013; Wu et al. 2014; Hashem et al. 2016). In addition, ROS involvement also suggests in providing tolerance against nematode (*Meloidogyne javanica*) infection in soybean (Beneventi et al. 2013). Similarly, root-knot nematode reduction was linked with ROS metabolism (*Meloidogyne incognita)* infection in mycorrhizal tomato roots (Vos et al. 2013). Increased antioxidant enzymes

including SOD, POD, CAT, APX, and GR were also argued to enhance cadmium (Cd) tolerance in tomato through AMF-mediated ROS scavenging activities (Hashem et al. 2016).

## 12.3 ROS Signaling

In order to adapt with various biotic and abiotic stresses, plants possess a highly complex signaling pathway. In addition, plants also utilize ROS as the major signaling agent and activate various adaptive defense mechanisms under stress conditions (Baxter et al. 2014; Xu and Brosche 2014; Sewelam et al. 2016). Higher expression of ROS scavenging-related genes, e.g., glutaredoxin, thioredoxin, and GPX, was correlated with herbicide (atrazine) stress tolerance in *Glomus mosseae*/*Medicago sativa*. Besides this, higher atrazine degradation was observed in *G. mosseae* (mycorrhizal)-treated *M. sativa* plants as compared with non-treated plants (Song et al. 2016).

However, ROS production is the most common response triggered which initiates signaling pathway under stress environment (Sewelam et al. 2016). A limited research related to ROS modulation during initial microbial interaction with plant root is available. ROS generation and cell death are also reported at the interaction site during early host-microbe association (Puppo et al. 2013). An active ROS component $H_2O_2$ is involved in adaptive defense mechanism which is also responsible for initiating signaling pathways in response to stressed environment (Xia et al. 2009). Due to its membrane-permeable nature, $H_2O_2$ further control the specific biological reactions providing stress tolerance in several components (Neill et al. 2002; Yan et al. 2007). The functional role of ROS through the role of exogenous $H_2O_2$ in regulating rhizobial symbiosis-related genes was demonstrated in *Medicago truncatula-Sinorhizobium meliloti* interactions (Andrio et al. 2013). On the other hand, a temporary ROS increase was also observed in root hairs of *Phaseolus vulgaris*, and specific ROS signature involvement was proposed during symbiotic association (Cardenas and Quinto 2008).

## 12.4 ROS Metabolism and Plant-*P. indica* Interaction Under Stress

A group of soil-dwelling fungi constitute AMF which are symbiotically associated with roots of many plants. Moreover, *P. indica*, AMF-like fungi, is an obligate biotroph which is able to be grown in pure culture and does not need the presence of the plant (Foley et al. 2011). It is well reported that *P. indica* has been found to improve plant growth and survival in agricultural, horticultural, and medicinal crops under stress condition (Verma et al. 1998; Waller et al. 2005; Baltruschat

et al. 2008; Vadassery et al. 2009a, b; Prasad et al. 2013; Jogawat et al. 2013; Lahrmann et al. 2013; Ye et al. 2014; Johnson et al. 2014; Gill et al. 2016; Trivedi et al. 2016). Interestingly, a potential role of *P. indica* has been also documented in plants in response to various kinds of biotic and abiotic stress including salt, drought, nutrient, and nematode stress tolerance (Sherameti et al. 2008; Cruz et al. 2013; Bakshi et al. 2014; Nath et al. 2016). In addition, *P. indica* was also reported to increase the alkaline phosphatase and acid phosphatase and consequently contributes for higher uptake of phosphate in plants (Malla et al. 2004).

In plant roots, ROS generation as a defense-related response was reported during initial plant-mycorrhizal associations (Pozo and Azcón-Aguilar 2007). Moreover, biotic stress tolerance was also linked with the ROS metabolism and modulation of antioxidant defense pathway in *P. indica*-inoculated plants, viz., wheat, maize, and barley (Waller et al. 2005; Serfling et al. 2007; Kumar et al. 2009). Besides this, ROS was also observed before *P. indica* physical contact with plant roots, though $H_2O_2$ was not reported after symbiotic relationship establishment (Vadassery et al. 2009a; Camehl et al. 2011; Vahabi et al. 2015). $H_2O_2$ was also found to encourage *OXI1* (*oxidative signal-inducible1*) gene and further trigger defense response under pathogen infection (Rentel et al. 2004; Anthony et al. 2006; Petersen et al. 2009). Additionally, OXI1 (a serine/threonine kinase) was also demonstrated as a requirement for ROS-mediated responses and oxidative burst for disease tolerance against pathogens in *Arabidopsis* (Rentel et al. 2004; Petersen et al. 2009). $H_2O_2$ generation was also found to repress in *P. indica*-colonized *Arabidopsis* roots and growth stimulation mediated through PLD-PDK1-OXI1 pathway under favorable cocultivation conditions (Camehl et al. 2011). Exudates are released during initial interaction of *P. indica* with plant, which further leads to ROS accumulation and stomatal closure and induces defense-responsive genes in *Arabidopsis*. On the other hand, after the establishment of *P. indica*-plant interaction, the stomata are reopened, while ROS generation decreased and defense-responsive gene expression turned down (Vahabi et al. 2015).

Enhanced antioxidant system and glutathione-ascorbate cycle activation were observed in in *P. indica*-colonized barley root (Waller et al. 2005). Similarly, *P. indica*-mediated enhancement of antioxidants was also linked with salinity stress tolerance in barley (Baltruschat et al. 2008). In another similar report in wheat, co-inoculation of *Azotobacter chroococcum* and *P. indica* indicated higher antioxidant enzyme activities including peroxidase and APX in colonized plants in response to zinc-deficient condition (Abadi and Sepehri 2016). Recently, candidate effector (PIIN_08944) expression of *P. indica* was reported to decline the ROS burst in barley (Akum et al. 2015). Here, we are summarizing the ROS metabolism link with plant-mycorrhiza interaction under stress (Fig. 12.1).

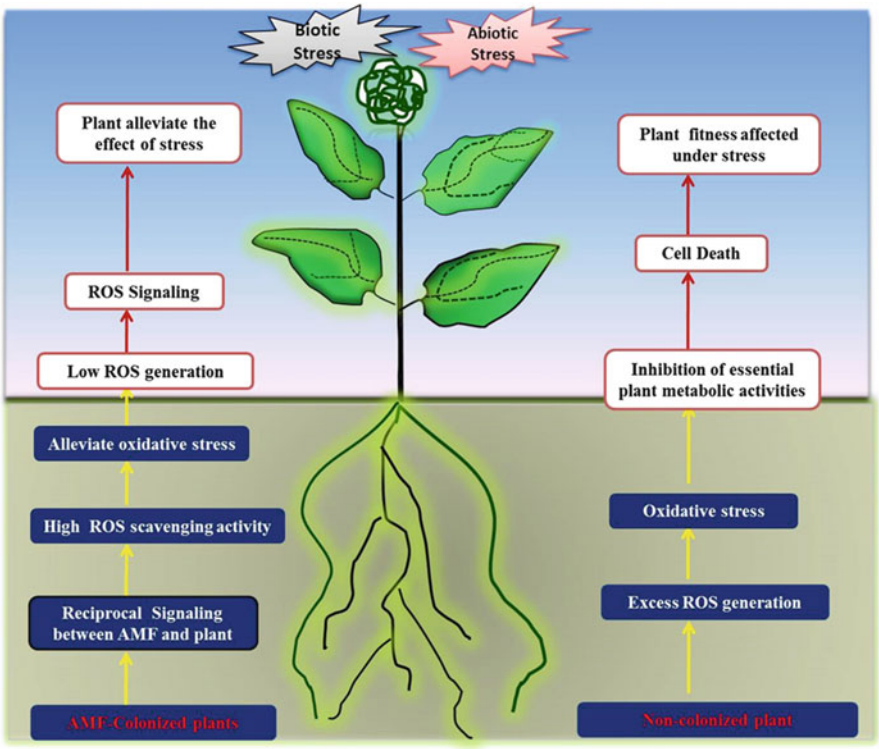


**Fig. 12.1** An overview of ROS metabolism and plant-mycorrhizal association under stress. In response to biotic and abiotic stress, ROS metabolism and modulation offer adaptive defense stress response in mycorrhizal-colonized plants and further provide stress tolerance. On the other hand, in absence of colonization, high ROS generated and inhibit the essential cellular activities; therefore, the plant fitness is compromised. AMF, arbuscular mycorrhizal fungi

## 12.5 Conclusions

The capability of ROS metabolism defense system enhancement via symbiotic microbial interactions ultimately improves plant fitness under stress. A detailed ROS signature kinetics during initial plant-mycorrhiza interaction will enhance the basic understanding of mycorrhizal link with ROS metabolism. On the other hand, molecular insights of ROS metabolism in plant-mycorrhizal especially *P. indica* interaction will be very helpful to plan innovative approaches and ultimately to improve plant growth and yield under stress conditions.

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# Chapter 13
# Stimulated Growth of *Lycopersicum esculentum* CLA 1131 in Presence of *Piriformospora indica* and Vermicompost

**Reshma Tuladhar, Kenneth Shahi, Sujen Man Shrestha, Anjana Singh, and Ajit Varma**

**Abstract** In the mutualistic association between plant and mycorrhiza, plant benefits by gaining an improved nutrient and water acquisition through fungal hyphae and/or an enhanced abiotic stress tolerance. Since mycorrhiza facilitates the plant in the nutrient uptake from the soil, fertility of soil is necessary for the availability of the essential nutrients. The axenically cultivable root-colonizing endophytic fungi *Piriformospora indica* treated tomato plant (*Lycopersicum esculentum* CLA 1131); when supplemented with vermicompost, the growth and biomass were enhanced. Mycorrhizal colonization was improved in the presence of vermicompost. The amount of essential nutrient nitrogen, phosphorous, and potassium content in plant was improved by the colonization with *P. indica* and influenced by the nutrient conditions in the soil. The efficiency of nutrient uptake by *P. indica* is complemented by vermicompost.

## 13.1 Introduction

The increase in crop production adopting environment-friendly strategy is necessary to address the demand of food for growing human population and need of sustainable agriculture. The green revolution, which was active during the 1940s and 1960s, supported the use of chemical fertilizers and pesticides to increase the yield (Tilman 1998). However, the negative impact of these chemicals in human health and environment has necessitated the use of alternative solutions to increase

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crop yields sustainably. The application of biological solution, which includes the manipulation and exploitation of beneficial plant-microbe interactions, is the sustainable approach.

The consortium of plant-microbe interaction is highly complex, comprising diverse microbial species. The complex relationship based on mutual interaction between diverse microbial population and plants proliferates in the rhizosphere and within the plant itself (Evangelisti et al. 2014). The root fungus mycorrhiza is an intriguing component which develops within the rhizosphere and associates symbiotically promoting the growth and health of the plant (Malla et al. 2002; Goltapeh et al. 2008). The diverse mycorrhiza includes ectomycorrhiza, endomycorrhiza, ectendomycorrhiza, and arbuscular mycorrhizal fungi (AMF) (Gosal et al. 2013). AMF occur on a vast taxonomic range of plants and hence most commonly reported group (Malla et al. 2002). The multitude benefits from arbuscular mycorrhizal association improved nutrient uptake, mineralization of organic nutrients, resistance to abiotic and biotic stress, etc. (Cruz et al. 2013; Prasad et al. 2015; Gill et al. 2016). Despite the plethora of benefits, AMF use is limited in agriculture owing to its difficulty in producing inoculums (DeClerk et al. 2005). It is obligate and thrives only on the living cells of the host plant.

However, *Piriformospora indica*, a non-obligate biotroph discovered by Varma and his co-worker (Verma et al. 1998), has potential for agricultural application for its ability to grow in synthetic medium. This axenically cultivable arbuscular mycorrhiza-like fungus endophyte similar to AMF in many aspects (Varma and Schuepp 1994; Varma et al. 1999; Singh et al. 2000; Rai and Varma 2005; Prasad et al. 2005) establishes mutualistic interactions with a broad variety of plant species (Jacobs et al. 2013). It has the ability to colonize the roots of wide host range from medicinal plants and ornamental plants to economically important crops (Singh et al. 2000; Prasad et al. 2008a, b, 2013). The host plants benefited by the colonization of this endophyte include crops like wheat, maize, and sugarcane (Rai et al. 2001; Waller et al. 2005; Baltruschat et al. 2008); legume crops like soybean, pea, and bean; and several medicinal plants (Kumari et al. 2004; Oelmüller et al. 2009; Das et al. 2013; Bagde et al. 2010, 2014).

Root colonized by *P. indica* provides various benefits to host plant which include growth promotion and enhancement for better biomass and yield (Gosal et al. 2013) and tolerance to abiotic stress (Bagyaraj and Varma 1995) and limit severity of plant disease (Fakhro et al. 2010). *P. indica* promote the plant with its ability to extract, mobilize, and transport phosphorous as well as several micronutrients from soil (Gosal et al. 2013). The fungus possesses positive phyto-promotional effects due to plant bio-regulation ability, apart from its role in mobilization and transportation of the plant unavailable phosphorous in soil (Gosal et al. 2013; Malla et al. 2004). The plant physiology is stimulated increasing vegetative growth, inducing resistance against plant pathogens, and increasing yield.

The effect of the interaction, however, depends on various factors such as amount of inoculums, the time point of inoculation, and nutrient content in the environment (Andrade-Linares et al. 2013). When high amounts of *P. indica* were inoculated in soil under nutrient-limiting conditions of low amounts of nitrogen and phosphorous, negative effect was observed in tomato plant (Andrade-Linares et al. 2013). Tomato is one of the most consumed vegetable crops worldwide. Researchers have demonstrated that growth of tomato plant is improved by colonization of its root with *P. indica* (Fakhro et al. 2010; Andrade-Linares et al. 2013). In this chapter, we review the impact of dual inoculation of endophyte *P. indica* and vermicompost on the growth of tomato plant.

## 13.2 Effect of Dual Inoculation on Vegetative Growth

Vermicompost is produced by earthworms in the form of worm cast upon feeding on biodegradable materials. This product of biodegradation of organic materials through interactions between earthworms and microorganisms (Sallaku et al. 2009) is rich in nitrogen, phosphorous, and potassium (NPK).

Tomato plant (*Lycopersicum esculentum* CLA 1131) inoculated with *P. indica* and grown in soil supplemented with vermicompost increased the length and dry weight of shoot and root compared to the tomato plant treated with *P. indica* or vermicompost alone (Fig. 13.1). The experiment was carried out in earthen pot filled with soil supplemented with vermicompost. The leaf number per plant was highest in the plant grown in presence of both *P. indica* and vermicompost in soil (Fig. 13.2). Increased biomass of the leaves by up to 20% has been observed in tomato plants colonized by *P. indica* (Fakhro et al. 2010). The length and dry weight of root and shoot were measured following 90 and 120 days of transplantation. The growth of plant in terms of root and shoot parameters was highest in *P. indica*-inoculated plant supplemented with vermicompost compared to plant with single treatment of *P. indica* alone or vermicompost alone (Figs. 13.3 and 13.4).

Singh et al. (2001) and Malla et al. (2002) reported significant increment in shoot length when *S. calva* and *W. somnifera* were inoculated with *P. indica*. The promotion of early growth stages of plant is owed to accelerated root development, and age-dependent regulation of genes shifted to earlier time points in *P. indica*-colonized roots (Waller et al. 2005, 2008). In addition, *P. indica* promote plant growth by inhibiting the ethylene signaling which impedes the plant development (Barazani et al. 2005).

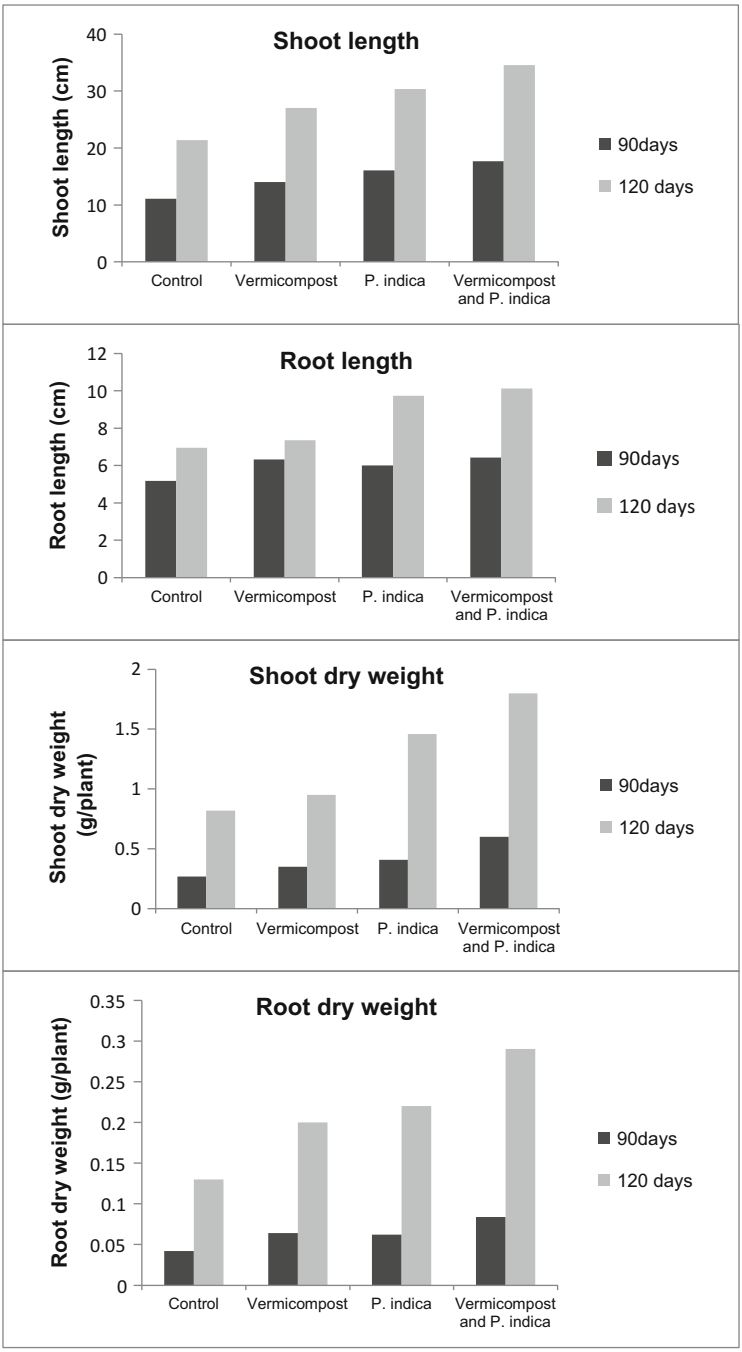


**Fig. 13.1** Impact of *P. indica* and vermicompost on vegetative growth on 90 and 120 days after inoculation. Tomato plants (*Lycopersicum esculentum* CLA 1131) in pot culture were inoculated with vermicompost only, *P. indica* alone, and dual inoculation of *P. indica* and vermicompost in three consecutive experiments. The plants harvested after 90 and 120 days of plantation were measured for root and shoot length and dry weight of root and shoot

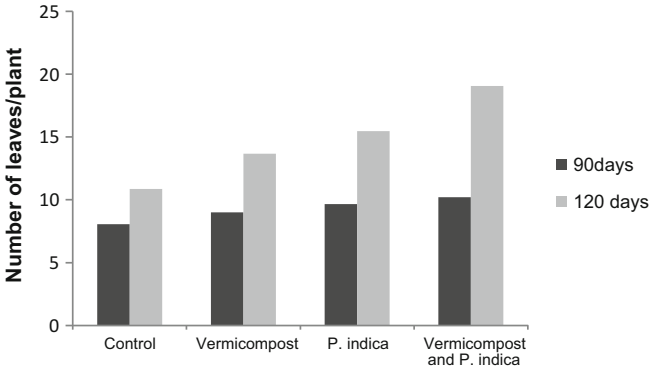


**Fig. 13.2** Impact of *P. indica* and vermicompost on the leaf number. Tomato plants (*Lycopersicum esculentum* CLA 1131) inoculated with vermicompost only, *P. indica* alone, and dual inoculation of *P. indica* and vermicompost were measured for the average number of leaves per plant after 90 and 120 days of plantation

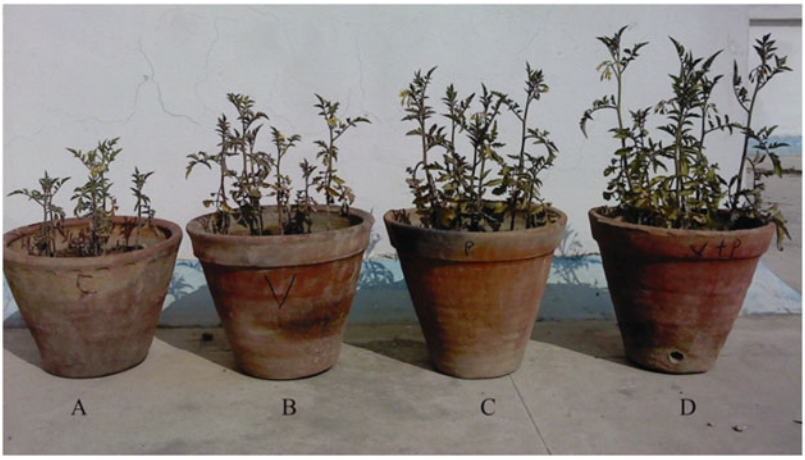


**Fig. 13.3** Effect of dual inoculation of *P. indica* and vermicompost on the growth of tomato plant (*Lycopersicum esculentum* CLA 1131) after 90 days of transplantation. A (control), B (vermicompost), C (*P. indica*), and D (*P. indica* and vermicompost)

## 13.3 Mycorrhizal Colonization in Presence of Vermicompost

The abundance of mycorrhizal fungi in soil has been indicated by the measurement of the extent to which the roots are colonized with mycorrhizae (Hayman and Stovold 1979; Sparling and Tinker 1978). The active symbiotic phase is reflected from the mycorrhizal root colonization. Dual inoculation of tomato plant with

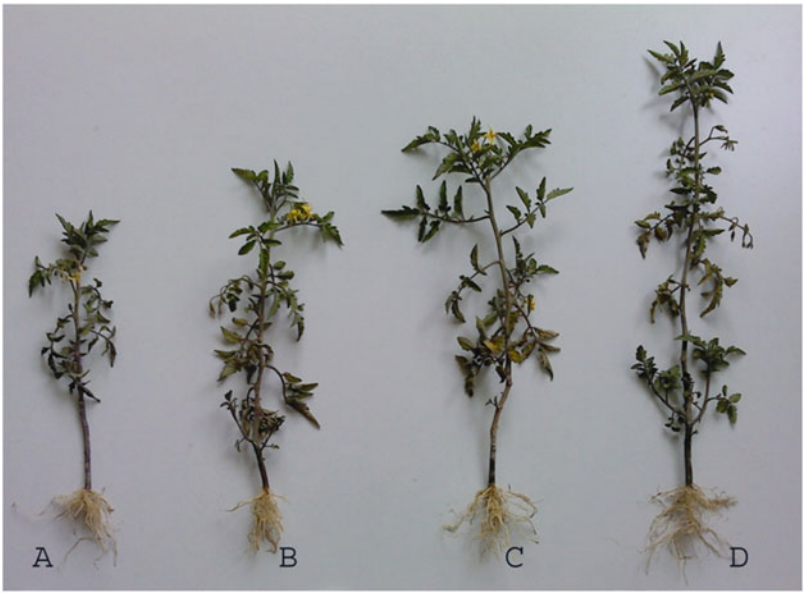


**Fig. 13.4** Effect of dual inoculation of *P. indica* and vermicompost on tomato plant (*Lycopersicum esculentum* CLA 1131) after 120 days of transplantation. A (control), B (vermicompost), C (*P. indica*), and D (*P. indica* and vermicompost)

*P. indica* and vermicompost improved mycorrhizal colonization compared to *P. indica* alone, while colonization was absent in control plant (Fig. 13.5). Vermicompost improves texture and properties of soil (Edwards and Burrows 1988) making it conducive for soil microflora. Mycorrhizal colonization has been significantly increased in presence of vermicompost (Kale et al. 1992). The chlamydospores colonized in the root of tomato plant in presence of vermicompost are greater than in absence of vermicompost (Fig. 13.6). This suggests that nutrient-rich soil facilitates the colonization of *P. indica* which in turn will provide enhanced benefit to the plant.

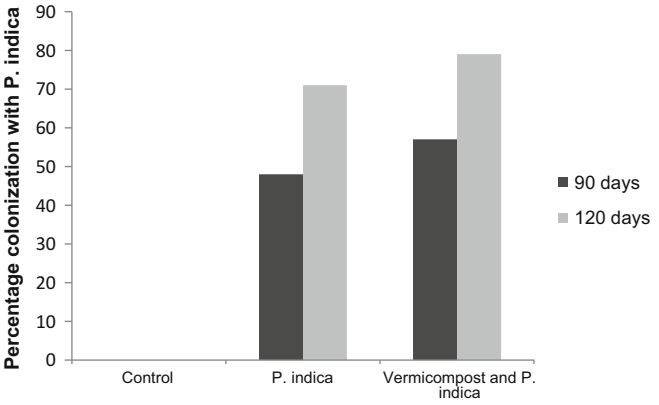


**Fig. 13.5** Mycorrhizal colonization percentage in the root of tomato plants (*Lycopersicum esculentum* CLA 1131) treated with *P. indica* alone and dual inoculation of *P. indica* and vermicompost

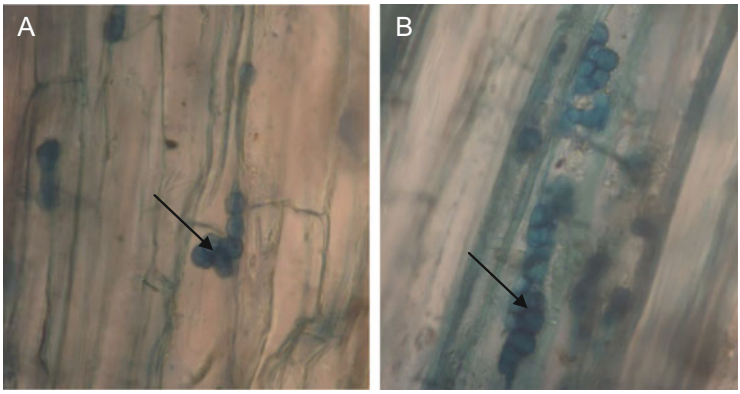


**Fig. 13.6** Tomato root colonized with *P. indica*. The roots of tomato plant in pot culture inoculated with *P. indica* alone and dual inoculation with *P. indica* and vermicompost were harvested and stained with *trypan blue*. (**a**) Chlamydospores in root of tomato plant inoculated with *P. indica* alone and (**b**) chlamydospores in dual inoculation with *P. indica* and vermicompost. *Arrow* indicates chlamydospores

## 13.4 Effect on Nitrogen, Phosphorous, and Potassium Content in the Plant

Organic fertilizers are known to contain nitrogen (N), phosphorous (P), and potassium (K), the essential macronutrients required for the growth and development of plant. Vermicompost, a superior organic manure, increases the soil fertility for its high percentage of NPK and water retention ability (Edwards and Burrows 1988; Acharya 1997) enhances biomass production of number of crops (Hidalgo 1999). Colonization of plant roots with *P. indica* increases the efficiency in the uptake of

these macronutrients by plant, aiding in the optimal growth of plant as well as ensuring the maximum utilization of the vermicompost.

Apart from its serving as an important building block of amino acids, nucleic acids, and chlorophyll in plant, nitrogen is an essential regulator in carbon and amino acid metabolism (Frink et al. 1999; Cai et al. 2012). Plants absorb N from soil in the form of nitrate and ammonia/ammonium (Kulcheski et al. 2015). Legumes are benefitted through the microbial symbiosis in acquiring nitrogen. The use of nitrogenous fertilizers is in practice for the plants that do not exhibit microbial symbiosis, but it has contributed to serious problems of soil and water pollution. Major portion of the nitrogenous fertilizers are lost due to incomplete capture by plant or through conversion to nitrous oxide (Montzka et al. 2011). Thus, the efficient uptake of N by plants is necessary to be established.

Mycorrhizal association is the best alternative for plants that do not symbiotically associate with N-fixing bacteria for the acquisition of N from soil. The mycorrhizal mycelium has the ability to transport organic and inorganic N sources from soil and export to the plant (Bonfante and Genre 2010). Symbiotic association with AMF and *P. indica* was found to improve the N acquisition by the plants, of which *P. indica* was more efficient (Cruz et al. 2013). The difference in their N uptake is that *P. indica* mediates nitrate uptake from soil, while AMF preferentially absorb ammonium (Gosal et al. 2013).

In most soil, large portion of P is unavailable to plant since they are immobilized (Marschner 1995). The soluble form is orthophosphate which is very low in concentration in soil. To acquire P under limiting condition, plant either explores for available P by extending root and extensively branching it off or enhances secretion of phosphatase and expression of new kind of Pi transporter in root cell (Johri et al. 2015).

*P. indica* is reported to enhance plant growth rate through an increase in nutrient uptake, especially P that is relatively immobile in soils (Singh et al. 2001; Varma et al. 2001). *P. indica* inoculation could have also induced soybean to absorb more nutrients by increasing the absorbing surface area. Enhanced activity of acid phosphatase and alkaline phosphatase was noticed in the rhizosphere soil of rice plants inoculated with *P. indica* (Das et al. 2014). A high-affinity phosphate transporter PiPT is present in *P. indica* which improve Pi nutrition levels in the host plant under P-limiting conditions (Johri et al. 2015). *P. indica* mediate more efficient phosphate (Pi) uptake by plant independent to the degree of root colonization suggesting it to be an alternative to other mycorrhizal fungi (Johri et al. 2015).

Potassium is essential for plant development and reproduction, yield, and responses to abiotic stress (Demidchik et al. 2014; Zorb et al. 2014; Zhang et al. 2015). Vermicompost enhances in the content of the essential nutrient in the soil as it led to significant increase in soil enzyme activities such as urease, phosphomonoesterase, and phosphodiesterase (Albiach et al. 2000). Besides, plant growth-promoting bacteria stimulate solubilization of nutrients (Rodriguez and Fraga 1999) and production of growth hormones (Correa et al. 2004). Increased nitrogen, phosphorous, and potassium content in plants has been resulted from the association of *P. indica* with plant roots. Higher NPK content in root and shoot of chick pea

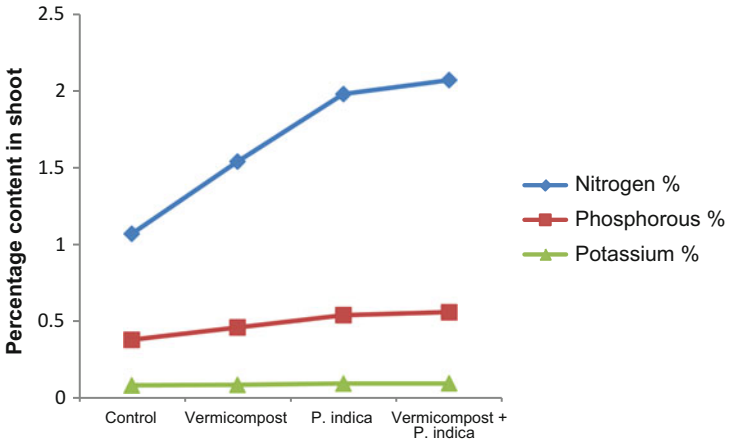


**Fig. 13.7** Effect of dual inoculation of *P. indica* and vermicompost on NPK content of shoot. Percentage of nitrogen (*diamond* marked), phosphorus (*square* marked), and potassium (*triangular* marked) present in the shoots of tomato plants after 120 days of transplantation

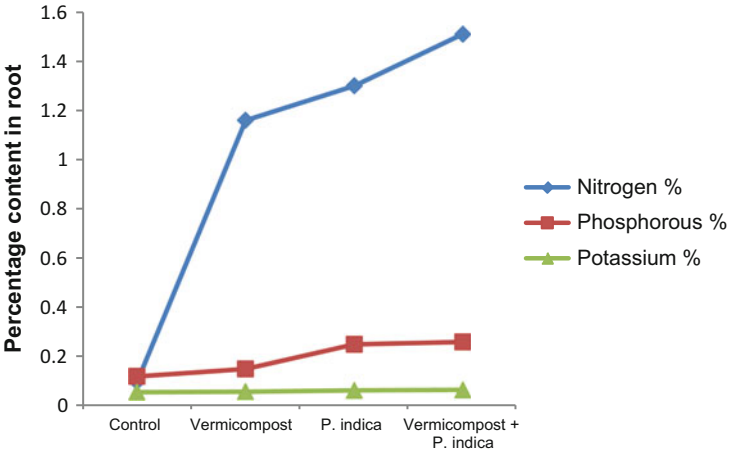


**Fig. 13.8** Effect of dual inoculation of *P. indica* and vermicompost on NPK content of shoot. Percentage of nitrogen (*diamond* marked), phosphorus (*square* marked), and potassium (*triangular* marked) present in the roots of tomato plants after 120 days of transplantation

and black lentil plants was established when inoculated with *P. indica* (Kumar et al. 2012; Nautiyal et al. 2010). The increased NPK in *Phaseolus* was associated with inoculation of *P. indica* and *Rhizobium* in presence of vermicompost (Tuladhar et al. 2013).

The association of *P. indica* in presence of vermicompost in soil has increased the NPK content in the tomato plant. Compared to phosphorous and potassium, the nitrogen uptake has been hugely elevated (Figs. 13.7 and 13.8). The P uptake is more efficient in soybean through tripartite association of *P. indica*, *Rhizobium*, and

vermicompost (Tuladhar et al. 2013) compared to dual effect of *P. indica* and vermicompost on tomato plant (Figs. 13.7 and 13.8), while the effect on N and K uptake is alike. It seems various factors are involved in the process of nutrient uptake leading to variation in the effect of mycorrhiza on the diverse plant species.

## 13.5 Conclusion

As in the circumstance of numerous types of plants, the vegetative growth of tomato plant is accelerated by *P. indica*. However, there are several conditions essential for the efficiency in the nutrient uptake. Supplementing vermicompost to enrich the soil has increased the efficiency of *P. indica* to promote the growth of tomato plant. Such effect may not be identical to every variety of plant and under different environmental conditions. It is necessary to identify the optimal conditions to augment the effectiveness of this mycorrhiza.

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# Chapter 14
# Promotion and Value Addition to Some Important Medicinal Plants Under Saline Condition by Intervention of a Novel Mycorrhizal Formulation

**Priyanka Sharma, Hemesh Joshi, Amit C. Kharkwal, Narendra Tuteja, and Ajit Varma**

**Abstract** The use of plants in the remediation of saline and sodic soils is an emerging low cost approach in the reclamation of abandoned irrigated fields. The present study focuses on use of a novel AM-like-Fungus *Piriformospora indica* for phtyoremediation by early intervention with selected medicinal plants. *Piriformospora indica,* a root endophytic fungus, has been reported to promote growth of many plants under normal condition and allow the plants to survive under stress conditions. The fungus is able to associate with the roots of various plant species in a manner similar to mycorrhiza and promotes plant growth. *P. indica* has been reported to induce resistance in the monocotyledonous plant barley to fungal diseases, along with tolerance to salt stress without affecting the plant productivity. The prospects for improved agriculture, by the use of microbial inoculants as biofertilizers or biological control agents, are particularly good in less intensive, low-input agricultural systems. Hence, in developing countries microbial inoculation of plants could be of great importance. The advantages are: better yields, lower costs, reduced dependence on chemicals, and sustainable environment. The production of microbial inoculants is not very difficult; unsophisticated fermentors of modest volume can be used to produce significant quantities of bioinoculants. Present study was undertaken to investigate the effect of consortium of *P. indica* and *Azotobacter chroococcum* on salinity stress tolerance of important medicinal plants. Both inoculated and non-inoculated plantlets were subjected to four levels of salinity treatment—0, 100, 200, and 300 mM NaCl. The salinity stress decreased the ability of the consortium to colonize roots of plants, but the interaction resulted in an overall increase in plant biomass and greater shoot and root length as well as number of shoots and roots. The inoculated plantlets had significantly higher secondary metabolite contents as determined using HPLC. The higher secondary

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metabolite content may help the plants ameliorate oxidative stress resulting from high salinity. This was achieved by early interaction of the selected medicinal plants, namely, *Glycyrrhiza glabra*, *Aloe vera*, *Bacopa monnieri*, *Asparagus racemosus*, *Coleus forskohlii*, *Withania somnifera*, *Vinca rosea*, and *Ocimum* sp. with *P. indica* and *A. chroococcum* under in vitro (plant tissue culture), in vivo (greenhouse), and field conditions in Amity University and Issapur, respectively. For development of different formulations, *P. indica* was grown in large-scale fermentor. The formulated inoculum produced along with the culture filtrate was used for early intervention during the growth of plants. The plants were intervened both under in vitro (tissue culture) and ex vitro (greenhouse) conditions. The treated plants were then transferred to field for further evaluation at Issapur farm in Delhi. Plant growth was assessed on the basis of plant biomass and other morphological parameters. The rejuvenation of soil with the microbes also makes it suitable for organic cultivation of crops in the forthcoming years hence reducing the impact of chemical fertilizers.

## 14.1 Introduction

In nature, plants are frequently exposed to adverse environmental conditions. Soil salinity is one of the major constraint that drastically affects the growth, yield, and survival of the plants (Ashraf and Harris 2004; Jin et al. 2007). Currently, high soil salinity occupies 7% of Earth's land surface, and it is predicted that by the end of twenty first century, 50% of arable land will be affected (Evelin and Kapoor 2014). Salt affected lands occur in almost all climatic regions, ranging from humid topics to the Polar Regions and from below sea level (around the Dead Sea) to mountains above 5000 m (Rocky Mountains) (Aggarwal et al. 2012). The presence of excess ions in the rhizosphere leads to lowering of soil water potential and limits the availability of water to the plant. This results in many detrimental effects such as growth reduction, injury of foliage, nutrient deficiencies, destruction of soil structure, cell death, and root necrosis, finally affecting the growth and survival of the plant (Aggarwal et al. 2012; Lozano 2003; Baltruschat et al. 2008). Soil salinity along with other abiotic stresses is responsible for more than 50% reduction in crop productivity. The common cations associated with soil salinity are $Na^+$, $Ca^{2+}$, and $Mg^{2+}$, whereas the major anions are $Cl^-$, $So_4{}^{2-}$, and $HCO_3{}^-$. However, the most damaging ions that deteriorate the soil structure and can be toxic to plants are $Na^+$ and $Cl^-$ (Hasegawa et al. 2000). Soil salinization results due to weathering of minerals, improper agricultural management practices, poor water management, low precipitation, limited rainfall, high evaporation, and heavy irrigation (Chen et al. 2011). This increase in soil salinization is against the needs of expanding global population, which requires an increase of 20% food production in developed countries and 60% in developing countries over next 30 years (Galvani 2007).

## 14.2 Effect of Salinity on Plant Growth

Increase in soil salinity results in reduction of osmotic potential of soil solution, which make it necessary for the plant to maintain a lower intracellular osmotic potential. The response of all plants to decreased osmotic potential is turgor loss, which results in stomatal closure, followed by reduction in gas exchange (i.e., transpiration and photosynthesis). Hence, the major cause of growth inhibition under salt stress is decreased turgor pressure (Ashraf and Tufail 1994). The stomatal closure in turn decreases the availability of $CO_2$ and reduces photosynthesis, which ultimately lead to production of Reactive Oxygen Species (ROS). Also, lower availability of NADP to accept electrons from PSI results in reduction of $O_2$ with a concomitant generation of ROS (Sudhakar et al. 2001; Gill and Tuteja 2010). The total ionic concentration adversely affects the growth of the plants. With increase in salinity, the uptake and translocation of ions get reduced. It has been reported that the salinity significantly increases $K^+$ and $Cl^-$ in the leaves and stems, while reduces $Ca^{2+}$, $K^+$, $Mg^{2+}$, and $NO_3^-$. The $K^+$ and $Ca^{2+}$ are required to maintain the selectivity and integrity of membrane. Thus, high $Na^+/K^+$ ratio alters the membrane selectivity and results in passive accumulation of $Na^+$ ion in the roots and shoots. It also affects other processes such as stomatal movement, photosynthesis, and transpiration (Hu and Schmidhalter 1997). Thus, plants need to adapt a strategy to protect themselves from such stresses.

## 14.3 Plant Microbe Interaction

Plant growth and development is synergistic combination of a number of environmental factors. Plants are being attacked by a number of microorganisms, with an aim to acquire nutrients from them. The consequences of the interactions can be harmful (as in parasitism), neutral, and beneficial (as in mutualism) (Shen et al. 2006; Thrall et al. 2007). The plants are in association with a number of microbes, including soil microbes, atmospheric microbes, plant colonizing microbes, plant surface microbes, and internal microbes. Most of the microbes act synergistically with plants and do not cause any ill effects to them. However, selection of appropriate microbe is important that can develop positive interaction with the plant and boost plant growth and productivity and can improve the soil health. Many of such microbes establish themselves in the rhizospheric regions in a large population and interact with the host plant in a number of ways, depending on the multitude of signals and signal perception between both partners (Petrini 1986; Jain et al. 2016). In a recent review conducted by Jia et al. (2016), the relationship of endophytic fungi and medicinal plant has been very well explained.

The symbiotic interaction of plant and fungi provides a promising strategy for the better establishment of tissue culture raised plants, enhancing various phytochemicals and reducing yield losses in cultivated crops grown under saline

environment (Varma et al. 2012b; Bajaj et al. 2014; Sharma et al. 2014a, b, 2015; Rodriguez et al. 2004). The use of plants in the remediation of saline and sodic soils is an emerging low cost approach in the reclamation of abandoned irrigated fields (Kumar and Abrol 1984; Qadir et al. 2002; Tokhtarov 2004). The creation of highly productive cultivation systems through the establishment of palatable plants has been shown to remediate saline/sodic soils as well as provide an income to resource poor farmers (Dagar et al. 2004). In this respect, the importance of arbuscular mycorrhizal (AM) fungi in restoration of denuded habitats has been recognized (St. John 1998). AM fungi support plant and bacterial performance and improve soil structure (Heinonsalo et al. 2000; Meharg and Cairney 2000).

## 14.4 Mycorrhizal Symbiosis and Alleviation of Salt Stress

Mycorrhizas are the symbiotic association between plants and fungi that colonize the root cortical cells during periods of active plant growth. Approximately, 80% terrestrial plants and 92% terrestrial plant families have this association in their natural habitats, surveyed by Wang and Qiu (2006). These associations are thought to exist since at least 400 million years (Rodriguez and Redman 2008; Harley 1989). The symbiotic association is characterized by translocation of sugars and other carbon compounds from the plant to fungus and, in turn, the fungus facilitates the plant with acquisition of mineral nutrients from the soil, thereby providing a critical linkage between the plant, root, and the soil (Brundrett 2004). The mycorrhization improves the nutrient and water uptake, enhance resistance to soil borne diseases, and impart tolerance against extreme environments (Smith and Read 1997). The mutualism once established changes the physiology and/or morphology of roots and plants significantly, altering the root exudation (Linderman 1988; Bansal and Mukerji 1994). More than 6000 fungal species are capable of establishing mycorrhizal relationship with approximately 2,40,000 plant species (Sharma 2001). The synergism provides enormous benefits to the plant, such as absorption of toxic elements from soil, soil restoration, establishment of green cover, disease resistance, drought tolerance, etc. These microorganisms enrich the soil and provide nutrition to the plant for better growth and development, by sequestering the nutrients from soil and translocating to the plant, in turn acquiring carbon input from the plant. This reduces the dependence on chemical fertilizers. Thus, mycorrhization is a mutually beneficial process where both the partners get benefit from one another. These associations are extremely important for the countries, where large chunks of land is degraded and is not fit for cultivation, such as South Asian Association for Regional Cooperation (SAARC) countries like Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan, and Sri Lanka.

### *14.4.1 Arbuscular Mycorrhizal (AM) Fungi*

The AM fungi are ubiquitous soil microbes that form an integral component of terrestrial ecosystem. They form symbiotic association with plant roots of over 90% terrestrial plant species, including Angiosperms, Gymnosperms, Pteridophytes, Lycopods, and Mosses (Smith and Read 1997). AM fungi got its name from the distinct fungal tree-shaped structure that develops in plant root cells (arbus + tree). The Arbuscules are relatively short lived (less than 15 days). AM fungi are found under all climates and in all ecosystems, regardless of the type of soil, vegetation, or growing conditions. AM fungi, which are microscopic soil fungi, colonize the roots and rhizosphere and the hyphae being thinner, branch more frequently than plant roots and spread out over several centimeters in the form of ramified filaments. This extended network increases the absorptive capacity of roots and allows the plant to have better access to a greater quantity of water and minerals required for nutrition. The association thus increases water uptake and availability of nutrients to the plant, especially insoluble soil phosphate (Clark and Zeto 2000), which in turn benefits the fungus by supply of carbohydrates derived from photosynthesis (Harrison 1999). The importance of this synergism lies in enhanced nutrient availability to the plant, resulting in better growth and improved vigor of plant and translocation of carbon to the fungus in the form of sugars, amino acids, and vitamins essential for its growth (Harley and Smith 1983). Armada et al. (2015) investigated the effectiveness of drought-adapted autochthonous microorganisms [*Bacillus thuringiensis* (Bt) and a consortium of arbuscular mycorrhizal (AM) fungi] from a degraded Mediterranean area to improve plant growth and physiology in *Zea mays* under drought stress. They found that autochthonous microorganisms were useful to protect not only native plants against drought but also an agronomically important plant such as maize. Mycorrhization significantly alters the morphology and physiology of roots and plants leading to altered root exudation.

### *14.4.2* Piriformospora indica

The present chapter focuses on use of a novel AM-like-Fungus *Piriformospora indica* for phtyoremediation by early intervention with selected medicinal plants. *Piriformospora indica*, an endophytic fungus of the Sebacinaceae family, was isolated from the rhizosphere soils of the woody shrubs *Prosopis juliflora* and *Zizyphus nummularia* in the sandy desert soils of Rajasthan, India (Weiß et al. 2004; Verma et al. 1998). The fungus is easily cultivable and colonizes the roots of a wide variety of plant species through directly manipulating plant hormone-signaling pathway during the course of mutualism (Varma et al. 1999). *P. indica* is able to associate itself with roots of various plant species in a manner similar to AM fungi (Varma et al. 1999, 2001; Sharma et al. 2014a; Das et al. 2012a, b; Singh et al. 2003; Franken 2012). *P. indica* promotes nutrient uptake and enhances the

growth and biomass of the plants, including monocots and dicots (Yadav et al. 2010 and Varma et al. 2000; Trivedi et al. 2016), induces early flowering (Das et al. 2012b), increases the resistance against fungal pathogens, and allows the plant to survive under stressed environment (Das et al. 2012a; Harman 2011). Ansari et al. (2013) highlighted the ethylene- and cyclophilin A (CypA)-mediated response of *P. indica* for sustainable crop production under adverse environmental conditions. The fungus grows inter- and intracellularly and forms pear-shaped, auto-fluorescent chlamydospores within the cortex of the colonized roots and in the rhizosphere zone, but it does not invade the endodermis and the aerial parts of the plants (Varma et al. 2012a, b; Siddhanta et al. 2017). Hence, *P. indica* is a novel mutualistic symbiont in contrast to known mycorrhizas, and root nodulating bacteria. *P. indica* contains a high affinity Pi transporter (PiPT) involved in improving Pi nutrition levels in the host plant under phosphorus limiting conditions (Johri et al. 2015). This fungus provides a promising model organism for the investigations of beneficial plant microbe interaction and enables the identification of compounds which improve plant growth and productivity.

The prospects for improved agriculture, by the use of microbial inoculants as biofertilizers or biological control agents, are particularly good in less intensive, low-input agricultural systems (Bhardwaj et al. 2014). Hence, in developing countries, microbial inoculation of plants could be of great importance. The production of microbial inoculants is not very difficult; unsophisticated fermentors of modest volume can be used to produce significant quantities of bioinoculants. Present study was undertaken to investigate the effect of consortium of *P. indica* and *Azotobacter chroococcum* on salinity stress tolerance of important medicinal plants. Both inoculated and non-inoculated plantlets were subjected to four levels of salinity treatment—0, 100, 200, and 300 mM NaCl. This was achieved by early interaction of the selected medicinal plants, namely, *Glycyrrhiza glabra*, *Aloe vera*, *Bacopa monnieri*, *Asparagus racemosus*, *Coleus forskohlii*, *Withania somnifera*, *Vinca rosea,* and *Ocimum* sp. with *P. indica* and *A. chroococcum* under in vitro (plant tissue culture), in vivo (greenhouse), and field conditions in Amity University, Noida, UP, India and Issapur, New Delhi, respectively. For development of different formulations, *P. indica* was grown in large-scale fermentor. The formulated inoculum produced along with the culture filtrate was used for early intervention during the growth of plants. The plants were intervened both under in vitro (tissue culture) and ex vitro (greenhouse) conditions. The treated plants were then transferred to field for further evaluation at Issapur. Plant growth was assessed on the basis of plant biomass and other morphological parameters. Quantity of formulation required for treatment has been standardized for large number of plants. The plants were also transplanted onsite at Issapur in saline field. The plants treated with *P. indica* (primed with *Azotobacter*) performed better as compared to control (Tables 14.1, 14.2, 14.3, 14.4, 14.5, 14.6, 14.7).

**Table 14.1** Growth parameters for tissue culture raised plants grown on 100 ppm salt stress in modified medium

| S. No. | Plant | | Shoot length (cm) | Root length (cm) | No. of leaves | Fresh wt (gm) | Dry wt (gm) | Moisture content (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | *Coleus forskohlii* | Treated | 2.1 | 0.8 | 6.1 | 0.95 | 0.12 | 87.36 |
| | | Control | 1.6 | 0.5 | 4.3 | 0.82 | 0.09 | 73.00 |
| 2 | *Bacopa monnieri* | Treated | 3.6 | 0.6 | 15.55 | 0.98 | 0.15 | 82.69 |
| | | Control | 2.2 | 0.4 | 9.6 | 0.89 | 0.11 | 76.40 |
| 3 | *Ocimum sanctum* | Treated | 1.6 | 0.4 | 6.1 | 0.65 | 0.10 | 84.61 |
| | | Control | 1.2 | 0.15 | 4.2 | 0.58 | 0.12 | 79.31 |
| 4 | *Aloe vera* | Treated | 3.7 | 1.05 | 6.1 | 1.25 | 0.10 | 92.00 |
| | | Control | 3.2 | 0.91 | 4.3 | 1.11 | 0.20 | 81.98 |
| 5 | *Withania somnifera* | Treated | 1.45 | 0.44 | 6.2 | 0.56 | 0.06 | 89.28 |
| | | Control | 1.25 | 0.32 | 4.3 | 0.49 | 0.11 | 77.55 |
| 6 | *Catharanthus roseus* | Treated | 1.85 | 0.32 | 6.3 | 0.58 | 0.09 | 84.48 |
| | | Control | 1.65 | 0.26 | 4.11 | 0.49 | 0.12 | 75.51 |

The treated plants were treated with *P. indica* primed with *Azotobacter*. Data was recorded after 2 weeks of salt treatment. Individual datum is replicate of 10 plants, $n = 3$

**Table 14.2** Growth parameters for tissue culture raised plants grown on 200 ppm salt stress in modified medium

| S. No. | Plant | | Shoot length (cm) | Root length (cm) | No. of leaves | Fresh wt (gm) | Dry wt (gm) | Moisture content (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | *Coleus forskohlii* | Treated | 1.8 | 0.6 | 4.2 | 0.87 | 0.15 | 82.75 |
| | | Control | 1.2 | 0.3 | 2.01 | 0.78 | 0.18 | 76.92 |
| 2 | *Bacopa monnieri* | Treated | 3.1 | 0.41 | 8.1 | 0.89 | 0.16 | 82.02 |
| | | Control | 1.6 | 0.26 | 4.7 | 0.80 | 0.20 | 75.00 |
| 3 | *Ocimum sanctum* | Treated | 1.12 | 0.23 | 4.12 | 0.61 | 0.10 | 83.60 |
| | | Control | 0.97 | 0.09 | 3.09 | 0.49 | 0.11 | 77.55 |
| 4 | *Aloe vera* | Treated | 3.11 | 0.85 | 4.2 | 0.98 | 0.18 | 81.63 |
| | | Control | 2.78 | 0.66 | 3.12 | 0.78 | 0.18 | 76.92 |
| 5 | *Withania somnifera* | Treated | 1.15 | 0.23 | 3.67 | 0.51 | 0.10 | 80.39 |
| | | Control | 0.95 | 0.16 | 2.42 | 0.44 | 0.11 | 75.00 |
| 6 | *Catharanthus roseus* | Treated | 1.16 | 0.28 | 5.51 | 0.51 | 0.10 | 80.39 |
| | | Control | 1.01 | 0.21 | 3.54 | 0.44 | 0.12 | 72.72 |

The treated plants were treated with *P. indica* primed with *Azotobacter*. Data was recorded after 2 weeks of salt treatment. Individual datum is replicate of 10 plants, $n = 3$

**Table 14.3** Growth parameters for tissue culture raised plants grown on 300 ppm salt stress in modified medium

| S. No. | Plant | | Shoot length (cm) | Root length (cm) | No. of leaves | Fresh wt (gm) | Dry wt (gm) | Moisture content (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | *Coleus forskohlii* | Treated | 1.44 | 0.32 | 3.12 | 0.69 | 0.12 | 82.60 |
| | | Control | 0.61 | 0.11 | 1.01 | 0.49 | 0.12 | 75.51 |
| 2 | *Bacopa monnieri* | Treated | 2.14 | 0.21 | 3.23 | 0.61 | 0.12 | 80.32 |
| | | Control | 0.89 | 0.06 | 0.54 | 0.44 | 0.14 | 68.18 |
| 3 | *Ocimum sanctum* | Treated | 0.62 | 0.13 | 1.12 | 0.52 | 0.10 | 80.76 |
| | | Control | 0.47 | 0.03 | 0.59 | 0.48 | 0.15 | 68.75 |
| 4 | *Aloe vera* | Treated | 2.16 | 0.65 | 2.12 | 0.78 | 0.13 | 83.33 |
| | | Control | 1.18 | 0.46 | 1.16 | 0.66 | 0.15 | 77.27 |
| 5 | *Withania somnifera* | Treated | 0.92 | 0.19 | 0.97 | 0.42 | 0.08 | 80.95 |
| | | Control | 0.89 | 0.12 | 0.52 | 0.32 | 0.09 | 74.19 |
| 6 | *Catharanthus roseus* | Treated | 1.04 | 0.21 | 1.11 | 0.46 | 0.08 | 82.60 |
| | | Control | 0.98 | 0.17 | 1.01 | 0.41 | 0.10 | 75.60 |

The treated plants were treated with *P. indica* primed with *Azotobacter*. Data was recorded after 2 weeks of salt treatment. Individual datum is replicate of 10 plants. $n = 3$

**Table 14.4** Growth parameters for cutting raised plants grown on 100 ppm salt stress

| S. No. | Plant | | Shoot length (cm) | Root length (cm) | No. of leaves | Fresh wt (gm) | Dry wt (gm) | Moisture content (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | *Coleus forskohlii* | Treated | 15.81 | 7.67 | 21.66 | 28.91 | 4.85 | 83.22 |
| | | Control | 13.65 | 6.32 | 18.88 | 26.21 | 4.99 | 80.96 |
| 2 | *Bacopa monnieri* | Treated | 12.08 | 3.22 | 45.11 | 14.85 | 2.67 | 82.02 |
| | | Control | 11.02 | 2.45 | 39.61 | 13.12 | 2.77 | 78.88 |
| 3 | *Ocimum sanctum* | Treated | 10.12 | 5.67 | 22.81 | 19.26 | 4.20 | 78.19 |
| | | Control | 8.98 | 4.98 | 19.12 | 18.43 | 4.98 | 72.97 |
| 4 | *Aloe vera* | Treated | 16.96 | 6.61 | 7.81 | 33.89 | 6.10 | 82.00 |
| | | Control | 14.12 | 5.87 | 6.78 | 31.26 | 6.91 | 77.89 |
| 5 | *Withania somnifera* | Treated | 12.12 | 7.88 | 41.22 | 14.23 | 2.59 | 81.79 |
| | | Control | 11.19 | 6.99 | 38.54 | 12.98 | 2.97 | 77.11 |
| 6 | *Catharanthus roseus* | Treated | 12.11 | 6.82 | 36.61 | 16.66 | 2.98 | 82.11 |
| | | Control | 11.18 | 6.23 | 33.86 | 14.94 | 3.01 | 79.85 |
| 7 | *Glycyrrhiza glabra* | Treated | 9.35 | 8.22 | 14.61 | 12.65 | 2.63 | 79.20 |
| | | Control | 8.78 | 7.98 | 12.32 | 11.18 | 2.69 | 75.93 |
| 8 | *Asparagus racemosus* | Treated | 18.21 | 10.11 | 96.12 | 16.16 | 2.89 | 82.11 |
| | | Control | 16.56 | 9.87 | 85.26 | 14.85 | 2.87 | 80.67 |

The treated plants were treated with *P. indica* primed with *Azotobacter*. Data was recorded after 4 weeks of salt treatment. Individual datum is replicate of 6 plants, $n = 3$

**Table 14.5** Growth parameters for cutting raised plants grown on 200 ppm salt stress

| S. No. | Plant | | Shoot length (cm) | Root length (cm) | No. of leaves | Fresh wt (gm) | Dry wt (gm) | Moisture content (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | *Coleus forskohlii* | Treated | 12.65 | 6.12 | 14.91 | 19.67 | 3.81 | 80.63 |
| | | Control | 10.11 | 5.11 | 9.81 | 17.41 | 3.92 | 77.48 |
| 2 | *Bacopa monnieri* | Treated | 10.11 | 3.02 | 21.15 | 10.63 | 2.57 | 75.82 |
| | | Control | 9.02 | 2.17 | 19.52 | 9.18 | 2.68 | 70.80 |
| 3 | *Ocimum sanctum* | Treated | 8.63 | 4.17 | 12.31 | 14.26 | 3.02 | 78.82 |
| | | Control | 7.21 | 3.18 | 10.11 | 13.13 | 3.98 | 69.68 |
| 4 | *Aloe vera* | Treated | 14.12 | 5.16 | 5.62 | 26.49 | 5.80 | 78.10 |
| | | Control | 12.73 | 4.65 | 4.14 | 23.11 | 5.81 | 74.85 |
| 5 | *Withania somnifera* | Treated | 10.02 | 5.34 | 21.44 | 11.15 | 2.89 | 74.08 |
| | | Control | 9.01 | 3.88 | 19.02 | 10.21 | 2.98 | 70.81 |
| 6 | *Catharanthus roseus* | Treated | 9.12 | 4.12 | 24.21 | 12.55 | 2.76 | 78.00 |
| | | Control | 8.11 | 3.06 | 20.23 | 11.12 | 2.91 | 73.83 |
| 7 | *Glycyrrhiza glabra* | Treated | 7.30 | 6.09 | 12.51 | 10.31 | 2.46 | 76.13 |
| | | Control | 6.12 | 5.87 | 10.13 | 9.12 | 2.56 | 71.92 |
| 8 | *Asparagus racemosus* | Treated | 14.11 | 9.01 | 63.34 | 13.28 | 2.89 | 78.23 |
| | | Control | 12.21 | 7.78 | 55.78 | 11.45 | 2.87 | 74.93 |

The treated plants were treated with *P. indica* primed with *Azotobacter*. Data was recorded after 4 weeks of salt treatment. Individual datum is replicate of 6 plants, $n = 3$

**Table 14.6** Growth parameters for cutting raised plants grown on 300 ppm salt stress

| S. No. | Plant | | Shoot length (cm) | Root length (cm) | No. of leaves | Fresh wt (gm) | Dry wt (gm) | Moisture content (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | *Coleus forskohlii* | Treated | 9.01 | 4.02 | 5.31 | 10.18 | 3.88 | 61.88 |
| | | Control | 6.97 | 3.41 | 3.21 | 7.32 | 3.17 | 56.69 |
| 2 | *Bacopa monnieri* | Treated | 2.21 | 1.01 | NA | NA | NA | NA |
| | | Control | 1.23 | 0.97 | NA | NA | NA | NA |
| 3 | *Ocimum sanctum* | Treated | 4.12 | 2.11 | NA | NA | NA | NA |
| | | Control | 3.01 | 1.38 | NA | NA | NA | NA |
| 4 | *Aloe vera* | Treated | 7.34 | 4.19 | 3.22 | 16.19 | 4.12 | 74.55 |
| | | Control | 6.67 | 3.46 | 2.44 | 14.04 | 4.91 | 65.02 |
| 5 | *Withania somnifera* | Treated | 7.21 | 4.32 | 12.43 | 8.09 | 2.28 | 71.81 |
| | | Control | 5.91 | 2.67 | 9.01 | 6.91 | 2.45 | 64.54 |
| 6 | *Catharanthus roseus* | Treated | 8.11 | 3.82 | 8.13 | 9.66 | 2.26 | 76.60 |
| | | Control | 7.41 | 3.06 | 6.21 | 8.34 | 2.43 | 70.86 |
| 7 | *Glycyrrhiza glabra* | Treated | 4.31 | 1.01 | NA | NA | NA | NA |
| | | Control | 3.92 | 0.88 | NA | NA | NA | NA |
| 8 | *Asparagus racemosus* | Treated | 9.12 | 6.01 | 21.15 | 9.09 | 2.54 | 72.05 |
| | | Control | 7.09 | 3.18 | 12.09 | 7.15 | 2.47 | 65.45 |

The treated plants were treated with *P. indica* primed with *Azotobacter*. Data was recorded after 4 weeks of salt treatment. Individual datum is replicate of 6 plants, $n = 3$

**Table 14.7** Growth parameters for cutting raised plants grown on saline soil at Issapur

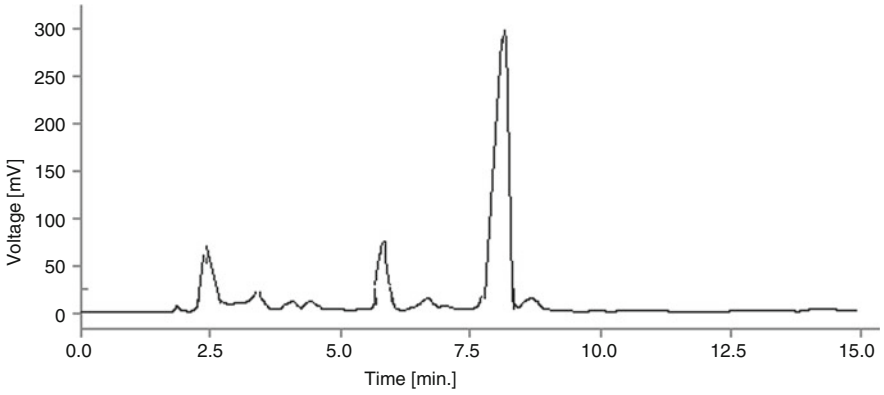
| S. No. | Plant | | Shoot length (cm) | Root length (cm) | No. of leaves | No. of flowers | Fresh wt (gm) | Dry wt (gm) | Moisture content (%) | % Survival |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Coleus forskohlii* | Treated | 9.01 | 4.02 | 5.31 | 0 | 10.18 | 2.88 | 71.65 | 21.6 |
| | | Control | 6.97 | 3.41 | 3.21 | 0 | 7.32 | 2.84 | 61.20 | 7.2 |
| 2 | *Bacopa monnieri* | Treated | NA | NA | NA | 0 | NA | NA | NA | 0 |
| | | Control | NA | NA | NA | 0 | NA | NA | NA | 0 |
| 3 | *Ocimum sanctum* | Treated | 4.12 | 2.11 | 8.23 | 0 | 7.45 | 2.42 | 69.37 | 2.8 |
| | | Control | 3.01 | 1.38 | 7.01 | 0 | 6.24 | 2.16 | 65.38 | 27.14 |
| 4 | *Aloe vera* | Treated | 7.34 | 4.19 | 3.22 | 0 | 16.19 | 3.12 | 80.72 | 56 |
| | | Control | 6.67 | 3.46 | 2.44 | 0 | 14.04 | 4.01 | 71.43 | 38.6 |
| 5 | *Withania somnifera* | Treated | 7.21 | 4.32 | 12.43 | 0 | 8.09 | 2.28 | 71.81 | 29.2 |
| | | Control | 5.91 | 2.67 | 9.01 | 0 | 6.91 | 2.45 | 64.54 | 18.5 |
| 6 | *Catharanthusroseus* | Treated | 8.11 | 3.82 | 8.13 | 8.22 | 9.66 | 2.26 | 76.60 | 21.66 |
| | | Control | 7.41 | 3.06 | 6.21 | 3.11 | 8.34 | 2.43 | 70.86 | 6.12 |
| 7 | *Glycyrrhiza glabra* | Treated | NA | NA | NA | NA | NA | NA | NA | 0 |
| | | Control | NA | NA | NA | NA | NA | NA | NA | 0 |
| 8 | *Asparagus racemosus* | Treated | 9.12 | 6.01 | 21.15 | 0 | 9.09 | 2.54 | 72.45 | 56 |
| | | Control | 7.09 | 3.18 | 12.09 | 0 | 7.15 | 2.47 | 65.45 | 10 |

The treated plants were treated with *P. indica* primed with *Azotobacter*. Data was recorded after 26 weeks onwards of salt treatment. Individual datum is replicate of 6 plants, $n = 3$

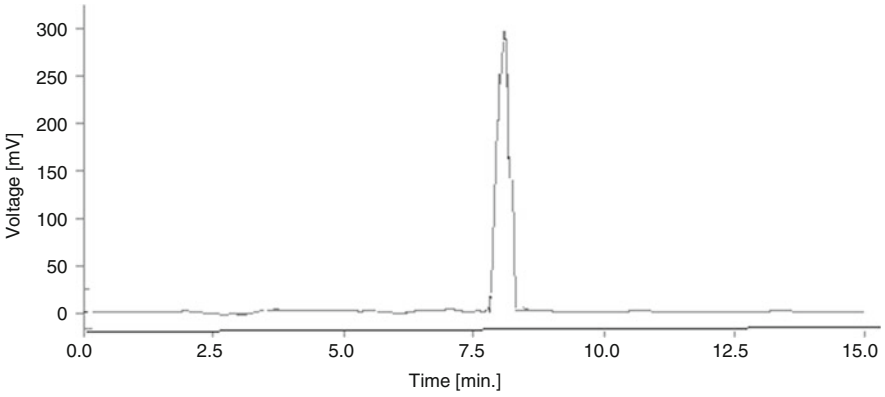
## 14.5 Chemical Analysis of Secondary Metabolites

### *14.5.1* Coleus forskohlii*: Root Harvested from Saline Soil After 6 Months Were Subjected to Forskoline Analysis*

**Standard**

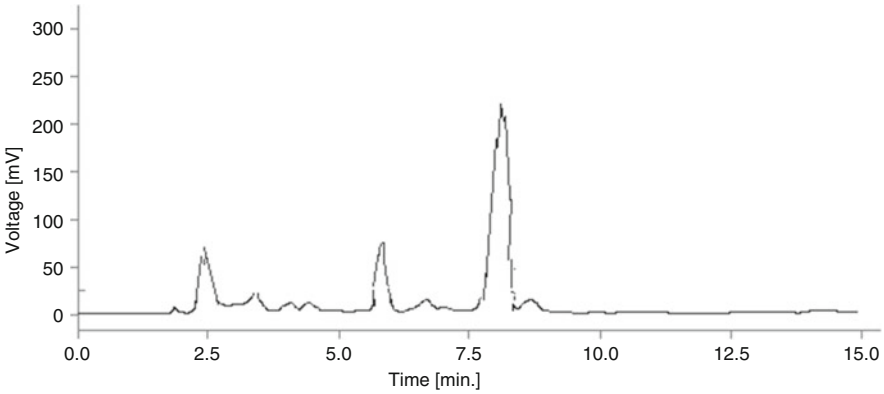



**Treated**




Details: *Coleus forskohlii* roots in HPLC for Forskoline
Conc. of sample 1.56512 gm/100 ml
Loss on drying = 40.1%
Forskoline content on L.O.D. basis: 0.85%
Forskoline content on fresh weight basis 8.19%

**Control**

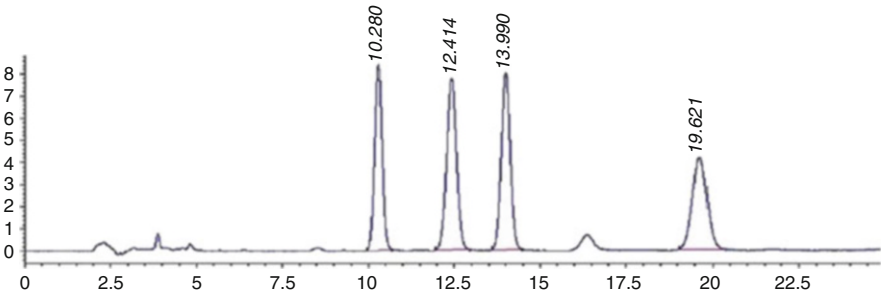



*Coleus forskohlii* Roots in HPLC for Forskoline Details: Conc. of sample 1.56891 gm/100 ml Loss on drying = 4.27% Forskoline content on L.O.D. basis: 0.68% Forskoline content on fresh weight basis 6.53%

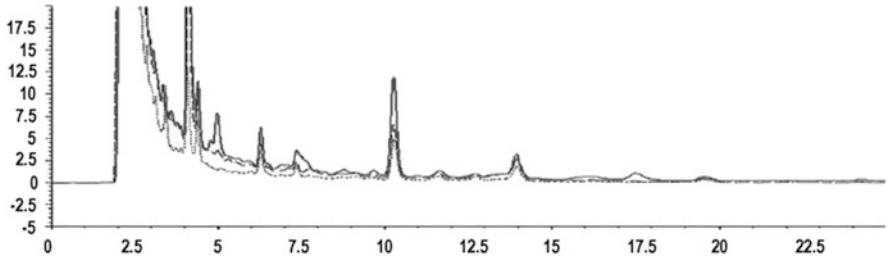
**The treated plants produced 25.47% more forskoline as compared to untreated control under saline conditions.**

### *14.5.2* Vinca rosea*: Leaves were Harvested from Plants Growing in Saline Soil After 6 Months and Subjected to Analysis of Vindoline, Vincristine, Catharanthine, and Vinblastine*

**Standard**



**Treated**




*Vinca rosea* leaves in HPLC for Vindoline, Vincristine, Catharanthine, and Vinblastine Details: Conc. of sample 0.5871 gm/100 ml

Loss on drying = 3.72%

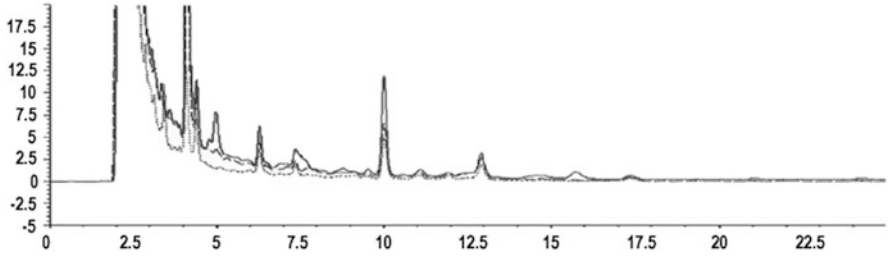
Alkaloid content on L.O.D. basis:

| Vindoline | Vincristine | Catharanthine | Vinblastine |
|---|---|---|---|
| 0.056 | 0.0013 | 0.016 | 0.0017 |

Alkaloid content on fresh weight basis:

| Vindoline | Vincristine | Catharanthine | Vinblastine |
|---|---|---|---|
| 0.51% | 0.013% | 0.15% | 0.018% |

**Control**




*Vinca rosea* leaves in HPLC for Vindoline, Vincristine, Catharanthine, and Vinblastine

Details: Conc. of sample 0.5831 gm/100 ml

Loss on drying = 3.69%

Alkaloid content on L.O.D. basis:

| Vindoline | Vincristine | Catharanthine | Vinblastine |
|---|---|---|---|
| 0.033 | 0.0007 | 0.0087 | 0.0012 |

Alkaloid content on fresh weight basis:

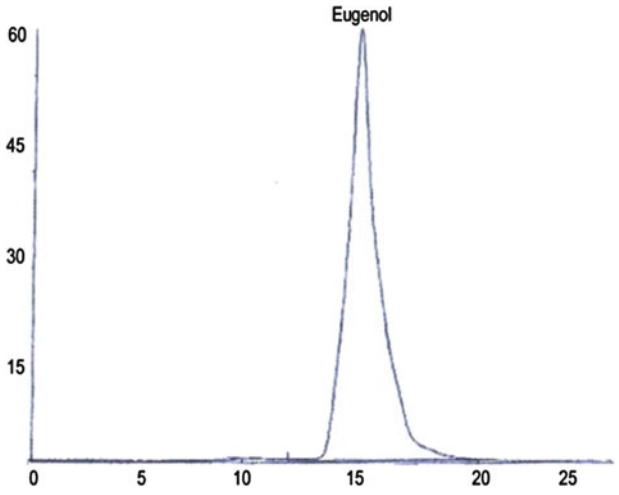
| Vindoline | Vincristine | Catharanthine | Vinblastine |
|---|---|---|---|
| 0.32% | 0.008% | 0.09% | 0.011% |

Alkaloid content treated vs control:

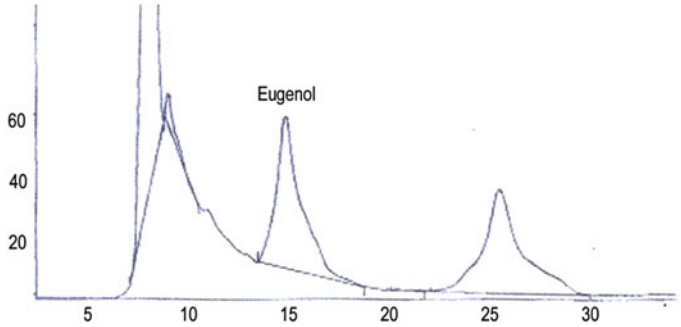
| S.No | Alkaloid | % Change |
|---|---|---|
| 1 | Vindoline | 59% |
| 2 | Vincristine | 62.5% |
| 3 | Catharanthine | 66.6% |
| 4 | Vinblastine | 63.6% |

### *14.5.3* Ocimum sanctum*: Leaves Were Harvested from Plants Growing in Saline Soil After 6 Months and Subjected to Analysis of Eugenol*

**Standard**

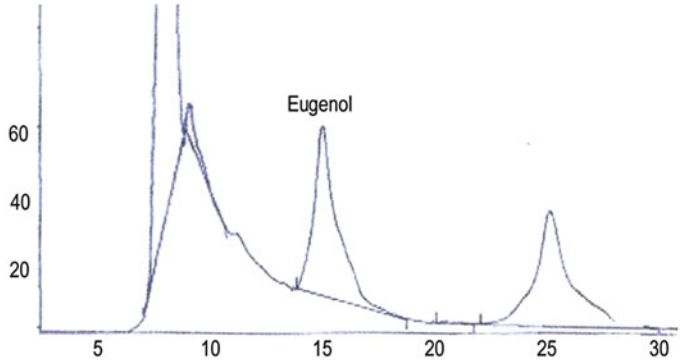


**Treated**




*Ocimum sanctum* leaves in HPLC for Eugenol analysis
Details: Conc. of sample 1.2865 gm/100 ml
Loss on drying = 3.93%
Eugenol content on L.O.D. basis: 0.27%
Eugenol content on fresh weight basis: 2.53%

**Control**

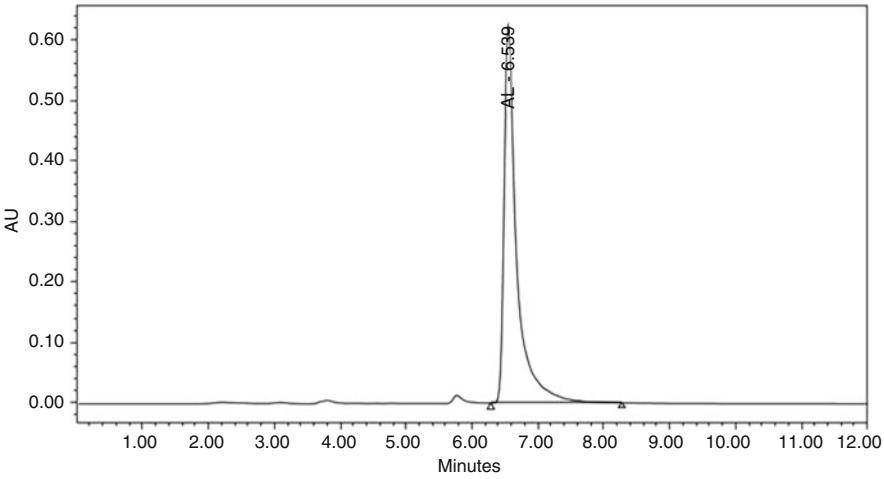



*Ocimum sanctum* leaves in HPLC for Eugenol analysis
Details: Conc. of sample 1.2845 gm/100 ml
Loss on drying = 3.87%
Eugenol content on L.O.D. basis: 0.21%
Eugenol content on fresh weight basis: 2.18%

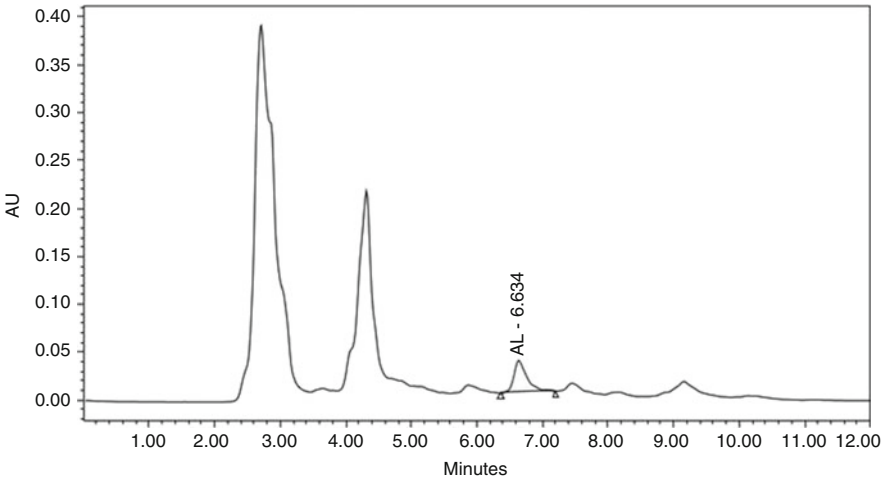
**The treated plants produced 28.57% more Eugenol as compared to untreated control under saline conditions.**

### *14.5.4* Aloe vera*: Leaves Were Harvested from Plants Growing in Saline Soil After 12 Months and Subjected to Analysis of Aloin*

**Standard**

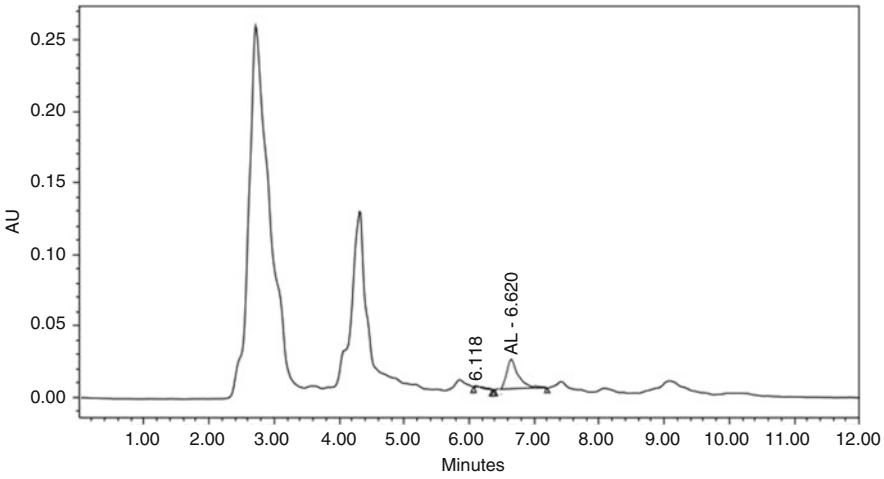



**Treated**



*Aloe vera* leaves in HPLC for Aloin analysis
Details: Conc. of sample 0.4365 gm/100 ml
Loss on drying = 2.05%
Aloin content on L.O.D. basis: 0.11%
Aloin content on fresh weight basis: 1.03%

**Control**

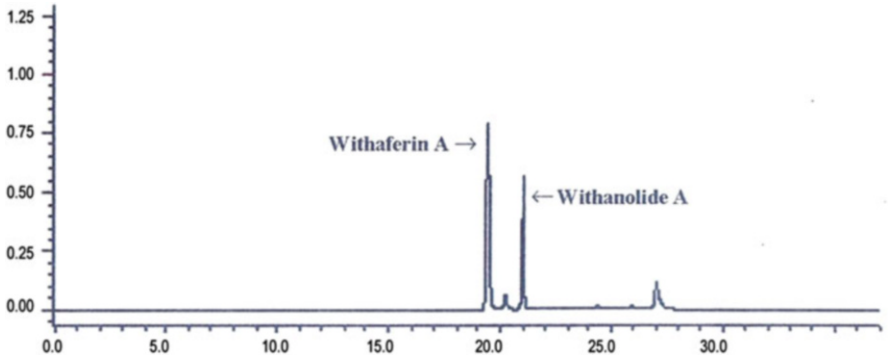



*Aloe vera* leaves in HPLC for Aloin analysis
Details: Conc. of sample 0.4123 gm/100 ml
Loss on drying = 2.01%
Aloin content on L.O.D. basis: 0.06%
Aloin content on fresh weight basis: 0.66%

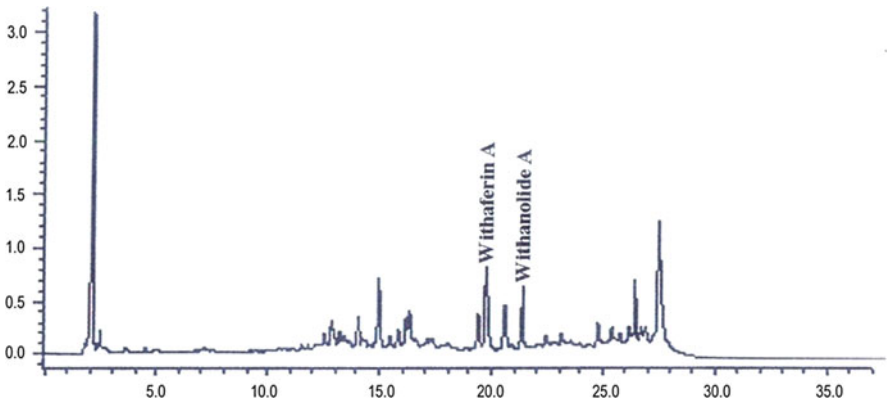
**The treated plants produced 56.06% more Aloin as compared to untreated control under saline conditions.**

### *14.5.5* Withania somnifera*: Roots Were Harvested from Plants Growing in Saline Soil After 4 Months and Subjected to Analysis of Withaferin A and Withanlide A*

**Standard**




**Treated**



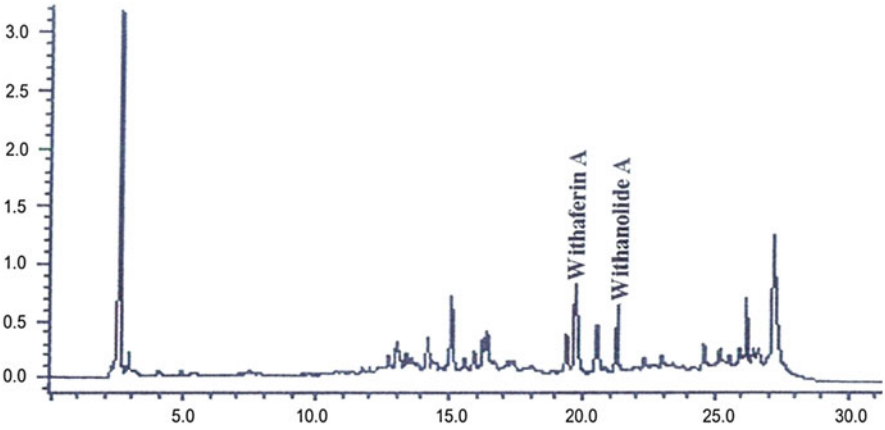

*Withania somnifera* roots in HPLC for Withaferin A and Withanolide A analysis
Details: Conc. of sample 1.4213 gm/100 ml
Loss on drying = 3.51%
Alkaloid content on L.O.D. basis:

| S.No | Alkaloid | Content |
|---|---|---|
| 1 | Withaferin A | 0.073 |
| 2 | Withanolide A | 0.055 |

Alkaloid content on fresh weight basis:

| S.No | Alkaloid | Content |
|---|---|---|
| 1 | Withaferin A | 0.72 |
| 2 | Withanolide A | 0.56 |

**Control**




*Withania somnifera* roots in HPLC for Withaferin A and Withanolide A analysis
Details: Conc. of sample 1.3917 gm/100 ml
Loss on drying = 3.45%
Alkaloid content on L.O.D. basis:

| S.No | Alkaloid | Content |
|---|---|---|
| 1 | Withaferin A | 0.055 |
| 2 | Withanolide A | 0.041 |

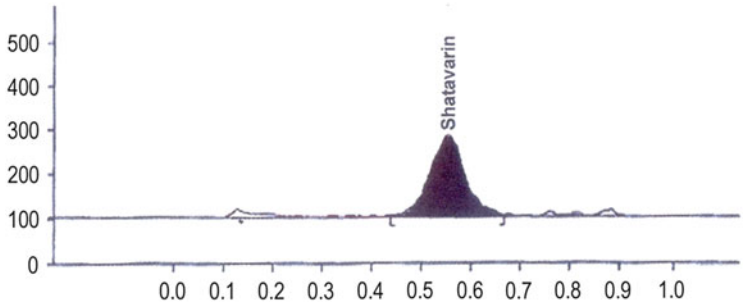
Alkaloid content on fresh weight basis:

| S.No | Alkaloid | Content |
|---|---|---|
| 1 | Withaferin A | 0.54 |
| 2 | Withanolide A | 0.41 |

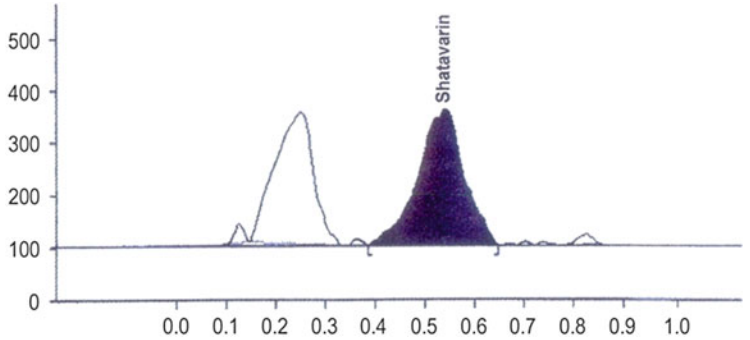
**The treated plants produced 33.3% more Withaferin A and 36.5 % more Withanolide A as compared to untreated control under saline conditions.**

### *14.5.6* Asparagus racemosus*: Roots Were Harvested from Plants Growing in Saline Soil After 18 Months and Subjected to Analysis of Shatavarin*

**Standard**

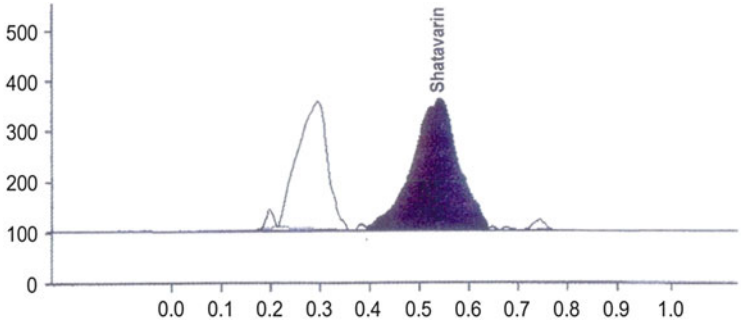



**Treated**




*Asparagus racemosus* roots in HPLC for Shatavarin analysis
Details: Conc. of sample 1.631 gm/100 ml
Loss on drying = 3.74%
Shatavarin content on L.O.D. basis: 0.41%
Shatavarin content on fresh weight basis: 4.21%

**Control**




*Asparagus racemosus* roots in HPLC for Shatavarin analysis
Details: Conc. of sample 1.541 gm/100 ml
Loss on drying = 3.68%
Shatavarin content on L.O.D. basis: 0.32%
Shatavarin content on fresh weight basis: 3.19%

**The treated plants produced 31.97% more Shatavarin as compared to untreated control under saline conditions.**

## 14.6 Discussion and Analysis

On interaction of *P. indica* with different strains of Plant Growth Promoting Rhizobacteria (PGPRs), it was observed that the growth was completely blocked by *Pseudomonas fluorescence*; however, *Azotobacter chroococcum* promoted the growth of the fungus (Pham et al. 2004). In the present study, *A. chroococcum* primed *P. indica* has considerable improvement in plant biomass and productivity as compared to untreated control both under simulated saline conditions and in field. *P. indica* has been reported to induce resistance in the monocotyledonous plant barley to fungal diseases, along with tolerance to salt stress without affecting the plant productivity (Waller et al. 2005; Tuteja 2007).

Soil salinity is one of the major constraints that affect the growth, yield, and survival of plants. The lower water potential in the roots under salinity stress causes stomatal closure and suppression of mesophyll conductance. This negatively affects the photosynthetic rate and limits $CO_2$ assimilation (Ashraf and Harris 2013; Fletcher et al. 2007). The immediate effect of salt stress is a lower biomass, stunted growth, and reduced chlorophyll content. Low and moderate salinity stress (100 and 200 mM NaCl) caused a low, but not significant reduction in growth parameters and biomass. High salinity stress (300 mM) in contrast caused considerable disturbance

of the physiology of both inoculated and non-inoculated plantlets. The enhancement in growth parameters of inoculated plantlets may partially be attributed to obligatory endomycotic bacteria, associated with *P. indica* (Sharma et al. 2008).

The significant increase in length of roots of consortium inoculated plantlets can be attributed to production of auxin by *P. indica* (Sirrenberg et al. 2007; Dong et al. 2013). Waller et al. (2005) found that *P. indica* inoculated barley plantlets could tolerate moderate salinity stress (100 mM) in hydroponic culture. In the present study, it was found that *P. indica* was able to protect plantlets to some degree even at high salinity (300 mM). An earlier study showed that *A. vera* plantlets inoculated with *P. indica* had appreciably higher gel and aloin (anthraquinone derivative) content (Sharma et al. 2014a, 2015). *Aloe vera* plantlets inoculated with *P. indica* had significantly higher secondary metabolite content and antioxidant activity (Sharma et al. 2016).

There are only a few reports on the potential application of *P. indica* for enhancing secondary metabolite content of plants (Satheesan et al. 2012; Bajaj et al. 2014; Sharma et al. 2014a, 2015; Kilam et al. 2015; Gill et al. 2016) under salinity stress. Thus, efforts have been made with the consortium of *P. indica* and *Azotobacter* with various plants having great medicinal importance, under saline stress.

In the present study, the increase in secondary metabolites could be due to elicitation of plant defense in response to fungal elicitors like lipopolysaccharides and glycoproteins formed by the action of plant-derived hydrolases secreted in response to endophyte colonization (Gao et al. 2010). The inoculated plantlets also had significantly higher radical scavenging activity in terms of greater inhibition of free radicals, which could be due to greater secondary metabolite content, i.e., phenolics, flavonoids, and flavonols. This imparts greater ability to reduce oxidative damage associated with many phyto-pathogenic diseases (Teshome et al. 2015; Nath et al. 2016)). The results presented in this study confirm that NaCl stress disrupts nutrient and water acquisition, resulting in reduced growth and biomass of plantlets. However, plant tolerance to salinity stress is improved by its association with symbiotic fungus *P. indica*. The interaction of Plantlets with the consortium resulted in improved morphological features, enhanced biomass, greater host defense, more survival, and increased photosynthetic activity. This interaction has been found successful in terms of protecting the plant not only at moderate but even at higher concentrations of NaCl. The mechanisms of stress alleviation by the consortium could be due to improved nutrient uptake leading to growth promotion and enhanced radical scavenging capacity and secondary metabolites that help the plant resist the damaging effects of salt stress. The potential of consortium of *P. indica* and *Azotobacter* in reducing the problems caused by salinity stress and protecting the crops in arid and semi-arid agricultural regions is worthy of more detailed research.

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# Chapter 15
# Cocultivation of *Piriformospora indica* and *Azotobacter chroococcum* for Production of Artemisinin

**Prasun Bandyopadhyay, Monika Arora, M.Z. Abdin, and Ajit Varma**

**Abstract** Artemisinin is one of the major active ingredients used in artemisinin combination therapies (ACTs) used in malarial treatment. It is produced from *Artemisia annua* L. Malaria being one of the most severe tropical diseases, dependency on the production of artemisinin has been increasing. Lower yield (0.01–1.1%) further complicates the production process. This has led to the development of alternate strategy to improve plant productivity and enhance the active ingredient. Biostimulants like *Piriformospora indica* and *Azotobacter chroococcum* have been well known for their beneficial interaction with plants. Here, we studied the impact of dual inoculation of these stimulants in the growth and productivity of artemisinin in the poly house condition. The plant growth was monitored by measuring parameters like height of plant, total dry weight, and leaf yield with an increase of 63.51, 52.61, and 79.70%, respectively, for treatment with dual biological consortium, as compared to that of control plants. This significant improvement in biomass was associated with higher total chlorophyll content (59.29%) and enhanced nutrition (especially nitrogen and phosphorus, 55.75 and 86.21%, respectively). The concentration of artemisinin along with expression patterns of artemisinin biosynthesis genes was appreciably higher in dual treatment, which showed positive correlation. The study suggested the potential use of the consortium *P. indica* strain DSM 11827 and *A. chroococcum* strain W-5 in *A. annua* L.

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## 15.1 Introduction

*Artemisia annua* L. (sweet wormwood) is an important medicinal plant due to presence of artemisinin. It belongs to genus *Artemisia*, family Asteraceae (Compositae) with an annual growth cycle (Willcox et al. 2004). The phytomolecule artemisinin, sesquiterpene lactone containing endoperoxide bridge, is obtained from aerial parts of *A. annua* L. plants (Mandal et al. 2015). Artemisinin is an effective anti-malarial drug discovered by Miller and Su (2011). It has been also reported that artemisinin is not only effective against malaria but also for human cancer (Singh and Lai 2004) and hepatitis B virus (Romero et al. 2005). So far artemisinin-based combination therapies (ACTs) have been the choice for the treatment of people worldwide (Abdin et al. 2003). *A. annua* L. produces small amount of artemisinin (0.01–1.1%). Such low yields of artemisinin results in relatively high cost for isolation and purification of the useful chemical. Also, the demand of artemisinin production from dried plant material of *A. annua* L. has been estimated to about 289 tons as against the annual production of about 232–262 tons (Arora et al. 2016).

Rhizosphere microbiota like arbuscular mycorrhizal fungi (AMF) are well-known plant beneficial soilborne microsymbionts. They significantly contribute toward improved agricultural performance by triggering diverse plant physiological responses. Hence, these have been employed for many agricultural production systems as well as for medicinal and endangered plant species (Pozo et al. 2010). The symbiotic association of arbuscular mycorrhizal fungi (AMF) with the plant is in synergistic coordination with the plant growth-promoting rhizobacteria (PGPR) (Bandyopadhyay et al. 2016a; Bandyopadhyay et al. 2016b; Bakker et al. 2013; Berendsen et al. 2012; Bhuyan et al. 2015). The overall plant performance relies on both bacteria and the fungi whereby the nitrogen-fixing ability of bacteria is stimulated by improved phosphate uptake due to AMF association and vice versa (Javot et al. 2007). PGPRs show phosphate-solubilizing mechanisms, enhancement in phytohormone production, increased antifungal activity, etc. (Awasthi et al. 2011; Prasad et al. 2015). The synergistic interaction between plant and microbes in rhizosphere critically improves growth and productivity of plants through an array of processes like increased nutrient uptake, availability, nitrogen fixation, nutrient recycling, photosynthetic rate, and pathogen resistance (Jeffries et al. 2003).

*P. indica* as well as arbuscular mycorrhiza fungi individually have also been shown to enhance artemisinin content in *A. annua* L. plants (Kapoor et al. 2007; Rapparini et al. 2008; Chaudhary et al. 2008; Sharma and Agrawal 2013). Kapoor et al. (2007) reported an increase in artemisinin concentration in leaves of *A. annua* from 0.1% (control) to 0.3% (*Glomus fasciculatum* treated) while investigating the effect of two AMF *Glomus fasciculatum* and *Glomus macrocarpum* singly and along with addition of phosphorous. The increased artemisinin concentration was attributed to high leaf yield and shoot growth which was further validated by high glandular trichome (artemisinin biosynthesis and assembly sites) density in the

mycorrhizal-treated plants. *Azotobacter* is a Gram-negative aerobic soil-dwelling nitrogen-fixing bacteria (Lakshminarayana et al. 1992). It is found in soil and water systems and in association with plants (Martyniuk and Martyniuk 2003). Only, recently studies analyzing synergistic effect of PGPRs and AMF on medicinal and crop plants have been conducted (Awasthi et al. 2011; Walker et al. 2012; Vafadar et al. 2014).

## 15.2 Effect of *P. indica* and *A. chroococcum* on Plant Growth Parameters

Inoculation of *A. annua* L. plants with *Piriformospora indica* and *A. chroococcum* either singly or in combination under poly house conditions improved the growth of plants in terms of plant height, biomass, and total leaf yield per plant as compared with control plants (Table 15.1). *A. annua* L. plants treated with either *P. indica* or *A. chroococcum* enhanced the growth compared with control. When combined, inoculation of plants with both *P. indica* and *A. chroococcum* was highly effective in improving the plant height, biomass, and leaf yield with an observed increase of 63.51, 52.61 and 79.70% respectively, compared with control (Table 15.1).

Rhizospheric soil from *A. annua* L. plants treated with *A. chroococcum* alone or in combination with *P. indica* was used for determination of the viable count of *A. chroococcum* by using standard serial dilution pour plate method. *A. annua* L. plants treated only with *A. chroococcum* showed $18.33 \times 10^5$ CFU/g soil, whereas dual treated plants exhibited high population of *A. chroococcum* ($21.12 \times 10^5$ CFU/g soil) in the rhizospheric soil. *P. indica* colonization was evaluated by randomly selected fine roots from 2-month-old *A. annua* L. as method followed by Phillips and Hayman (1970), and percentage colonization of *P. indica* was calculated using the formula as described by McGonigle et al. (1990). *A. annua* L. plants cocultivated with *P. indica* resulted in 50.23% colonization, while dual treated plants have better root colonization of 78.99% by *P. indica* (Arora et al. 2016).

**Table 15.1** Effect of *P. indica* and *A. chroococcum* alone or in combination on plant growth

| Parameters | Control | *P. indica* | *A. chroococcum* | *P. indica* + *A. chroococcum* |
|---|---|---|---|---|
| Plant height | $60.4 \pm 3.36^a$ | $79.37 \pm 2.76^b$ | $74.74 \pm 4.42^b$ | $98.76 \pm 2.68^c$ |
| Plant biomass | $57.71 \pm 3.23^a$ | $76.14 \pm 2.47^b$ | $64.84 \pm 3.56^b$ | $88.07 \pm 4.53^c$ |
| Leaf yield | $7.93 \pm 1.26^a$ | $12.13 \pm 1.03^b$ | $10.04 \pm 1.05^b$ | $14.25 \pm 1.14^c$ |

Plants were grown with *P. indica*, *A. chroococcum*, both *P. indica* + A. *chroococcum*, and control plant without *P. indica* or *A. chroococcum*. Values are presented as means ($n = 8$) ± SD. Different letters (a,b,c) indicate significant differences between each treatment ($P \leq 0.05$) by Tukey's post hoc test

## 15.3 Effect of *P. indica* and *A. chroococcum* on Nitrogen and Phosphorus

Phosphorus and nitrogen are the important macromolecules that are responsible for increased growth, yield, and quality of plant. Concentrations of phosphorus and nitrogen were significantly higher in those plants cocultivated with dual treatment (Fig. 15.1). On individual basis, plants treated with *P. indica* significantly increased P content by 65.95% and with *A. chroococcum* resulted in 31.90% higher P content in *A. annua* L. plants compared to the control plants, respectively. Likewise, plants treated with *P. indica* significantly increased N content by 13.27% and with *A. chroococcum* resulted in 29.20% higher N content in *A. annua* L. plants compared to the control plants, respectively. The colonization of *A. annua* L. with dual treatment resulted in 86% increase in P content and 55.75% increase in N content (Fig. 15.1). *P. indica* is known to enhance phosphorous uptake in plants, which in turn might enable more energy available for nitrogen fixation by *A. chroococcum*; this could be the reason for higher P and N content in dual treated plants (Arora et al. 2016).

## 15.4 Effect of *P. indica* and *A. chroococcum* on Chlorophyll Content

Chl a, chl b, and total chlorophyll content was quantified in leaves of *A. annua* L. and found significantly increased in plants treated with *P. indica*, *A. chroococcum* alone, or in combination as compared to the control plants. Chl a showed values of 4.5 and 4.7 mg/g, respectively, for plant treated with *A. chroococcum* and *P. indica*, separately, and 5.6 mg/g fresh weight for plant dual treated with *P. indica* and *A. chroococcum* together. Similarly, the content of chl b exhibited values of 0.7

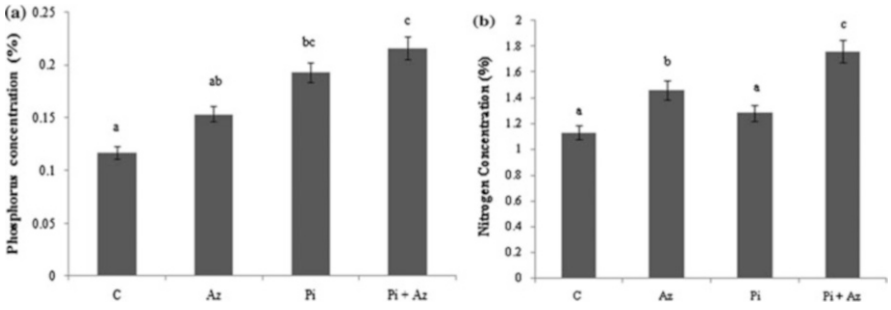


**Fig. 15.1** Phosphorus (**a**) and nitrogen (**b**) concentration (%) in leaves of *A. annua* L. plants, grown for 2 months after transplanting, under poly house conditions. Columns with different letters are indicating significant differences between each treatment at 5% probability level according to Tukey's post hoc test, and the error bars represent the standard error

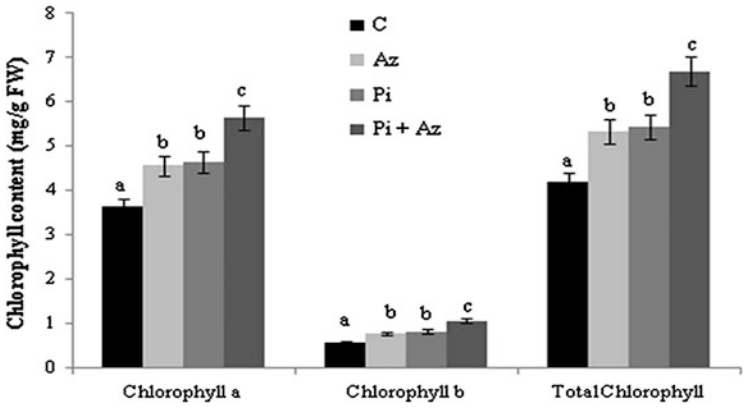


**Fig. 15.2** Chlorophyll content (mg/g fresh weight) in leaves of *A. annua* L. plants, grown for 2 months after transplanting, under poly house conditions. Columns with different letters are indicating significant differences between each treatment at 5% probability level according to Tukey's post hoc test, and the error *bars* represent the standard error

and 0.8 mg/g, respectively, for plant treated with *A. chroococcum* and *P. indica*, separately, and 1.0 mg/g fresh weight for plant dual treated with *P. indica* and *A. chroococcum* together. The plants dual treated with *P. indica* and *A. chroococcum* together also enhanced total chlorophyll content by 57.91% than control plants (Fig. 15.2). However, the chlorophyll content of *A. annua* L. plants treated with *P. indica* and *A. chroococcum*, separately, was not significantly different. More chlorophyll content in the plants is attributed to the fact that an increase in plant nutrition by an increase in P and N uptake will optimize the rate of photosynthesis by increasing the amount of plant chlorophyll, which will lead to an increase in biomass production by C fixation from $CO_2$. Nitrogen is part of the chlorophyll molecule, which gives green color to plants and is involved in creating food for the plant through photosynthesis.

## 15.5 Effect of *P. indica* and *A. croococcum* on Artemisinin Content

One gram of dry leaf material was used for the estimation of artemisinin using the method as described by Zhao and Zeng (1986). Derivatized artemisinin was analyzed and quantified through reverse phase column (C18, 5 μm, 4.6 × 250 mm) using premix methanol: 100 mM K-phosphate buffer, pH, 6.5 (60:40), as mobile phase at constant flow rate of 1 ml $min^{-1}$ with the detector set at 260 nm. Artemisinin was quantified with the help of standard curve prepared by HPLC (Fig. 15.3). An overlay of the results obtained with preparative HPLC of a

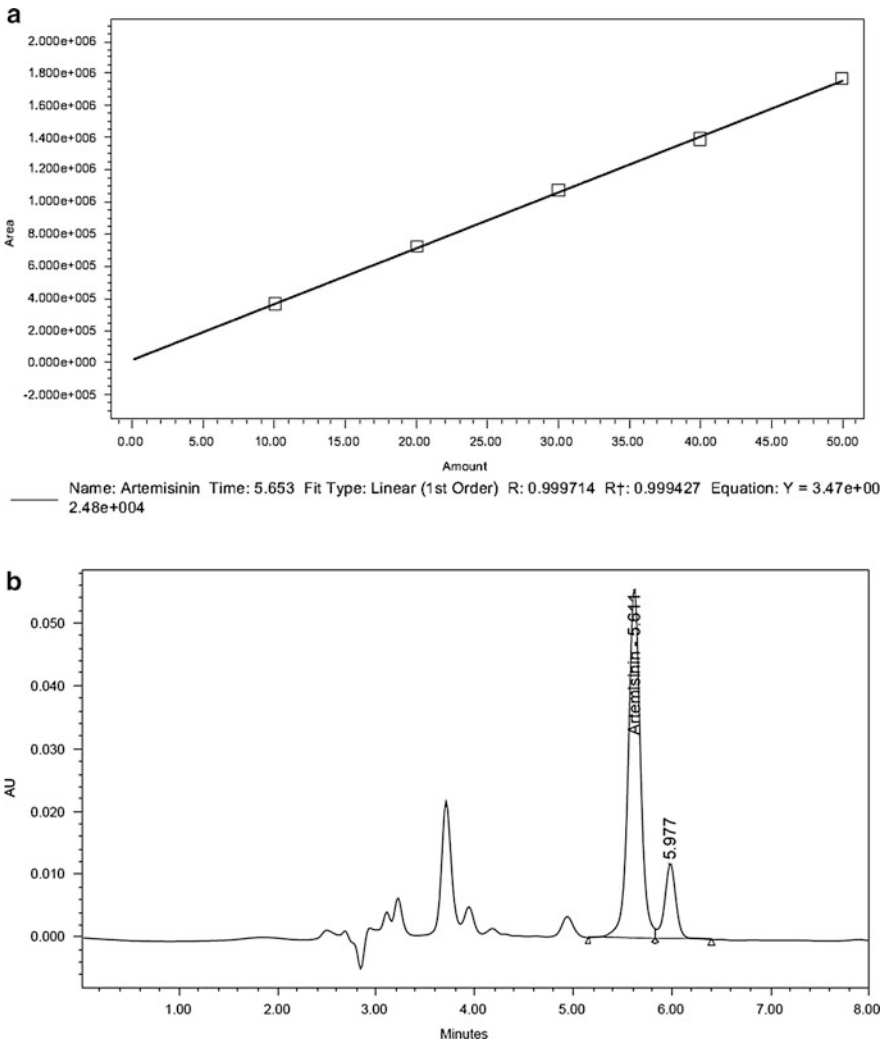


**Fig. 15.3** (**a**) Calibration curve of artemisinin standard. (**b**) Chromatogram of a standard solution of artemisinin after process prior to analysis (RT = 5.611)

standard solution of artemisinin prior to analysis of samples is shown in Fig. 15.3. Artemisinin content was expressed as % as well as mg $g^{-1}$ dw of leaves.

The symbiotic effectiveness was much evident when artemisinin content was recorded 70% higher in *A. annua* L. plants subjected to dual inoculation (Fig. 15.4). *P. indica* colonization or *A. croococcum* inoculation independently enhanced artemisinin content to approximately similar levels. The enhanced concentration of artemisinin by dual treatment may be due to improved growth and nutrient status of the plants (Arora et al. 2016; Davies et al. 2009).

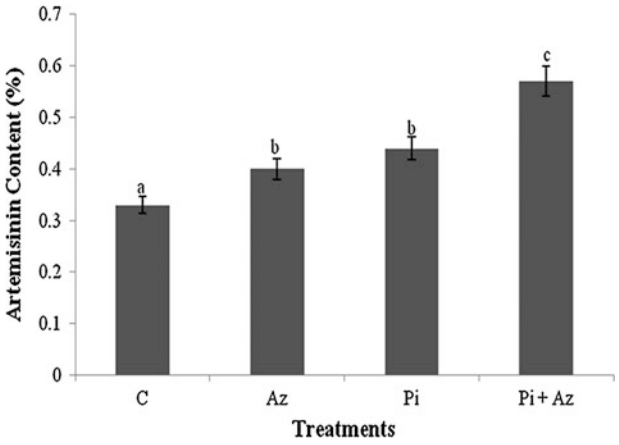


**Fig. 15.4** Artemisinin content (%) in leaves of *A. annua* L. plants, grown for 2 months after transplanting, under poly house conditions. Columns with different letters are indicating significant differences between each treatment at 5% probability level according to Tukey's post hoc test, and the error *bars* represent the standard error

## 15.6 Conclusion

Interaction of *A. annua* L. with both *P. indica* and *A. chroococcum* in cocultivation resulted in improved plant biomass and concentration of artemisinin in the plant as compared to control and singly treated plants. The combinatorial application of *P. indica* with *A. chroococcum* induces reprogramming of many cellular activities like phytohormone biosynthesis, nutrient acquisition, and secondary metabolite synthesis in *A. annua* L. leading to higher biomass and enhanced artemisinin content and yield. The use of this microbial consortium as bio-fertilizer in place of chemical fertilizers, hence, presents a viable option for increased artemisinin availability.

## 15.7 Future Prospects

The current study provides a perspective into study of combined inoculation of symbiotic fungus and nitrogen-fixing bacteria and their interaction with plants. Different beneficial and symbiotic bacterial fungal associations can also be studied with plants to check their effect on plant yield, disease resistance, abiotic and biotic stress response, production of important molecules, and plant products. It will also help to understand the molecular mechanism between the microorganisms and determine the active compounds released that help in plant trait enhancement. Proteomic studies can also be carried out to check the effect of consortium on plants. Hence, this consortium can also be used to check their effect on other plant

species. Further study is also required to check the effectiveness of microbial consortia in making the plant resistant to pathogens through systemic induced resistance.

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# Chapter 16
# Microbial Symbiosis and Bioactive Ingredients of Medicinal Plants

**Divya Kilam, Priyanka Sharma, Abha Agnihotri, Amit Kharkwal, and Ajit Varma**

**Abstract** Medicinal plants have been used widely for their bioactive ingredients as they are highly potent and have least side effects. This has led to a surge in demand for medicinal plants for producing higher quantity and good quality bioactive compounds. Symbiotic association of microorganisms with plants has been shown to affect the production and quality of active ingredients. However, the effect is not consistent and is seen to vary under different microbial associations. This chapter elucidates the studies on microbial symbiosis with medicinal plants and the effect of this interaction on medicinally important bioactive ingredients. The role of both nutritional and non-nutritional pathways in this interaction has also been discussed.

## 16.1 Introduction

Plants have been used as an important source of medicine in pharmaceutical biology since thousands of years. As per WHO estimates, even today, up to 80% of population rely on traditional medicines for primary healthcare needs (Sieniawska et al. 2013). Medicinal plants are a rich source of bioactive compounds, which are widely used as potent drugs for their therapeutic properties (Gu et al. 2014). They are also used in pharmaceuticals, food additives, fragrances and industrially important compounds. The use of medicinal plants for the cure of ailments has a long history in cultures across the globe. The Chinese book on roots and grasses, written around 2500 BC, mentioned 365 drugs from dried parts of medicinal plants (Bottcher 1965). The Indian Vedas also mention about treatment with plants and their products (Tucakov 1971). The Ebers Papyrus, written around circa 1550 BC, refers to 700 plant species and drugs used for therapy (Glesinger 1954). About 7000 species of medicinal plants have been reported in China alone ("Center for Traditional Medicinal Plants," 2016). In India, around 25,000 effective plant-based

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formulations are used in traditional medicines to cure different diseases (Pandey et al. 2013). Natural medicines have gained popularity among consumers as they are effective and safe to use and have lesser side effects. This increasing demand for plant-based medicines has resulted in large-scale production of medicinal plants using modern techniques. Innovative strategies are also required to maintain the quality of medicinal plant products as they get affected due to pests, diseases and excessive use of pesticides.

Plant growth and development is synergistic combination of a number of environmental factors. Plants being the motionless entities are confronted with a number of unfavourable conditions, e.g. salinity, drought, pathogen attacks, etc. However, with the course of evolution, they have developed a number of mechanisms to protect themselves from the attack of such stresses (Kogel et al. 2006). They produce bioactive compounds as a survival mechanism against biotic and abiotic stresses (Großkinsky et al. 2016). These stress reactions are triggered by elicitors that can be abiotic, such as metal ion or inorganic compounds or biotic, derived from a biological source or their products. Studies have shown that treatment of plants with biotic elicitor can be employed for the production of plant secondary metabolites (Gorelick and Bernstein 2014). This can lead to activation of defence reactions which would cause production of bioactive molecules like phytoalexins in the plants. Elicitation is thus being used to induce production of secondary metabolites in plants. The three major groups of plant secondary metabolites, terpenoids, phenolics and alkaloids, are used in preparation of medicinal products. Essential oils from medicinal plants, mostly consisting of monoterpenes, sesquiterpenes and phenylpropanoids, are used as flavours, fragrances, antioxidants and antimicrobial agents (Guenther 2013). The study of interaction of microbial diversity with medicinal plants is thus important as it will help determine their impact on bioactive compounds and also help improve the quality of the produce. The best strategy adapted by plants is to form a mutualistic association with beneficial microorganisms to protect themselves from stresses (Lum and Hirsch 2003). However, the most difficult task is to distinguish between mutualistic partners and parasites (Kogel et al. 2006; Schulz and Boyle 2005), because both of these interactions share a number of common signalling pathways (Paszkowski 2006). This chapter gives an account of the studies on accumulation of plant bioactive compounds in a variety of medicinal plants upon association with beneficial microorganisms and the proven or putative mechanisms by which these microbes promote the production of bioactive compounds in plants.

## 16.2 Bioactive Constituents of Medicinal Plants

The damaging effects of all environmental stresses on plants can be observed as either death of the plant or decrease in productivity. The response generated by plant correlates well with that of oxidative stress. Oxidative stress results in the generation of free oxygen radicals or reactive oxygen species (ROS), which are

cytotoxic at high concentrations (McKersie 1996). These radicals catalyse self-propagating autoxidation reactions that lead to formation of other organic peroxides, which cause major damage to biological system. Various cellular locations and the environments where the free oxygen species are formed demand for a scavenging system for uninterrupted growth and survival of plants (Nath et al. 2016). To fight against the deleterious effects of reactive oxygen species, plants are endowed with several antioxidants and metabolites in different plant cell compartments (Ashraf and Harris 2004). These active compounds are mainly secondary metabolites or their derivatives like alkaloids, glycosides, terpenes, flavonoids, tannins, phenolics, anthraquinones, saponins and essential oils (Croteau et al. 2000; Bagde et al. 2010). More than 12,000 alkaloids are known to be present in 20% of plant species, and over 4000 flavonoids are reported in nearly 70% of plant species, which suggests the vastness of these compounds (Heim et al. 2002; Ziegler and Facchini 2008). Essential oils or volatile oils are also present in medicinal plants and are known to comprise of more than 200 different chemical components (Martinez et al. 2008). Medicinal plants, in particular, have been exploited by humans for this pool of phytochemicals. Thus the primary focus of research on medicinal plants has been on the bioactive compounds with therapeutic properties.

## 16.3 Microorganisms for Plant Secondary Metabolite Enhancement

Soil microflora consists of a range of microorganisms, such as algae, bacteria and fungi. These microorganisms contribute actively in almost all the chemical processes that occur within the soil. They participate in carbon and nitrogen cycling, nutrient acquisition, tolerance to various stresses and other processes that are important for the survival and growth of the plant. In contrast, plants can have enormous effects on soil microbial communities especially those colonizing the rhizospheric region. This is because of increasing the availability of carbon in the soil due to the release of root exudates and decaying plant material, which will act as growth substrate, structural material or signal for them (Barea et al. 2005).

### *16.3.1 Arbuscular Mycorrhizal Fungi*

Arbuscular mycorrhizal fungi (AMF) are known to form symbiotic association with more than 80% of vascular plants on the earth. They are found under all climates and in all ecosystems, regardless of the type of soil, vegetation or growing conditions. AM fungi colonize the roots and rhizosphere, and the hyphae being thinner branch more frequently than plant roots and spread out over several centimetres in the form of ramified filaments. This extended network increases the absorptive

capacity of roots and allows the plant to have better access to a greater quantity of water and minerals required for nutrition. Their association thus increases water uptake and availability of nutrients to the plant, especially insoluble soil phosphate (Clark and Zeto 2000), and the fungus in turn is benefitted by supply of carbohydrates derived from plant photosynthesis (Harrison 1999; Gianinazzi et al. 2010). The fungi provide nourishment, increase the reproductive potential, improve root performance and provide a natural defence against invaders, like pests and pathogens (Singh et al. 2000). The use of AMF was proposed for a number of medicinal plant species (Toussaint et al. 2007; Jurkiewicz et al. 2010; Copetta et al. 2006), endangered plants (Sharma et al. 2007; Bothe et al. 2010) and for the restoration of devastated habitats (Turnau and Haselwandter 2002).

The potential of AMF to promote accumulation of bioactive compounds in medicinal plants has been investigated by several researchers. The first studies of AMF affecting medicinal plants were conducted by Honggang (1989). They studied the effect of *Glomus mosseae* and *Glomus epigaeum* on the growth, nutrient uptake and synthesis of effective compounds, hyoscine in medicinal herb *Datura stramonium* L. The hyoscine content was found to increase significantly by 103.2–117.2% (Honggang 1989). Another study by the same group showed enhanced growth, nutrient uptake and volatile oil synthesis in *Schizonepeta tenuifolia* upon inoculation with AMF, wherein the volatile oil content increased by 163.6–209.1% as compared to control (Wei and Wang 1991). Since then symbiosis between many different medicinal plants and AMF has been studied for accumulation of important bioactive compounds (Table 16.1). AM symbiosis has also shown to change the composition of bioactive compounds in medicinal plants. AM symbiosis in *Salvia officinalis* showed changes in the composition of essential oils with enhanced quantities of bornyl acetate, 1,8-cineole, α-thujones and β-thujones (Geneva et al. 2010). The composition of essential oils in *Origanum onites* and *Mentha viridis* was also seen to differ upon mycorrhization (Karagiannidis et al. 2011). Contrasting results have also been seen in some of the studies, with no change in the chemical composition of these bioactive compounds upon inoculation with AMF. *Origanum vulgare* plants inoculated with AMF showed a similar chemical profile of essential oils as seen in control plants (Morone Fortunato and Avato 2008). Studies by Lermen et al. (2015) showed similar results with no significant change in composition of essential oils in *Cymbopogon citratus* upon mycorrhization.

### *16.3.2 Root Endophytic Fungus*

Another type of symbiotic relationship that the plants form is with the endophytes. The non-mycorrhizal microbes such as dark septate endophyte, *Phialocephala fortinii*, *Cryptosporiopsis* spp., *Piriformospora indica*, *Fusarium* spp. and *Cladorrhinum foecundissimum* have been shown to improve the growth of their hosts after colonization (Schulz 2006; Chadha et al. 2014). Unlike mycorrhizal fungi, endophytes are known to reside and grow within plant tissues, leaves, bark, stems and roots (Carroll 1988). Root endophytes inhabit the roots without forming the

**Table 16.1** Effect of inoculation of *Arbuscular mycorrhizal* fungi on secondary metabolite content of medicinal plants

| Medicinal plant | Arbuscular mycorrhizal fungi | Secondary metabolite content | Reference |
|---|---|---|---|
| *Anadenanthera colubrine* | *Glomus* spp. | Enhanced phenol, flavonoids and total tannins | Pedone-Bonfim et al. (2013) |
| *Anethum graveolens* | *Glomus macrocarpum*, *Glomus fasciculatum* | Essential oil increased up to 90% | Kapoor et al. (2002) |
| *Angelica archangelica* L. | *Glomus intraradices*, *Glomus mosseae* | Enhanced monoterpenoids and coumarins | Zitterl-Eglseer et al. (2015) |
| *A. dahurica* | *Glomus* spp. | Increased total coumarin content | Zhao et al. (2009), Zhao and He (2011) |
| *Arnica montana* | *G. intraradices* | Increased secondary metabolite content | Jurkiewicz et al. (2010) |
| *A. montana* | *G. intraradices* | Increased phenolic acids in roots | Jurkiewicz et al. (2010) |
| *Artemisia annua* L. | *G. macrocarpum*, *G. fasciculatum* | Enhanced artemisinin and essential oil content | Kapoor et al. (2007), Chaudhary et al. (2008) |
| *A. annua* | *Rhizophagus intraradices* | Enhanced artemisinin content | Mandal et al. (2015) |
| *Bupleurum scorzonerifolium* | *G. mosseae* | Enhanced flavonoid content | Teng and He (2005) |
| *Camptotheca acuminate* | *G. intraradices* | Increased camptothecin content | Zhao et al. (2006) |
| *Castanospermum australe* | *Glomus* spp. | Increased castanospermine content | Abu-Zeyad et al. (1999) |
| *Catharanthus roseus* | *Glomus* spp. | Increased phenolic compound and vinblastine content | De la Rosa-Mera et al. (2011) |
| *Coleus forskohlii* | *Glomus bagyarajii* | Increased forskolin content | Sailo and Bagyaraj (2005) |
| *Cymbopogon citratus* S. | *Rhizophagus clarus* | Increased essential oil content | Lermen et al. (2015) |
| *Echinacea purpurea* | *G. intraradices* | Enhanced phenolics in roots | Araim et al. (2009) |
| *Foeniculum vulgare* | *G. macrocarpum* and *G. fasciculatum* | Essential oil increased by 78% | Kapoor et al. (2004) |
| *Glycyrrhiza uralensis* | *G. mosseae*, *G. versiforme* | Enhanced glycyrrhizin concentration | Liu et al. (2007) |
| *Hypericum perforatum* | *G. intraradices*, *G. mosseae*, *G. constrictum*, *G. geosporum* | Higher hypericin and pseudohypericin content | Zubek et al. (2012) |

(continued)

**Table 16.1** (continued)

| Medicinal plant | Arbuscular mycorrhizal fungi | Secondary metabolite content | Reference |
|---|---|---|---|
| *Inula ensifolia* | *Glomus clarum* | Increased thymol derivative content | Zubek et al. (2010) |
| *Mentha arvensis* | *G. fasciculatum* | Significantly increased oil content and oil yield | Gupta et al. (2002) |
| *M. crispa* | *Glomus etunicatum*, *R. clarus* | Enhanced essential oil content | Urcoviche et al. (2015) |
| *M. viridis* | *G. etunicatum*, *Glomus lamellosum* | High levels (>5%) of limonene, 1,8-cineole, linalool, carvone, eugenol, (E)-methyl cinnamate | Karagiannidis et al. (2011) |
| *Ocimum basilicum* | *G. intraradices* | Enhanced anthocyanin concentration | Lee and Scagel (2009) |
| *Origanum onites* | *G. etunicatum*, *G. lamellosum* | High levels (>5%) of sabinene, terpinene, trans-sabinene hydrate, terpinen-4-ol, carvacrol | Karagiannidis et al. (2011) |
| *Origanum* sp. | *G. mosseae* | Increased essential oil concentration | Copetta et al. (2006), Khaosaad et al. (2006) |
| *Passiflora alata* C. | *Gigaspora albida* | Increased flavonoids | Oliveira et al. (2015) |
| *Phellodendron amurense* | *G. mosseae*, *G. etunicatum*, *G. versiforme*, *G. diaphanum* | Increased berberine, jatrorrhizine, palmatine content | Fan et al. (2006) |
| *Phellodendron chinense* | *Acaulospora laevis*, *Acaulospora mellea*, *Glomus ditum*, *G. intraradices*, *G. mosseae*, *G. versiforme* | Increased berberine content | Zhong and Fan (2007) |
| *Pinellia ternata* | *G. mosseae*, *G. intraradices* | Increased guanosine and alkaloid content | Guo et al. (2010) |
| *Pogostemon cablin* | *Acaulospora laevis*, *Gigaspora margarita*, *G. bagyarajii*, *G. etunicatum*, *G. fasciculatum*, *G. intraradices*, *G. leptotichum*, *G. macrocarpum*, *G. monosporum*, *G. mosseae*, *S. calospora* | Enhanced essential oil content | Arpana et al. (2008) |
| *Prosopis laevigata* | *G. rosea* | Increased trigonelline content | Rojas-Andrade et al. (2003) |
| *Salvia miltiorrhiza* | *G. mosseae* | Enhanced essential oil content | Meng and He (2011) |

(continued)

**Table 16.1** (continued)

| Medicinal plant | Arbuscular mycorrhizal fungi | Secondary metabolite content | Reference |
|---|---|---|---|
| *S. officinalis* | *G. intraradices* | Enhanced essential oils, 1,8-cineole, α- and β-thujones | Geneva et al. (2010) |
| *S. officinalis* | *G. intraradices*, *G. mosseae* | Enhanced phenolic and rosmarinic acid content | Nell et al. (2009) |
| *Stevia rebaudiana* | *R. fasciculatus* | Increase in steviol glycoside content | Mandal et al. (2013) |
| *Trachyspermum ammi* | *G. macrocarpum*, *G. fasciculatum* | Essential oil increased by 72% | Kapoor et al. (2002) |
| *Viola tricolor* L. | *Rhizophagus irregularis*, *Funneliformis mosseae* | Increased phenolic acid and flavonoid concentration | Zubek et al. (2015) |

typical anatomical features of mycorrhiza and without showing the signs of pathogenesis. The fungal root endophytes involve a diverse group of fungi. The most-studied taxa have dark pigmented hyphal walls and are referred as the dark septate endophytes (DSE). They are morphologically defined fungi belonging to the group of ascomycetes. They are frequently observed in cortex, epidermis as well as the root surface (Knapp et al. 2015). Colonization by DSE has shown to increase growth of the plants (Mayerhofer et al. 2013). *Trichoderma* is another true endophyte which is mainly found in the root ecosystems. In the last few decades, there has been a spurt of research focused on plant-endophyte associations and their application as a potential bio augmenting agent. Besides imparting beneficial effects on plant growth and health, the association also provides tolerance against various biotic and abiotic stresses (Hermosa et al. 2012). Another well-studied group of the root endophytes are the Sebacinales. *Piriformospora indica* is an extensively studied member of this group. *P. indica* is able to associate itself with roots of various plant species in a manner similar to AM fungi (Varma et al. 1999, 2001; Sharma et al. 2014; Das et al. 2012, 2013; Singh et al. 2003). *P. indica* promotes nutrient uptake and enhances the growth and biomass of the plant, including monocots and dicots (Yadav et al. 2010; Varma et al. 2000, 2012), induces early flowering (Das et al. 2012, 2013), increases the resistance against fungal pathogens and allows the plant to survive under stressed environment (Das et al. 2012; Harman 2011; Gill et al. 2016). It has been established as a bio fertilizer, bio protectant and biological hardening agent (Johnson et al. 2014; Varma et al. 2012). Sharma et al. (2014) reported higher antioxidant activity and greater secondary metabolite content in *P. indica*-colonized *A. vera* plantlets thus enhanced ability to reduce oxidative stress and fight against various phytopathogenic diseases. The phylogenetic relationship of another new species, *Piriformospora williamsii* with Sebacinales, has been studied by Basiewicz et al. (2012). The study also showed biphasic lifestyle of mutualistic symbiont *P. indica*. Researchers have endeavoured to know the molecular mechanisms during plant-endophyte association and their effect on plants. However, only few documents refer

**Table 16.2** Effect of inoculation of root endophytic fungi on secondary metabolite content of medicinal plants

| Root endophytic fungus | Medicinal plant | Secondary metabolite content | Reference |
|---|---|---|---|
| Dark septate endophytic (DSE) fungi (seven isolated strains) | *Epimedium wushanense* | One of the strains (DSE8) showed improved flavonoid and icariin content | Zhu et al. (2015) |
| *Piriformospora indica* | *Aloe vera* L. | Increased aloin content and anti-oxidant activity | Sharma et al. (2014) |
| | *Aristolochia elegans* | Enhancement in aristolochic acid | Bagde et al. (2014) |
| | *Artemisia annua* | Enhancement in artemisinin content | Sharma and Agrawal (2013) |
| | *Bacopa monnieri* | Higher bacoside content | Prasad et al. (2013) |
| | *Centella asiatica* | Enhanced asiaticoside content | Satheesan et al. (2012) |
| | *Coleus forskohlii* | Enhancement in essential oils (p-cymene, nonanal) | Das et al. (2012) |
| | *Curcuma longa* | Increased curcumin content | Bajaj et al. (2014) |
| | *Linum album* | Increase in podophyllotoxin and 6-methoxpodophyllotoxin | Baldi et al. (2008) |
| | *Withania somnifera* | Increased withaferin A content | Ahlawat et al. (2016) |

secondary metabolite accumulation in plants upon interaction with fungal root endophytes (Table 16.2).

### *16.3.3 Plant Growth-Promoting Rhizobacteria*

Plant growth-promoting rhizobacteria (PGPRs) are a specific group of soil bacteria that colonize the rhizosphere and rhizoplane of plants. They are known to enhance plant growth through mechanisms like nitrogen fixation, phosphate solubilization and quorum sensing (Prasad et al. 2015; Goswami et al. 2016). Rhizobacteria have the ability to successfully colonize the plant roots and positively enhance plant growth. They can also improve the secondary metabolite content in plants by improving the phosphorus status or by altering the hormonal balance of the plants (Köberl et al. 2015). Till date, several studies have reported the ability of PGPRs to promote growth of cereals, vegetable and food crops. However, there are limited reports on interaction of PGPRs with medicinal plants for accumulation of secondary metabolites, as summarized in Table 16.3.

**Table 16.3** Effect of inoculation of plant growth-promoting rhizobacteria on secondary metabolite content of medicinal plants

| Medicinal plant | PGPR | Secondary metabolite content | Reference |
|---|---|---|---|
| *Anoectochilus roxburghii* | *B. subtilis* | Increase in flavonoids and essential oil content | Refish et al. (2016) |
| *Bacopa monnieri* | *Bacillus pumilus*, *Exiguobacterium oxidotolerans* | Increase in bacoside-A content | Bharti et al. (2013) |
| *Catharanthus roseus* | *Pseudomonas fluorescens* | Increased production of ajmalicine | Jaleel et al. (2007) |
| *C. roseus* | *Azotobacter chroococcum*, *P. fluorescens*, *Bacillus megaterium* | Enhanced alkaloid content | Karthikeyan et al. (2010) |
| *C. roseus* varieties 'rosea' and 'alba' | *Azospirillum brasilense*, *P. fluorescens* | Enhanced ajmalicine content | Karthikeyan et al. (2009) |
| *Glycine max* | *P. fluorescens* | Enhanced isoflavone content | Algar et al. (2012) |
| *Hyoscyamus niger* L. | *Pseudomonas putida*, *P. fluorescens* | Increase in tropane alkaloids; hyoscyamine, scopolamine | Ghorbanpour et al. (2011, 2013) |
| *Matricaria chamomilla* L. | *Azospirillum lipoferum*, *A. chroococcum* | Increased essential oil content | Dastborhan et al. (2011) |
| *Mentha piperita* | *P. fluorescens*, *B. subtilis*, *A. brasilense* | Increased essential oil (pulegone, menthone) content | Santoro et al. (2011) |
| *M. piperita* | *B. subtilis*, *P. fluorescens*, *P. putida* | Marked changes in monoterpene accumulation | Del Rosario et al. (2015) |
| *Ocimum basilicum* | *B. subtilis* | Increase in essential oil (α-terpineol and eugenol) content | Banchio et al. (2009) |
| *Origanum majorana* L. | *P. fluorescens*, *B. subtilis*, *Sinorhizobium meliloti*, *Bradyrhizobium* sp. | Increased essential oil content | Banchio et al. (2008) |
| *Salvia miltiorrhiza* B. | *Bacillus cereus* | Increased accumulation of tanshinone | Zhao et al. (2010) |
| *S. officinalis* L. | *P. putida*, *P. fluorescens* | Increased essential oils content | Ghorbanpour et al. (2014) |
| *Tagetes minuta* | *P. fluorescens*, *A. brasilense* | Enhanced essential oil content | Del Rosario et al. (2013) |
| *Trigonella foenum-graecum* | *Bacillus* sp. | Enhancement in diosgenin levels | Jasim et al. (2015) |
| *Withania somnifera* | *Azospirillum*, *A. chroococcum*, *P. fluorescens*, *Bacillus megaterium* | Enhanced alkaloid content | Rajasekar and Elango (2011) |

### 16.3.4 Microbial Cocultures

Soil microorganisms influence the plant health by increasing the extent of their root system and in turn the ability to acquire nutrients from the soil (Bucio et al. 2007). Engineering the plant rhizosphere through inoculation of specific microorganisms can provide cumulative benefits to the plant. Several beneficial microorganisms have been studied for their potential as a biocontrol agent and are being used as bio fertilizers (Bhardwaj et al. 2014). The study of antagonistic or synergistic effects of different microbial inoculants is however critical for development of an effective host-microbe interaction. Several studies have been undertaken to see the effect of symbiotic fungi and plant growth-promoting rhizobacteria on plant. Toro et al. (1997) reported that dual inoculation of AM fungus, *Glomus intraradices*, and PGPR, *Bacillus subtilis*, increased the plant biomass and tissue phosphorus accumulation. Synergistic effect of *G. intraradices* and *B. subtilis* was also seen in *Lactuca sativa*, where combined inoculations resulted in 77% enhanced plant growth as compared to control plants (Kohler et al. 2007). The potential benefits of dual inoculation of endophytic fungus, *P. indica*, with AM fungi and PGPR (s) have also been reported. Meena et al. (2010) reported that combined inoculations of *P. indica* and *Pseudomonas striata* lead to a significant increase in plant biomass and grain yield of *Cicer arietinum* L. (chickpea). Another study by Nautiyal et al. (2010) showed enhanced nodulation and plant growth promotion in chickpea upon inoculation with *P. indica* and *Paenibacillus lentimorbus*. Recent studies have shown that microbial coculture systems can act as effective tools for biotic elicitation of plant secondary metabolites (Table 16.4).

Green house studies on *Solanum viarum* seedlings showed enhancement in secondary metabolite content, when plants were inoculated with AMF, *Glomus aggregatum*, and PGPR(s), *Bacillus coagulans* and *Trichoderma harzianum* (Hemashenpagam and Selvaraj 2011). Synergistic effect of four different AM fungi, *G. aggregatum*, *Glomus fasciculatum*, *G. intraradices* and *G. mosseae* with PGPR, *B. subtilis*, showed an increase in herb biomass and total oil yield in *Pelargonium graveolens*. The herb yield increased by 59.5% when a combination of *G. mosseae* and *B. subtilis* was used for inoculation experiments (Alam et al. 2011). Endophytic fungus, *P. indica*, is known to form a synergistic association with PGPR, *A. chroococcum*. Studies by Bhuyan et al. (2015) showed that *A. chroococcum* (WR5 and M4 strains) influences the overall growth and physiology of *P. indica* to enter into a symbiotic association. The potential of *P. indica* in combination with *A. chroococcum* (WR5 strain) on secondary metabolite content of *Stevia rebaudiana* has been reported by our laboratory. The study showed a marked increase in total flavonoid and phenolic content in plants treated with dual inoculations of *P. indica* and *A. chroococcum* as compared to singly inoculated and control plants. The major steviol glycosides, stevioside and rebaudioside-A, also showed significant enhancement in plants given combined inoculation (Kilam et al. 2015).

**Table 16.4** Effect of inoculation of microbial cocultures on secondary metabolite content of medicinal plants

| Microbial coculture | Medicinal plant | Secondary metabolite content | References |
|---|---|---|---|
| AMF + PGPR(s) | | | |
| *Glomus aggregatum*, *Bacillus coagulans* + *Trichoderma harzianum* | *Solanum viarum* | Increase in total phenols, flavonoids, alkaloids, saponins, tannins | Hemashenpagam and Selvaraj (2011) |
| *G. aggregatum*, *Glomus fasciculatum*, *G. intraradices*, *G. mosseae* + *B. subtilis* | *Pelargonium graveolens* | Increase in the total oil yield | Alam et al. (2011) |
| *G. intraradices* + *Bacillus polymyxa*, *P. putida*, *A. chroococcum* | *S. rebaudiana* | Enhanced stevioside content | Vafadar et al. (2014) |
| *G. mosseae* + *B. subtilis*, *P. fluorescens* | *Thymus daenensis* | Increased concentrations of thymol | Bahadori et al. (2013) |
| *Glomus*, *Gigaspora*, *Acaulospora* sp. + *Bacillus megaterium*, *Azospirillum amazonense*, *Azotobacter* sp. | *Curcuma longa* L. | Increased flavonoids, phenolic and curcumin content | Dutta and Neog (2016) |
| Fungal endophyte + PGPR(s) | | | |
| *P. indica* + *A. chroococcum* | *Stevia rebaudiana* | Enhanced steviol glycoside (stevioside and rebaudioside-A) content | Kilam et al. (2015) |
| *P. indica* + *A. chroococcum* | *Artemisia annua* | Enhanced artemisinin content | Arora et al. (2016) |

## 16.4 Effect of Microbial Symbiosis on Plant Bioactive Compounds

The increase in the concentration of plant bioactive compounds upon symbiotic association of microorganisms has in general been considered a defence response of the host plant. However, it is not clear how microbial association causes changes to the phytochemicals in the plant (Toussaint 2007). The improved quantity and quality of bioactive compounds can be attributed to changes in the plant nutritional and non-nutritional pathways.

Phosphorus is an important constituent of the intermediates of secondary metabolite biosynthetic pathways. It is also an important constituent of nucleic acids and bio membranes (Marschner 2011) and an important source for essential oil synthesis by plants (Lichtenthaler 2009). Phosphorus fertilization has shown to increase the secondary metabolite content in both mycorrhizal and non-mycorrhizal plants, indicating its role in secondary metabolite accumulation (Abu-Zeyad et al. 1999). Bacterial and mycorrhizal fungi symbioses have shown to increase the phosphorus acquisition efficiency of plants by releasing phosphatase enzymes or organic acids that make phosphorus available in the organic form (Malla et al. 2004; Smith and

Read 2008). However, the microbes in the rhizosphere have different capacities to solubilize phosphorous and therefore could differently affect its availability to plants. Mycorrhizal inoculation at higher levels of phosphorus in medicinal plant *Anadenanthera colubrina* showed increased concentration of secondary metabolites (Pedone-Bonfim et al. 2013). Study in *Salvia officinalis* has shown that phosphorus fertilization alone has a better enhancement effect on secondary metabolite content as compared to AMF-inoculated plants (Nell et al. 2009). Another study in *Ocimum bascilicum* showed higher production of antioxidants, rosmarinic and caffeic acids in mycorrhizal plants, as compared to non-mycorrhizal plants with higher phosphorus amendments (Toussaint et al. 2007). Plant-associated symbionts are also known to maintain the availability of other plant nutrients such as nitrogen, potassium, magnesium and microelements (Maheshwari et al. 2012). Sharma et al. (2014) reported significant increase in phytochemical content and radical-scavenging activity upon inoculation of *A. vera* plantlets with *P. indica*. In another study, Selvaraj and Sumithra (2011) also showed an increase in root phosphorus, potassium, zinc, copper and iron contents after inoculation with consortium of AM fungi and PGPR(s). Similar results were observed by Singh et al. (2013) where macronutrients increased upon inoculation with *Pseudomonas monteilii* and *G. fasciculatum*.

Medicinal plants are widely affected by abiotic stress which has a direct impact on plant growth and production of bio active compounds. Microbial mediated alleviation of abiotic stress helps in reducing this negative effect on plants. Medicinal plant, *Hyoscyamus niger* which is a source of important tropane alkaloids, showed reduced plant growth and development under water stress. To alleviate the water stress, *Pseudomonas putida* and *P. fluorescens* were inoculated, which led to increase in plant growth and alkaloid content in *H. niger* (Ghorbanpour et al. 2013). Similar study was also undertaken in *Pelargonium* sp. where salinity stress decreased the plant growth and essential oil content in plants. Combined effects of AMF, *G. intraradices*, and PGPR, *P. fluorescens*, were studied which showed increased plant growth, nutrient uptake and essential oil contents (Prasad et al. 2012).

## 16.5 Conclusion and Future Prospects

This chapter highlights the use of microbial symbionts to improve the quantity and quality of bioactive compounds in medicinal plants. The plant-microbe symbiotic interaction provides a promising strategy for the better establishment of plants and also for enhancement of various phytochemicals present in the plant. A wide variety of fungi including AM fungi, root endophytes, and bacteria is recognized in the rhizosphere that has significant effect on the bioactive constituents of medicinal plants. However, selection of an efficient bacteria/or fungi for a specific medicinal plant is mandatory for the best response to obtain higher quantity and quality of their products. Therefore, further research is recommended to better understand the

function and diversity of interaction between symbiotic microbes and medicinal plants and also to elucidate the involved mechanisms.

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# Chapter 17
# Cultivation of *Piriformospora indica* with Nanomaterial in Bioreactor

**Uma and Ajit Varma**

**Abstract** *Piriformospora indica* is an axenically cultivable root endophytic fungus which exerts plant growth-promoting effects on its host plants. To enable commercial production of its spores, *Piriformospora indica* was cultivated in association of nanostructured materials "zinc oxide" in a 7 L batch bioreactor called "nanoembedded fungus" a novel nano-tool such that they result in maximum biomass during growth phase and in maximum spore yield during subsequent sporulation phase. An enhancement in overall biomass productivity of about 50% when *P. indica* was grown with zinc oxide nanorods and also the maximum spore yield ($9.25 \times 10^9$ spores/mL) was achieved in comparison to the cultivation of *P. indica* alone (without inclusion of nanomaterials) in bioreactor. The high spore yield obtained when cultivated with zinc nanomaterials in the chapter seems to be economical for commercial production of *P. indica*.

## 17.1 Introduction

Nanotechnology is an enabling technology dealing with nanometer-sized particles. A decade ago, nanoparticles were studied because of their size-dependent physical and chemical properties (Prasad et al. 2016). Now they have entered a commercial exploration period in the area of biotechnology, leading to the development of a new field of science nanobiotechnology (Murray et al. 2000). Understanding of biological processes at the nanoscale level is a strong driving force behind the development of nanobiotechnology (Whitesides 2003) as it is well known; living organisms are built of cells that are typically 10 km across. However, the biomolecules are much smaller and are in the submicron size domain. Even smaller are the proteins with a typical size of just 5 nm, comparable with the smallest man-made nanoparticles. This simple size comparison gives an idea of using nanoparticles as nanoprobes that would allow us to spy at the cellular machinery without too much interference (Taton 2002).

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In order to enhance the utilization of nanomaterials in biological systems, it is important to understand the influence they have on the cellular health and function (Suman et al. 2010). Nanomaterials present a research challenge as little is known about how they behave in relation to microorganisms, particularly at the cellular level. Most of the nanomaterials reported earlier have been demonstrated to be efficient antimicrobial agents (Raffi et al. 2008; Aziz et al. 2015, 2016). There are only a few or no reports on the growth-promoting role of the nanomaterials, especially with respect to microbes (Suman et al. 2010). It has been reported that nanoparticles possess more surface area than microparticles, thus improving the physical and chemical characteristics of a particle, which may also influence the biological property of the material. Zinc oxide (ZnO), which can exhibit a wide variety of nanostructures, possesses unique semiconducting and optical properties. One of the most important features of ZnO nanomaterials is low toxicity and biodegradability. $Zn^{2+}$ is an indispensable trace element for adults (~10 mg of $Zn^{2+}$ per day is recommended), and it is involved in various aspects of metabolism. Chemically, the surface of ZnO is rich in –OH groups, which can be readily functionalized by various surface decorating molecules. ZnO nanomaterials are important for its biomedical applications, such as biomedical imaging (which includes fluorescence, magnetic resonance, positron emission tomography, as well as dual-modality imaging), drug delivery, gene delivery, biocompatible/biodegradable, biosensing, photocatalysis, and antibacterial applications (Bhuyan et al. 2015a, b; Hatamie et al. 2015).

In the present chapter, the interaction of nanomaterials with the fungal endophyte *Piriformospora indica* and their effect on the growth processes at different stages of development have been studied (Suman et al. 2010). The fungus was chosen as a representative model to observe the effect of nanoparticles on growth enhancement. This property has already been patent granted (Varma et al. 2015, Patent Number: 267958).

## 17.2 Methods

### *17.2.1 Synthesis of Zinc Oxide Nanorods*

Zinc oxide nanorods were synthesized by using mechanically assisted thermal decomposition method described by Bhuyan et al. (2015a) and characterized by XRD, UV-Vis, and scanning electron microscopy.

### *17.2.2 Microorganism, Culture Maintenance, and Inoculum Preparation*

Usually the stock culture is maintained on slants containing Hill and Kaefer medium (Prasad et al. 2005, 2013) supplemented with 15 g/L agar. Inoculate the slants, incubate at 30 °C for 8 days, and then store at 4 °C. For the preparation of inoculum, grow *P. indica* on Kaefer medium in a petri dish (Hill and Kaefer 2001).

During the interaction between nanomaterials and *P. indica*, a zinc oxide (ZnO) nanomaterial was used. ZnO was mixed (50 mg/100 mL) in Hill and Kaefer broth with 2% glucose, in separate Erlenmeyer flasks with initial pH adjusted to 6.4. Usually 4–5 fully grown fungal agar discs (4 mm in diameter) were inoculated into each 500 mL Erlenmeyer flask containing 100 mL of Hill and Kaefer broth medium. Flasks were incubated for 7–10 days at 28 + 2 °C with constant shaking at 100 rotations/min on a rotary shaker.

## 17.3 Production of Fungal Culture with Nanomaterial in Bioreactor

Bioreactor provides optimized environmental and nutritional conditions for the large-scale production of microbial cultures. The constant administration of conditions at variable stages in bioreactor enables a more efficient scale-up of microbial cultures. The submerged conditions enhance the uptake of nutrients resulting in stimulation of the biochemical processes. Batch culture comprises of a closed system which encompasses an initial restricted availability of nutrient. The batch fermentation is employed for the production of nanoembedded fungal biomass.

### *17.3.1 Medium for Optimal Growth*

The media used for fermentation greatly influences the nutritional requirements as well as physiochemical environment and thus directly affects productivity and process economics (Zhang and Greasham 1999; Bagde et al. 2010). Therefore, a suitable medium should invariably support vegetative growth and production of spores. The optimum growth conditions are observed in a modified Kaefer media containing (50 mg/100 mL) zinc oxide nanomaterial with peptone, 3.0; yeast extract, 3.0; $KH_2PO_4$, 1.83; and $MgSO_4.7H_2O$, 0.65 g/L. The concentration of other components was the same as in the original Kaefer medium without $NaNO_3$ and KCl, while the glucose concentration was 20 g/L (Kumar et al. 2011).

### 17.3.2 Sterilization of the Fermenter

Prior to the initiation of the production process, the fermenter needs to be sterilized. The fermentation media and the fermenter can be sterilized together or separately. The fermenter is sterilized by channeling steam into the vessel *via* all entries and releasing the steam slowly through air outlet. The jackets or coils of the fermenter are sterilized by heating them with stream. Also the steam pressure is maintained at 15 psi inside the vessel for 20 min approximately for thorough sterilization.

### 17.3.3 Cultivation in Bioreactor

For nanoembedded fungal biomass production in bioreactor, an active 2% inoculum raised in an optimized medium is used. The initial pH is calibrated at 6.4. As the biomass production is initiated, there is an uptake of glucose which decreases the pH to between 5.5 and 6.0 in late log phase. Since the optimum pH for sustainable growth of nanomaterial-infused *P. indica* is 5.8, there is no requirement for pH control in fermenter systems where the fungal cultures are grown on media containing complex nitrogen sources. The temperature range is in between 20 and 35 °C. However, for optimized growth the fungal cultures are grown at a temperature of 30 °C. The fungus grows best at lower agitation and low oxygen concentrations (Varma et al. 2001). Thus, the cultures are grown at 200 rpm and 20% working volume.

### 17.3.4 Recovery of Biomass Produced

After the desired biomass is obtained, the production process is terminated. The biomass produced in the fermenter vessel is removed. The produced biomass is then filtered, separating the filtrate from the biomass. After separation the biomass obtained is then formulated by mixing with sterilized magnesium sulfite, talcum powder, or vermiculite.

## 17.4 Cultivation in Bioreactor

Growth may be defined as an irreversible increase in the volume of an organism, usually accompanied by an increase in biomass. Mycelial fungi exhibit extension growth of hyphae, accompanied by an increase in biomass. Unicellular fungi (e.g., yeasts) may exhibit an increase in individual cell volume, accompanied by an increase in biomass. But collectively, the number of yeast cells within a culture

(i.e., cell concentration) may also increase, resulting in an increase in biomass of the culture as a whole.

### 17.4.1 Batch Fermentations

A tank of fermenter is filled with the prepared media to be fermented. The temperature and pH for microbial fermentation is properly adjusted, and occasionally nutritive supplements are added to the prepared media. The media is steam-sterilized in a pure culture process. The inoculum of a pure culture is added to the fermenter, from a separate pure culture vessel. Fermentation proceeds, and after the proper time, the contents of the fermenter are taken out for further processing. The fermenter is cleaned and the process is repeated. Thus, each fermentation is a discontinuous process divided into batches.

### 17.4.2 Mass Cultivation of P. indica with Nanomaterial in 7 L Fermenter

*P. indica*, which mimics AMF, represents a model system to understand the molecular basis of photo and mycobiont interaction. Its application in horticulture or agriculture as a potent biofertilizer and biocontrol agent is economically and practically feasible through the easy propagation of a fungal inoculum using liquid or axenic cultures.

It is shown that the fungus can be grown axenically on different synthetic media. Among the tested media, the best growth reported to be on Hill and Kaefer medium (2001) which is reported from different authors (Varma et al. 1999, 2001; Pham et al. 2004; Qiang et al. 2011). However, significant quantitative and morphological changes are detected when the fungus is grown on different nutrient composition with no apparent negative effect on plants (Kumar et al. 2011).

A 7 L bioreactor was used to grow *P. indica* on optimized Hill and Kaefer medium containing nanomaterial to establish the best conditions for a maximal biomass and spore production for scale-up studies.

When *P. indica* was grown in 7 L bioreactor on optimized Hill and Kaefer medium (containing 20.0 g/L glucose, 1.0 g/L peptone, 1.0 g/L yeast extract, 1.0 g/L Casein acid hydrolysate, 50.0 mL/L macroelement, 2.5 mL/L microelement stock solution, 1.0 mL/L vitamin stock solution, 1 mL/L $Cacl_2$, 0.1 M, 1.0 mL/L $Fecl_3$, and 50 mg/100 mL ZnO nanomaterial), a maximum dry cell weight of 8.45 g/L was obtained after 42 h of growth. The value of biomass yield and the specific daily growth rate were 0.87 and 2.05, respectively. The fungus initiated the sporulation after 48 h, and a spore yield of $9.25 \times 10^9$ spores/mL was achieved after 60 h of growth which is much higher in comparison to control. The early sporulation in this

case may be due to the varying and size-dependent interaction between the surface of the respective nanoparticle and the cell wall which has, e.g., an influence on the diffusion of nutrients and therefore affects the growth rate (Feng et al. 2013; Ren et al. 2011). Due to more efficient mixing and homogenized fungal suspension, the growth of fungus was faster in the bioreactor and resulted in early depletion of the carbon source and thereby early sporulation compared to a shake flask. A complete growth profile of *P. indica* on modified Hill and Kaefer medium has been depicted. The pattern of pH profile was quite similar in all these experiments where complex nitrogen sources were present in the growth medium. The uptake of glucose caused a decrease in pH of fermentation broth which might be due to the generation of acidic metabolites. The growth of fungus remained unaffected as long as the pH during the log phase was not reduced below 4.5.

### *17.4.3 Measurement of Cell Growth, Growth Yield, and Specific Growth Rate*

The growth of *P. indica* was expressed in terms of dry cell weight (DCW) per liter of culture broth, which was determined by filtering a known volume of culture broth through Whatman No. 1 filter paper, drying to a constant weight in vacuum oven at 60 °C for about 48 h and weighing the dry mass. It was found that there was about 50% increase in dry cell weight of treated *P. indica* in comparison to control. Growth yield ($YX/S$) was calculated as grams of biomass produced per gram of substrate consumed. The specific growth rate ($\mu$) was calculated from the equation $= 1/X \times dx/dt$, where $X$ is the biomass concentration (g/L) at time $t$.

### *17.4.4 Measurement of Spores*

*P. indica* produced pear-shaped chlamydospores, which were attached to the mycelium (Siddhantha et al. 2016). The spores were dislodged by adding 1 mL of Tween 80 to 100 mL of culture broth, vortexing, grinding in a mixer grinder, and sonicating for 5 min each. After their detachment, the spores were counted with a hemocytometer, and it was found that spore size was bigger in nanomaterial-treated culture as compared to control.

## 17.5 Conclusions

There was a significant increase, i.e., two- to threefold in fungal biomass in the presence of nanomaterial as compared to control using batch bioreactor for its mass cultivation. The fresh biomass was maximum in case of ZnO nanomaterial-infused medium. Colony morphology also differed; the medium appears useful for economical mass production of spore-rich *P. indica* biomass for agricultural and horticultural applications.

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# Chapter 18
# Understanding the Mycorrhiza-Nanoparticles Interaction

**Avinash Ingle, Dnyaneshwar Rathod, Ajit Varma, and Mahendra Rai**

**Abstract** Arbuscular mycorrhizal fungi (AMF) always associate with the roots of higher plants and form a mutualistic symbiosis with the roots of over 90% of plant species, including forest trees, wild grasses, and many crops. Recently, considerable efforts were put to revolutionize agricultural systems through the applications of nanotechnology in various ways. Nanoparticles exploited for plant growth promotion showed the controversial opinions. Similarly, the interaction of various nanoparticles with mycorrhizal fungi found to influence its growth and showed both positive and negative effects. Some of the nanoparticles helps in colonization of AMF, whereas some negatively affects the colonization. Therefore, understanding the exact mechanism of interaction between AMF and nanoparticles is necessary.

Hence, in this book chapter, we have focused on the influence of different nanoparticles on the growth of AMF. Moreover, we have also discussed the role of nanoparticle in plant growth promotion.

## 18.1 Introduction

Arbuscular mycorrhizal fungi (AMF) are the most widespread fungal symbionts of plants, being associated with more than 90% of current land plants (Bonfante and Genre 2010; Prasad et al. 2017a). All AMF belong to the phylum *Glomeromycota*, a monophyletic group that diverged from the same common ancestor as *Ascomycota* and *Basidiomycota* (Prasad et al. 2017a). AMF are common soil microbes whose association with roots can have extensive effects on growth of the host plant (Rathod et al. 2011). AMF associations enhance not only the uptake of nutrients and water but also increase the resistance of their host plant towards disease and drought. AMF-infected roots are less susceptible to certain types of pathogens. Therefore, in

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recent years, major interest has centered on AMF in the control of soilborne pathogens present in the rhizospheric region of plants. Many researchers reported that colonization of roots by AMF confers resistance to plants against invasion by pathogens (Caron et al. 1985; Prasad 1998; Schouteden et al. 2015). The AM fungal hyphae extend into the rhizosphere and improve the absorption of water and nutrients such as phosphate and nitrogen from the soil through arbuscules (Chalot et al. 2006; Prasad et al. 2017a).

Recently, tremendous efforts have been made by the researchers all over the world to revolutionize agricultural systems through the applications of nanotechnology. Working with the nanomaterials in nanotechnology raises hope for improving agricultural productivity by encountering challenges which are unsolved conventionally. Moreover, various nanomaterials such as nanoclays and zeolites can be used to increase the efficiency of applied fertilizer, and it also helps in restoration of soil fertility by releasing fixed nutrients (Manjunatha et al. 2016). Apart from this, various studies have been performed to demonstrate the efficacy of different metal nanoparticles in plant growth promotion, whereas the findings reported showed that nanoparticles can exert both positive and negative effects on plant growth. However, the efficacy of nanoparticles is found dependent on various parameters like concentration, size, and shape of nanoparticles (Yin et al. 2012; Syu et al. 2014). In addition, effect of nanoparticles may vary plant to plant and even in different species of the same genus.

Nanoparticles are also reported to have great influence on the growth of AMF and other soil microorganisms beneficial for plants which are generally present in soil. Primary studies showed that direct application of metal nanoparticles exert both positive (Suman et al. 2010; Feng et al. 2013) and adverse effects (Cao et al. 2016) on the growth of AMF which are colonized in the plant roots due to their accumulation. Hence, there is a need to understand the exact interaction of nanoparticles and AMF in plants.

Therefore, the main aim of the chapter is to focus on understating of interaction of AMF and different nanoparticles. Moreover, we have also discussed the most contradictory and important area of research, i.e., role of nanoparticles in plant growth promotion. Apart from this, the role of endo- and ectomycorrhizal fungi has been discussed.

## 18.2 Plant and Microbe Symbiosis

Arbuscular mycorrhizae are characterized by the formation of unique structures, arbuscules, and vesicles by fungi of the phylum *Glomeromycota*. AMF develop a mutualistic relationship with the roots of host plants. AMF help plants to uptake nutrients such as nitrogen, phosphorus, and sulfur from the soil. It is reported that the development of the arbuscular mycorrhizal symbiosis plays a crucial role in initial colonization of land by plants and evolution of the vascular plants (Brundrett 2002; Prasad et al. 2017a).

A minor group of fungi, the parasitic and mutualistic symbionts, feed on living organisms. Such a classification cannot be easily applied to mycorrhizal fungi, a heterogeneous group of species spread over diverse fungal taxa. AMF are always associated with the roots of higher plants; indeed over 90% of plant species, including forest trees, wild grasses, and many crops (Bonfante and Genre 2010). Therefore, both partners are benefited from the mutual relationship, that is, mycorrhizal fungi improve the nutrient status of their host plants, influencing mineral nutrition, water absorption, growth, and disease resistance, whereas in exchange, the host plant is necessary for fungal growth and reproduction.

Substantial progress has been made in exploring the use of microorganisms in control of plant diseases in integrated plant disease management. One such strategy is the better exploitation of microbes present in soil, which contribute to soil fertility. AMF are ubiquitous in nature and constitute an integral component of terrestrial ecosystems, forming symbiotic associations with plant root systems of over 80% of all terrestrial plant species, including many agronomically important species (Berruti et al. 2016). Therefore, AMF are considered natural biofertilizers, because they provide the host with water, nutrients, and pathogen protection, in exchange for photosynthetic products. AMF are particularly important in organic and or sustainable farming systems that rely on biological processes rather than agrochemicals to control plant diseases (Harrier and Watson 2004). Significant advances have been made in the last two decades to understand the potential of mycorrhizal fungi in suppression of plant pathogens especially soilborne pathogens in wide range of fruits and vegetable host plants (Naqvi and Naqvi 2007). Plants are generally affected by many pathogens, which cause disease to the plants and thereby reduce the crop productivity, but the plants which are inoculated with mycorrhiza exhibit increased resistance to the fungal root-rot disease (Cameron et al. 2013; George et al. 2016). Mycorrhizae enter into a mutualistic relationship with plant roots, in which the fungi actually become integrated into the physical structure of the roots. The fungus derives nutritional uptake from the plant roots, without causing any plant disease.

## 18.3 Mycorrhiza: As Plant Symbionts

Mycorrhizal fungi have a close symbiotic relationship with plant roots. Mycorrhizal fungi colonize the plant's root system and develop a symbiotic association called "mycorrhiza." The terms symbiotic and mutualistic have been used interchangeably to describe mycorrhizal associations (Kaur et al. 2014). There are two major types of mycorrhizal fungi, that is, endomycorrhizal and ectomycorrhizal fungi. Both groups play an important role in symbiotic association relationship between host and AMF. Ectomycorrhizae basically originate on trees and form visible structures. These fungi colonize in trees as well as shrubs and most herbaceous plants and do not form visible structures (Kaur et al. 2014).

Among the types of endomycorrhizal fungi, arbuscular mycorrhizal (AM) fungi are the most widespread in soils. The most important members of endomycorrhizal group are called arbuscular mycorrhizae (AM). Earlier AMF were also called as vesicular arbuscular mycorrhizae (VAM) as fungal hyphae insert into the cortical root cell wall and once inside the plant cell, form small hyphae branched structures known as arbuscules. This name is derived from the occurrence of two types of structure characteristics of the fungi which belong to the family *Endogonaceae*, i.e., arbuscules (arbuscules are finely branched structures that form within a cell and serve as a major metabolic exchange site between the plant and the fungus) and vesicles (sac-like structures, emerging from hyphae, which serve as storage organs for lipids). The endomycorrhizal fungi involved consist of septate hyphae which are members of the phycomycetes and basidiomycetes. The hyphae of fungi penetrate the cells of the root cortex forming an internal hyphae network. Numbers of plants including many agricultural crops have infection by fungi that are vesicular arbuscular mycorrhizae. The actual process for root colonization by AMF starts with germination of resting spores present in the soil after subjected to the favorable conditions which is followed by the production of a short explorative mycelium. The perception of plant secretions, released by the host root, induces recursive hyphal branching, increasing the probability of a direct contact between the symbionts. Meanwhile, fungal secretions are perceived by the root, where they trigger calcium spiking through the activation of the common symbiosis pathway. This symbiotic reaction leads to signal transduction which activates cellular and transcriptional responses (green cells and nuclei). The interaction between the plant and fungus is followed by the adhesion of a hyphopodium to the root surface. This stimulates the assembly of a broad aggregation of cytoplasm (yellow), named the prepenetration apparatus (PPA) in the contacted epidermal cell and underlying outer cortical cell. Subsequent intracellular fungal colonization strictly follows the route of PPAs from the epidermis to the inner cortex. Here, intercellular hyphae can develop along the root axis. The PPA mechanism is then replicated in the contacted inner cortical cells, both before fungal entry and on a smaller scale branching (Fig. 18.1).

AMF significantly enhanced the potential of plants to absorb phosphorus (P) and other nutrients that are relatively immobile and available in low concentration in the soil (Kaur et al. 2014). It also plays a significant role in P nutrition of crop. Schüßler et al. (2001) reported that AMF are obligate symbionts belonging to the phylum *Glomeromycota*. Smith and Smith (2011) stated that AMF provides mineral nutrients to the host plant in exchange for photosynthetic products from the host. AMF are potential to improve remarkably plant mineral nutrient gaining, mostly in low-nutrient conditions, and it has clearly been studied that plants retain a symbiotic P uptake pathway (Tawaraya 2003; Smith and Smith 2011; Berruti et al. 2016).

Ectomycorrhizal fungi (EMF) are mostly observed in forest ecosystems and also found in other natural environments. EMF grow between root cells without piercing them. The fungal hyphae grow outer side in thick manner called as fungal mantle

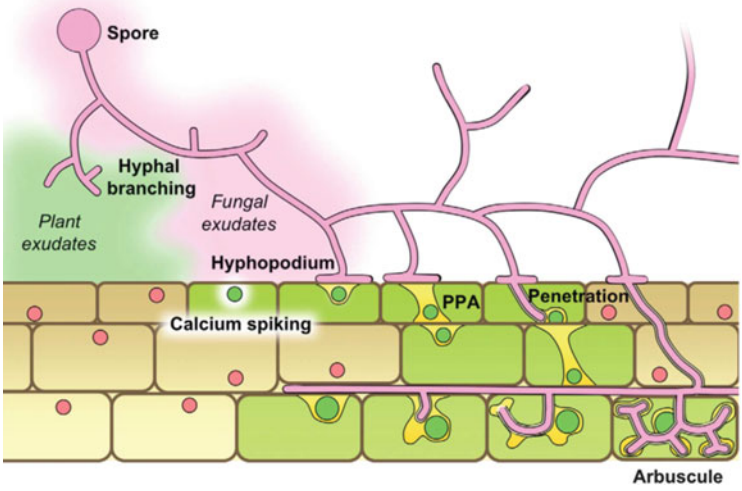


**Fig. 18.1** Schematic summary of the root colonization process by AMF [Reused from Bonfante and Genre (2010) with copyright permission from Nature Publishing Group]

which forms symbiotic relationship between plants and fungi. The EMF surrounds the root tip with a thick mantle of closely appressed hyphae, whereas the Hartig net develops around epidermal cells (green). In the case of arbuscular mycorrhizae, the root tip is usually not colonized. Hyphae develop from a spore and produce a hyphopodium on the root epidermis. Intraradical colonization proceeds both intra- and intercellularly and culminates with the formation of arbuscules, little fungal trees, and inside inner cortical cells (brown) (Fig. 18.2).

## 18.4 Role of Nanoparticles in Plant Growth

Nanotechnology has opened large scope of novel application in the various fields including agricultural sector, because the building blocks of nanotechnology, i.e., nanoparticles, have novel and unique physicochemical and biological properties. Various studies carried out in the past reported the potential use of some metal nanoparticles as nano-herbicides, nano-pesticides, and nano-fertilizers (Rai and Ingle 2012; Prasad et al. 2017b). In some cases, these nanomaterials were used as vehicle for the target-specific delivery in plants. Moreover, recently many researchers around the globe are focusing on one of the important applications of various nanoparticles, i.e., their role in plant growth promotion. In spite of the plenty of information available on the toxicity of nanoparticles to plant system, few studies have been conducted, and depending upon these studies carried out, there are distinct opinions (both positive and negative effects) of researchers regarding the role of nanoparticles in plant growth and development (Siddiqui et al. 2015).

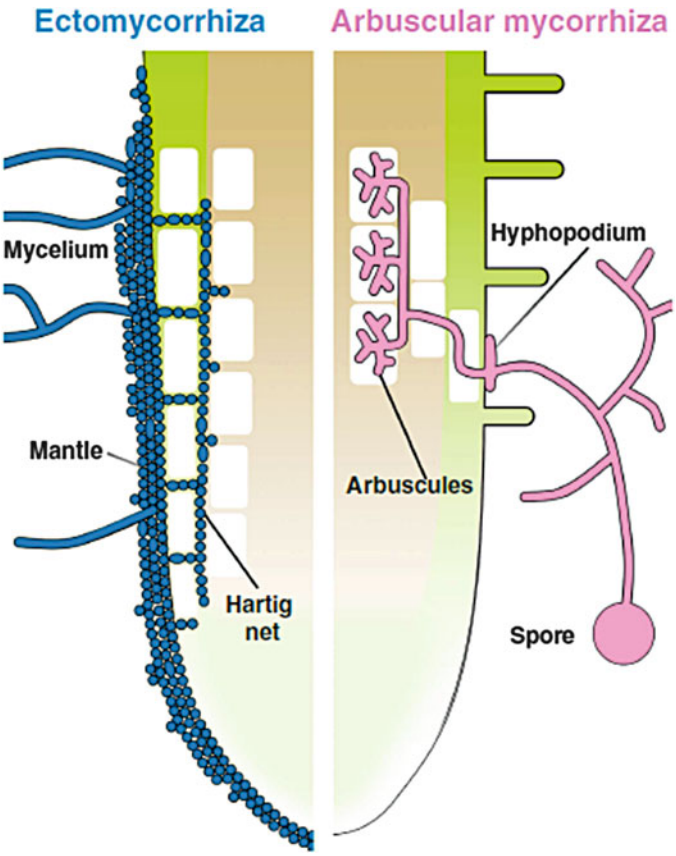


**Fig. 18.2** Schematic representation of root colonization structures in ectomycorrhizal (*blue*) and arbuscular mycorrhizal (*pink*) interactions [Reused from Bonfante and Genre (2010) with copyright permission from Nature Publishing Group]

Generally, germination of seeds is the first step toward the plant growth and development which is later followed by root elongation and shoot formation. Hence, the nanoparticles which help in the development of these parts will show positive effect in plant growth promotion. However, those which don't support the growth of these parts exert negative effects on plant growth. In this context, various studies have been performed which exploit different nanoparticles. Some of these studies are briefly discussed here.

Siddiqui and Al-Whaibi (2014) demonstrated the seed germination efficacy of silicon dioxide nanoparticles ($SiO_2$NPs) in tomato seeds (*Lycopersicum esculentum*). This was evident by studying various parameters such as percent seed germination, mean germination time, seed germination index, seed vigor index, fresh weight and dry weight of seedling, etc. The collective results obtained suggested that $SiO_2$NPs showed enhanced seed germination at lower concentration

(i.e., 8 g/L). In another study, Suriyaprabha et al. (2012) reported significant increase in seed germination in maize plant after application of $SiO_2$NPs due to sufficient availability of nutrients in the presence of $SiO_2$NPs. $SiO_2$NPs also reported to have improved seedling growth. Bao-shan et al. (2004) studied the effect of $SiO_2$NPs on the growth of seedlings in Changbai larch (*Larix olgensis*). The results showed the significant increase in the seedling growth which was confirmed from the improved mean height, root collar diameter, main root length, and the number of lateral roots of seedlings and also induced the synthesis of chlorophyll.

Similarly, there are many reports on zinc oxide nanoparticles (ZnONPs) which proved their ability in plant growth promotion in lower concentration; however, higher concentration exerts negative effects by impairing the seed germinations, and it also varies from plant to plant (de la Rosa et al. 2013; Raskar and Laware 2014). These include studies of Prasad et al. (2012) in peanut, Sedghi et al. (2013) in soybean, and Ramesh et al. (2014) in wheat. Moreover, studies carried out by Raliya and Tarafdar (2013) and Mahajan et al. (2011) demonstrated that ZnONPs showed significant growth and development in shoot and root length and biomass of *Cyamopsis tetragonoloba*, *Vigna radiata*, and *Cicer arietinum* plants, respectively. However, in another study, it was reported that ZnONPs do not show any improved efficacy in all parameters (viz., seed germination percentage, root length, and number of roots) studied in rice (*Oryza sativa* L.) (Boonyanitipong et al. 2011). They report stunt root length and reduce the number of roots and also detrimental effects on rice roots at early seedling stage.

Apart from these, other metal nanoparticles like gold (AuNPs) also reported to have positive effect on seed germination. Barrena et al. (2009), Arora et al. (2012), Savithramma et al. (2012), and Gopinath et al. (2014) demonstrated the increase in seed germination in lettuce and cucumber, *Brassica juncea*, *Boswellia ovalifoliolata*, and *Gloriosa superba*, respectively. Not only seed germination AuNPs also showed improved growth and development of other plant parts (Arora et al. 2012; Gopinath et al. 2014). On the contrary, Shah and Belozerova (2009) reported that AuNPs exert toxic effects on the various proteins which help in transportation of wide range of molecules including water, thereby interfering with aquaporin function of plant.

Like AuNPs, silver nanoparticles (AgNPs) are also found to have significant effects on seed germination of different plants including *Bacopa monnieri* (Krishnaraj et al. 2012) and *B. ovalifoliolata* (Savithramma et al. 2012). In addition, increased plant growth profile (shoot and root length, leaf area) was reported in *B. juncea* (Sharma et al. 2012). Various physical parameters such as concentration, shape, and size of AgNPs play key a role in the plant growth promotion (Syu et al. 2014). Recently, Razzaq et al. (2016) demonstrated the effect of different concentrations (0, 25, 50, 100, and 150 ppm) of AgNPs on growth of wheat plants. The results showed that plants treated with 25 ppm AgNPs showed more prominent growth. Apart from this, its effect was also found to be dependent on plants and their species (Yin et al. 2012). In contradiction to above studies, Gruyer et al. (2013) reported negative effect on root elongation of lettuce. Other nanoparticles like

titanium dioxide ($TiO_2$NPs) also showed improved seed germination and also promoted growth of radicle and plumule of canola seedlings (Mahmoodzadeh et al. 2013). Jaberzadeh et al. (2013) demonstrated that $TiO_2$NPs help in growth promotion of wheat plant growth and increased yield was reported even in water deficit stress condition.

In addition, studies carried out on the use of carbon nanotubes (CNTs) in plant growth and development showed both positive as well as negative effects. The reports by Gajanan et al. (2010), Mondal et al. (2011), Morla et al. (2011), and Nalwade and Neharkar (2013) demonstrated that multi-walled carbon nanotubes (MWCNTs) showed high germination rate in tomato, hybrid Bt cotton, *B. juncea*, *Phaseolus mungo*, and rice. However, Husen and Siddiqi (2014) and Lin and Xing (2007) reported that MWCNTs do not exhibit a positive influence on seed germination.

Overall, nanoparticles discussed above showed both positive and negative effects on the plant growth. Some of these play a key role in the growth promotion, and some of these are reported to exert toxic effects on plants. On one hand, the significant increase in seed germination and other parameters may be effective to increase the crop yield. On the other hand, direct exposure of plants to nanoparticles causes significant phytotoxicity (Tripathi et al. 2017). Hence, extensive care needs to be taken during the disposal of wastes containing nanoparticles, and also there is huge need to encourage the studies on toxicity assessment and impacts of nanoparticles on agricultural and environmental systems.

## 18.5 Mycorrhiza: Nanoparticle Interaction

It is a well-known fact that AMF are one of the most important members of soil microbial community which can form a mutualistic symbiosis with the roots of over 90% of land plants. AMF play key role in plant growth promotion directly or indirectly. It helps in proper aquaporin function (water uptake) of plant and uptake of many other nutrients required for plant growth. On the other side, applications of different nanoparticles in agriculture are increasing day by day; however, there are different positive and negative opinions of researchers about the role of nanoparticles in plant growth. Moreover, some of the attempts have been made to alleviate the negative effects of nanoparticles by using AMF. But, still there is no sufficient literature available regarding the studies on interaction of mycorrhizal fungi and nanoparticles.

As described earlier, direct application of some of the metal nanoparticles exerts toxic effects by accumulating in various parts of plants when used in higher concentration. Application of nanoparticles in higher doses also contaminate the soil which ultimately effects on crop yield and harms beneficial soil microorganisms, posing new concerns and challenges. According to the recent study, it was observed that the interaction of AMF with such nanoparticles help in the alleviation of negative effects exerted in plants. Wang et al. (2016) performed some

experiments, in which they demonstrated the interactions between maize plants treated with ZnONPs and inoculated with or without AMF. They reported that when the maize plants were treated with ZnONPs at the concentration of 800 mg/kg, decreased plant mineral nutrient acquisition, photosynthetic pigment concentrations, and root activity which were observed in plants without AMF. However, plants inoculated with AMF showed increased growth, nutrient uptake, photosynthetic pigment content, and superoxide dismutase activity in leaves. It is because of interaction of AMF with nanoparticles which helps in the decrease of Zn bioavailability and accumulation, thereby increasing mineral nutrients and antioxidant capacity of plant.

Moreover, available reports suggested that interaction of some nanoparticles with AMF helps in its colonization in plant roots and associated soil. Feng et al. (2013) studied the biological effects of AgNPs and iron oxide nanoparticles (FeONPs) and $Fe_2O_3$ and Ag in bulk material form on AMF in mycorrhizal clover (*Trifolium repens*) in dose-dependent manner. The results demonstrated the significant increase in AMF growth and function reported in plant treated with nanoparticles; however, their respective bulk material did not colonize AMF in plants. Improved colonization of AMF in plant roots and associated soil will automatically reflect in improved plant growth. On the contrary, Dubchak et al. (2010) evaluated the efficacy of AgNPs and TiNPs on the development of colonization of mycorrhiza in *Helianthus annuus* cultivated in the presence of radioactive $^{134}Cs$. It was reported that mycorrhizal colonization and uptake of $^{134}Cs$ were greatly affected in the presence of AgNPs and TiNPs. However, application of activated carbon reduced the effect of these nanoparticles and increases both colonization of AMF and uptake of $^{134}Cs$ by plants.

Li et al. (2015) also reported exactly opposite results in case of ZnONPs and $ZnSO_4$ (bulk form of Zn). In their study, biological effects of ZnONPs and $ZnSO_4$ alone and in combination on colonization of AMF (*Funneliformis mosseae*) in maize plants were studied. They reported that both ZnONPs and $ZnSO_4$ at the concentration of 500 mg/kg inhibited the AMF colonization and also the growth of maize plants, whereas improved colonization of AMF and growth of plants were reported when they were used in combination. It may be due to the decreased Zn concentrations and improved uptake of other nutrients in maize plants.

It is well documented that AMF not only itself help in plant growth promotion but also help to increase the activity of other soil microorganisms. In this context, recently, Cao et al. (2016) demonstrated the interaction of AMF and iron oxide nanoparticles ($Fe_3O_4$NPs) and studied the role of AMF in the alleviation of negative effects of these nanoparticles on other microbes present in rhizospheric soils. The results obtained revealed that $Fe_3O_4$NPs in higher concentration exert toxic effects which leads to significant decrease in soil bacterial abundance which further leads to decrease in soil-dissolved organic contents. However, no significant changes were reported in soil bacterial abundance and soil-dissolved organic after $Fe_3O_4$NPs treatment in the presence of AMF. It indicated that AMF alter the effects of $Fe_3O_4$NPs on soil microorganisms, possibly by influencing plant growth and organic matter released from plant roots.

Contamination of soil by various types of nanoparticles is a great challenge. Interaction of metal nanoparticles having broad-spectrum antimicrobial activities with soil microbes including various mycorrhizal fungi resulted in the depletion of useful microflora of soil (Aziz et al. 2016). Sweet and Singleton (2015) studied the effects of various concentrations of AgNPs (0, 350, and 790 mg/kg) on the growth of EMF which are equally important and responsible for enhancing growth of plants by nutrient transfer like AMF and on growth of pine plant. It was reported that AgNPs even at lower concentration, i.e., 350 mg/kg, showed significant reduction in growth of EMF in pine root and also it affects the fresh root and shoot biomass in pine plants.

The efficacy of $TiO_2$NPs was studied on the growth of maize and soybean plants and associated microbial community including AMF. The results revealed that there were no any significant effects on plant growth and the composition of bacterial communities within the rhizosphere. However, composition of AMF was greatly affected in the presence of these nanoparticles (Burke et al. 2014). The possible reason behind the interaction maybe the increase in the concentration of titanium (Ti) in the roots of plants due to binding of $TiO_2$NPs to plant roots. The increased concentration of Ti in roots affects the composition of AMF (Seeger et al. 2009) as these are colonized in the interior of the root system. However, as the other bacterial community is mainly present in the rhizosphere, it was not affected much.

Although the various studies have been performed to understand the interaction between different nanoparticles and mycorrhizal fungi, due to huge contradiction in the finding reported for each study, researchers have to wait for some more time period to understand the exact role of different nanoparticles and their actual interaction with mycorrhizal fungi.

## 18.6 Conclusions

Considering the facts described in the present book chapter, it can be concluded that mycorrhizal fungi are the most important symbiotic agents for plants that help in growth promotion through the uptake of various nutrients and water. Although, many researchers claimed application of nanoparticles in agricultural systems including plant growth promotion, the data generated from the previous studies set a question mark on such claims. The findings reported for the same are contradictory; some showed positive effects of nanoparticles, whereas some others showed negative effects on plant growth. Similarly, there are mixed opinions, in the context of the interaction of mycorrhizal fungi and nanoparticles. On one hand, AMF are found to alleviate the negative effects of nanoparticles and play a key role in the management of environmental risks posed by different nanoparticles in agriculture, while on the other hand, some nanoparticles are found to affect the colonization of AMF in plant roots, thereby affecting plant growth. Hence, there is

requirement of extensive studies on interaction of mycorrhizal fungi and nanoparticles to understand the exact mechanism involved in it.

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