Hinanit Koltai · Yoram Kapulnik

Editors

Arbuscular Mycorrhizas: Physiology and Function

Second Edition

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Preface

In the years since the first edition of "Arbuscular Mycorrhizas: Physiology and Function" was published, an exceptional proliferation of interest in mycorrhizal biology has developed. This has been associated with advances in different research disciplines such as genetics, genomics, proteomics, metabolomics and physiology, advances which have generated better insight into topics of mycorrhizal biology, including the mechanisms of host-mycorrhiza interactions pre- and post-penetration, the influence of the symbiosis on the host and its surroundings, and the evolution and diversity of mycorrhization. It therefore became necessary to both update and expand the book's coverage in this, its second edition.

In the second edition of this book, we wanted to retain the unique angle of our original goal of bringing the reader a state-of-the-art multidisciplinary view of arbuscular mycorrhiza (AM) – plant interactions, beginning with pre-penetration stages, through colonization, symbiotic function, influence of the symbiosis on the host and its surroundings, and ending with evolution and diversity of mycorrhization. To this aim, we have recruited leading scientists in their fields to provide a thorough and detailed review of the current status of scientific knowledge on mycorrhizal biology.

The subject of host–fungal interactions during the pre-penetration stage of AM is discussed in the first four chapters of the book. The first chapter, a review by Manuela Giovannetti, Luciano Avio and Cristiana Sbrana, presents the physiological and genetic aspects of AM fungal spore germination and pre-symbiotic mycelial growth. This chapter focuses on new insights into the main fungal developmental switches in the absence of the host; it promotes resolution of an interesting enigma: despite a putative strong selective disadvantage, how do these obligate symbionts compensate for the lack of host-regulated spore germination?

The chapter by Gerald Nagahashi, David D. Douds and Yurdagul Ferhatoglu presents a study of metabolites identified in separated exudate fractions of carrot roots grown under phosphorus stress, and their potential role in AM fungus-host interactions is suggested. This study provides a more general picture of the types of compounds exuded by host roots, which affect the growth of AM fungi.

Andrea Genre and Paola Bonfante discuss some of the responses that are induced in root cells during the different steps of AM colonization. Interestingly,

the establishment of a functional symbiosis appears to lie at the end of a series of plant-controlled checkpoints; each of these checkpoints is required but not sufficient to achieve the next one.

The chapter by Soizic Rochange focuses on strigolactones and their role in AM symbiosis. A group of carotenoid-derived metabolites exuded by plant roots, strigolactones, have recently been recognized as major contributors to the establishment of mycorrhizal symbiosis. This chapter presents recent advances and future prospects in our knowledge of the structural features of strigolactones, their biosynthetic pathway and their mode of action.

The next subject in the book is host–fungal interactions post-host penetration by the mycorrhizal fungi. The chapter by Philipp Franken presents molecular and physiological aspects of AM symbiosis post-penetration. Different strategies that have been taken to understand the molecular-physiological basis of the symbiosis are described, including non-targeted and function-targeted approaches. The described studies lead to new hypotheses concerning mycorrhizal functioning.

The chapter by Maria Harrison, Nathan Pumplin, Florence J. Breuillin, Roslyn D. Noar, and Hee-Jin Park focuses on a specific, yet crucial component of mycorrhizal symbiosis, that of phosphate transport. For across-membrane symbiotic Pi transfer, transport proteins of both the AM fungus and plant are needed. Recent progress in the identification of plant and fungal Pi-transporter proteins, their expression patterns and roles in AM symbiosis are presented.

Elke Neumann and Eckhard George present AM fungal symbiosis as a plant nutrient-acquisition strategy. Both direct and indirect effects of AM mycelia on plant nutrient availability and uptake are presented. The integration of AM fungal symbiosis into particular plant nutrient-acquisition strategies is discussed, as well as the contribution of the subject to our understanding of ecosystem functioning, and implications as to further development of mycorrhizal technology for plant production.

The chapter by Jutta Ludwig-Müller presents the subject of hormonal responses in host plants that are triggered by AM fungi. Major advances in our knowledge of the functions of phytohormones in AM development are presented, along with thoughts on the effects of belowground signals on aboveground tissues and the possible role of the effecting hormones in the upper part of the plant.

The subject of influences of AM symbiosis on the host and its surroundings is divided into two sub-topics: host responses to biotic stress, and host responses to abiotic stress. María J. Pozo, Sabine C. Jung, Juan A. López-Ráez and Concepción Azcón-Aguilar discuss the impact of AM symbiosis on plant defense responses to biotic stress. They highlight the mechanisms that may be involved in particular cases and elaborate on a general modulation of plant defense responses in mycorrhizal systems.

Another aspect of the effect of mycorrhiza on host responses to biotic stress is mediated via the biotic environment of AM fungi in soil. This subject, covered by Jan Jansa and Milan Gryndler, presents AM fungus interactions with soil and in particular with other soil microbes. It is suggested that AM fungi play an important role as a carbon and nutrient highway between the plants and the soil, whereas both

AM fungi and plant root physiology are affected by biologically active substances produced by the soil biota.

The influence of the symbiosis on host responses to abiotic stress is presented in the book in the following chapters. The chapter by Juan Manuel Ruiz-Lozano and Ricardo Aroca presents host responses to osmotic stresses, including stomatal behavior and water-use efficiency of AM plants. Here, a combination of physiological, biochemical and environmental conditions provides detailed insight into the mechanism of AM symbiosis's ability to protect host plants against the detrimental effects of water deficit, caused by osmotic stresses such as drought and salinity.

The chapter by Katarzyna Turnau, Przemysław Ryszka, and Grzegorz Wojtczak presents the issue of metal tolerance in mycorrhizal plants, in heavy-metal-rich, industrial waste lands in temperate regions. The role of mycorrhizal fungi in phytoremediation of these regions is presented, for both heavy-metal detoxification and the establishment of vegetation in strongly polluted areas; the importance of selection of both plant and fungal partners for the effectiveness of bioremediation is emphasized.

Evolutionary and diversity perspectives of mycorrhizal associations are presented in the last two chapters of the book. Victoria Estaún, Cinta Calvet and Amèlia Camprubí describe the effect of differences among crop species and cultivars on AM symbiosis. Interestingly, despite decades of breeding new varieties with no consideration for the presence or role of AM symbiosis, such symbiosis persists. However, differences in diverse plant species' responsiveness to, and dependence on the symbiosis have been found. A holistic approach to breeding crop cultivars is suggested that will make full use of AM symbiosis.

Roger T. Koide presents the subject of mycorrhizal symbiosis and plant reproduction. Several aspects of sexual reproduction that may be affected by mycorrhiza are presented. Interestingly, mycorrhiza are suggested to lead to variations among individual plants in their contributions to the next generation. As a result, mycorrhiza may control the genetic structures of populations and communities.

We feel that the unique combination of subjects brought together in this book provides a thorough and detailed picture of the updated knowledge on mycorrhizal symbiosis biology. Many new notions of mycorrhizal symbiosis are raised, as well as future prospects on mycorrhizal effects and their management. We are grateful to the authors who contributed these book chapters, for sharing their knowledge, vision and thoughts; we thank the dedicated anonymous reviewers who improved the book's quality. We hope that this book will serve as a basis for upcoming scientific advances in mycorrhizal studies, promoting future understanding of this symbiosis and utilization of its benefits.

Hinanit Koltai and Yoram Kapulnik

Cover: Micrographs showing differential staining of mycelium originated by *Glomus mosseae* spores growing in the absence of the host by Manuela Giovannetti, Luciano Avio and Cristiana Sbrana

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Chapter 1
Fungal Spore Germination and Pre-symbiotic Mycelial Growth – Physiological and Genetic Aspects

Manuela Giovannetti, Luciano Avio, and Cristiana Sbrana

Abstract Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs, living symbiotically in the roots of most land plants. They form spores in the soil, which are able to germinate and grow, but are unable to complete their life cycle without establishing a functional symbiosis with a host plant. In this chapter, results of recent studies providing new insights into the main developmental switches occurring in the fungal organism, from the relief of spore dormancy to the development of germlings and growth arrest in the absence of the host, are reviewed. The knowledge of environmental, cytological, biochemical and molecular events involved in early stages of AMF life cycle may reveal how these obligate symbionts compensate for the lack of host-regulated spore germination, possibly representing a strong selective disadvantage. Diverse scientific approaches showed multiple survival strategies, active during pre-symbiotic mycelial growth, contributing to the survival of AM fungal individuals and populations.

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1 Introduction

Arbuscular mycorrhizal (AM) fungi (AMF) are obligate biotrophs, which live symbiotically in the roots of about 80% of plant species. Most AMF form spores in the soil which are able to germinate and grow from a quiescent-like state in response to different edaphic and environmental conditions, but are unable to produce extensive mycelia and to complete their life cycle without establishing a functional symbiosis with a host plant (Mosse 1959; Hepper and Smith 1976). The key developmental switches occurring in the fungal organism, from the germination of an individual spore to the formation of an extensive hyphal network in the soil, involve a sequence of morphogenetic events represented by: spore germination and pre-symbiotic mycelial growth, differential hyphal branching pattern in the presence of host roots, appressorium formation, root colonization, arbuscule development, extraradical mycelial growth and spore production (Giovannetti 2000).

The lack of host-regulated spore germination, contrary to what happens with many pathogenic biotrophic fungi, could have represented a strong selective disadvantage. Nevertheless, AMF are considered evolutionary successful "living fossils", having survived and evolved for 460 millions years, their ancestral nature having been shown by diverse fossil records and DNA sequence data (Simon et al. 1993; Remy et al. 1994; Phipps and Taylor 1996; Redecker et al. 2000a, b). Their persistence indicates that they must have evolved efficient strategies to overcome the lack of spore germination regulation and to allow the survival of individuals and populations (Logi et al. 1998; Giovannetti et al. 2000; Giovannetti 2002).

The aim of this chapter is to review recent developments which contributed to our understanding of cellular and molecular events involved in the early stages of the life cycle of AMF, from relieving spore dormancy and triggering spore germination to germling growth and growth arrest in the absence of the host.

2 Spore Dormancy

The phenomenon of spore dormancy has concerned researchers since Godfrey's early studies on spore germination (Godfrey 1957). As early as 1959, Barbara Mosse suggested the storage of collected spores on damp filter paper at 5°C for 6 weeks in order to obtain the regular germination of resting spores of an *Endogone* sp. (presumably *Glomus mosseae*) (Mosse 1959). Eighty percent of spores treated in this way germinated within 3–4 days. The problem of erratic spore germination has been mentioned in many reports, and in 1983 Tommerup gave a clear-cut definition of spore dormancy, making a distinction between dormancy and quiescence (Tommerup 1983a). A dormant spore was defined as one failing to germinate when exposed to physical and chemical conditions which support germination of apparently identical spores, defined as quiescent spores. Differences in cytoplasmic organization between young and old resting spores were described in *Acaulospora laevis* and in *Glomus* species: in dormant spores the oil globules enlarged at the expense of the cytoplasm, which was restricted to small interstitial spaces (Mosse 1970a, b;

Meier and Charvat 1992; Maia and Kimbrough 1998). A fine network of cytoplasmic material interlaced between large lipid droplets was also described by Sward (1981a) in dormant spores of *Gigaspora margarita*.

The relief of dormancy by storage was reported by many authors. Hepper and Smith (1976) found that spores of *G. mosseae* from freshly harvested sporocarps germinated slowly compared to spores detached from sporocarps and stored at 6°C for 5 weeks. The same results were obtained with a North American isolate of *G. mosseae*, which showed a marked difference in germinability between freshly isolated and 10°C-stored spores (Daniels and Graham 1976). Diverse species of the genus *Glomus* exhibited spore dormancy, such as *Glomus intraradices, Glomus clarum, Glomus caledonium, Glomus monosporum* (Hepper 1979; Tommerup 1983b; Louis and Lim 1988; Douds and Schenck 1991; Juge et al. 2002). Other species, such as *Glomus coronatum*, showed erratic germination even after cold treatments lasting 1 year (Giovannetti et al. 1991).

A marked dormancy was shown by spores of *A. laevis*, which germinated after 6 months storage in two different experimental conditions (Tommerup 1983a; Gazey et al. 1993). Other species within the genus *Acaulospora* exhibited the same behaviour: in a laboratory experiment only a small proportion of spores stored for 2 months germinated, while most spores germinated well after storage for 4–6 months (Gazey et al. 1993). Similarly, *Acaulospora longula* showed complete relief of dormancy after 8 weeks storage at 23°C in soil (Douds and Schenck 1991).

Not all the species and genera of AMF show spore dormancy. Spores of *Gigaspora gigantea* collected throughout the year from sand dunes did not show any dormancy, and were able to germinate as early as 1 day after incubation, either when they had been surface sterilized or not (Koske 1981a), while newly formed spores showed a period of endogenous dormancy (Gemma and Koske 1988). Germ tubes of *G. margarita* emerged after 72 h incubation on water agar or within 3–5 days on agar media without any storage treatment (Sward 1981c; Siqueira et al. 1982). Similarly, spores of *Scutellospora fulgida* and *Scutellospora persica* did not possess any dormancy, showing mycelial growth and the formation of auxiliary cells after 2 weeks in the dark at 24°C (Turrini et al. 2008).

Propagule dormancy may contribute to the survival of AMF in adverse environments, but despite many different experimental reports on spore dormancy of many species of AMF a complete understanding of the phenomenon has not been obtained. We still do not know whether dormancy is more species or genus than isolate correlated, because experiments have often been carried out on different isolates. Moreover, no studies have been performed on the molecular bases of dormancy: we ignore whether it may be affected by the presence of compounds in young spores, which inhibit germination, or by the occurrence of compounds in mature, old spores, which enhance germination.

3 Triggers for Spore Germination

The molecular signals which relieve spore dormancy and activate the cell cycle still remain unknown, though different environmental conditions triggering the initiation of germination in genera and species of AMF have been investigated. In fact,

resting spores of many AM fungal species germinate both in soil and in agar under adequate physical, chemical and microbiological conditions.

Many germination factors have been identified which play important roles in growth activation of quiescent spores. Although complex interactions among different factors probably play the most important role in spore germination in nature, many investigators have studied germination factors such as pH, temperature, moisture, mineral and organic nutrients, host plants, and microorganisms as if they were independent triggers, and as such they will be considered here.

3.1   pH

Differences in spore germination among species and genera are often related to the environment where the endophytes live and to which they are ecologically adapted (Sylvia and Williams 1992; Clark 1997). For example, spore population surveys from different sites showed that *A. laevis* is the predominant AM fungus in low pH soils (Abbott and Robson 1977), or even the only species in soils at pH < 4.9 (Nicolson and Schenck 1979). Also data from INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, http://invam.caf.wvu.edu/) collection showed that 88.5% of *Acaulospora* isolates live at soil pH < 6.0 (Morton et al. 1993). Accordingly, results obtained in experimental conditions demonstrated that spore germination of *A. laevis* is strongly regulated by soil pH, being optimum between 4 and 5, decreasing at pH 6, and declining to less than 10% between pH 6.5 and 8 (Hepper 1984b). Similar results were exhibited by *Gigaspora coralloidea* and *Gigaspora heterogama* isolated from acidic soils, which germinated best at pH from 4 to 6 (Green et al. 1976). Other authors reported that *G. margarita* is less sensitive to acidic conditions than *G. mosseae* (Siqueira et al. 1984).

An isolate of *G. mosseae*, collected from a wheat field, showed a pH optimum for spore germination between 6 and 9 in water or in soil extract agar, and was not able to form germ tubes at pH 4 and 5 (Green et al. 1976). Another strain of the same species, isolated from an agricultural soil, failed to germinate at pH 4.5 (Mosse and Hepper 1975). Other species of the genus *Glomus* germinated best at pH ranging from 6 to 8 and were capable of producing root infection and multiplying in very alkaline soils (Daniels and Trappe 1980; Giovannetti 1983; Douds 1997).

The thorough surveys of Sieverding (1991) confirmed that *G. mosseae* does not occur in natural tropical soils with pH < 5.5. Thus, it may be that edaphic factors related to the environment from which the different species of glomeromycotan fungi were originally isolated play an important role in spore germination and pre-symbiotic hyphal growth (Giovannetti and Gianinazzi-Pearson 1994). However, it may also be that the optimal pH values attributed to each species are actually characteristic of the isolates used in each experiment and cannot be applied to all the isolates of the species. Each isolate originating from a specific environment could in fact represent an ecotype adapted to peculiar soil characteristics. This could apply in particular to *G. mosseae*, which has been shown to occur in

55 different countries throughout all continents and biomes (Avio et al. 2009). Different geographic isolates of the same species should be used to obtain new evidence on this point.

3.2 Temperature

The germination of AM fungal spores is greatly affected by temperature and the limits for germination exhibited by different species have been ascribed to their fundamental dissimilarity. Tommerup (1983b) reported that three fungal species isolated from the same source possessed different temperature limits for germination: *A. laevis* germinated best at temperature ranges of 15–25°C, *Gigaspora calospora* at 10–30°C, and *G. caledonium* at 10–25°C.

Some studies have suggested that many differences among glomeromycotan fungi in temperature ranges affecting spore germination reflect the differences in the environments from which the fungi were isolated. Accordingly, two Florida isolates of *G. coralloidea* and *G. heterogama* germinated best at 34°C, while *G. mosseae*, isolated from more northern latitudes, showed maximum germination at 20°C and failed to germinate at 34°C (Schenck et al. 1975). Also, an isolate of *Glomus epigaeum* from cool climates showed maximum germination at 22°C (Daniels and Trappe 1980). Most rapid germination of spores of *G. gigantea* was obtained at 30°C, whereas no germination occurred at 15°C, and only 6% spores germinated at 35°C (Koske 1981a).

It is interesting to note that the lethal exposure times to 60°C for *G. caledonium* and *A. laevis* spores were 5 min and 1 min, respectively (Tommerup and Kidby 1980). Viability of *G. intraradices* and *G. mosseae* was nil beyond 60°C, and that of *Glomus deserticola* beyond 54°C (Nemec 1987). Interestingly, an isolate of *G. intraradices* showed a great tolerance to 45°C for up to 24 h (Bendavid-Val et al. 1997).

Temperature optima for germination may be related to the environment to which each endophyte is indigenous. The demonstration of this requires the germination of different strains of the same species isolated from geographical areas with very dissimilar climates.

3.3 Moisture

Soil water content can have variable effects upon spore germination of species and genera of AMF. *G. margarita* spores germinated independently of soil water content, while germination of *G. intraradices, G. mosseae* and *A. longula* was strongly inhibited by matric potentials between −0.50 and −2.20 MPa (Douds and Schenck 1991). Other authors reported that spore germination of *G. epigaeum* and *G. gigantea* was increased at soil moisture near field capacity or above (Daniels and Trappe 1980; Koske 1981a). Three other *Glomus* species, *Glomus macrocarpum, G. clarum* and

G. etunicatum, showed tolerance to soil drying, maximum germination occurring at matric potential of −0.01 MPa (Sylvia and Schenck 1983). *G. epigaeum* spores germinated well when soil moisture content ranged from field capacity to soil saturation, and no germination was observed below −3.4 MPa (Daniels and Trappe 1980), while *G. gigantea* showed delayed germination at −1.0 Mpa (Koske 1981a).

As noted above for pH and temperature, differences in spore germination of AM fungal species and genera are often related to the moisture conditions of the environment to which they are ecologically adapted. No general conclusions can be made without knowing germination responses of several isolates of a species, each from environments with widely different moisture regimes, to different soil matric potentials. Moreover, it is probable that soil wetting and drying cycles are the most important factors affecting survival, germination and thus infectivity of AMF in nature; in particular in Mediterranean climates where glomeromycotan spores survive the hot and dry summers to colonize young emerging plants during the following seasons (Braunberger et al. 1996).

3.4   Mineral and Organic Nutrients

The germination of AM fungal spores is inconsistently affected by mineral nutrient content of soil. *G. gigantea* spores germinated at the same rate regardless of phosphorus concentrations (5–500 ppm) in sand plates (Koske 1981a). Germination of *G. mosseae* and *G. caledonium* spores was not affected by phosphorus concentrations in agar up to 30 mM, while above this level germination was reduced by 56% or more (Hepper 1983). Similar results were obtained with *G. margarita*, whose spores germinated well up to 16 mM phosphate solution (Tawaraya et al. 1996a) and with *G. epigaeum* spores, whose germination was not influenced by increasing levels of NH_4NO_3 and K_2SO_4, up to 200 ppm (Daniels and Trappe 1980). However, when phosphorus was added to soil, spore germination of different species of AMF decreased with soil P increments (De Miranda and Harris 1994). Investigations on the role of inorganic sulphur-containing compounds on the growth of *G. caledonium* mycelium showed that it was stimulated by the presence of thiosulphate, metabisulphite, sulphite and sulphate in the medium (Hepper 1984b).

Some inorganic ions completely inhibit spore germination of AMF. Hepper and Smith (1976) found that the inhibitory effects of different agar media on germination of *G. mosseae* spores was due to Mn and Zn. Toxicity of heavy metals such as Cu, Mn and Zn also affected spore germination of *G. caledonium* (Hepper 1979).

Germination and hyphal growth of different AM fungal species, evaluated in acidic soils with varying Al saturation, showed that most species of *Gigaspora* and *Scutellospora* were more tolerant than *Glomus* species (Bartolome-Esteban and Schenck 1994). However, no generalization is possible, since different isolates within a species showed varying responses to heavy metals (Gildon and Tinker 1981; Weissenhorn et al. 1993).

Studies on the effects of salinity on AM fungal spores showed inhibition of germination and hyphal growth by increasing concentrations of NaCl (Hirrel 1981; Estaun 1989; Juniper and Abbott 1993, 2006; McMillen et al. 1998). However, Koske et al. (1996) reported on the ability of *G. gigantea* spores to retain germinability upon exposure to natural conditions of immersion in sea water and stressed the importance of this feature for the dispersal of the species in coastal waters.

A range of organic substrates such as glucose, fructose, sucrose, L-arabinose and aspartic, succinic, malic, pyruvic acids, reduced germination and germ tube growth of *G. mosseae* spores (Siqueira et al. 1982). Accordingly, their germination was inhibited by excess nutrients, such as those provided by Potato Dextrose or Nutrient Broth Agars (Daniels and Graham 1976). However, hyphal growth of *G. mosseae* was stimulated by tartaric acid (Mosse 1959), and peptone, yeast extract, thiamine, cystine, glycine and lysine showed great growth promoting effects on *G. caledonium* hyphae (Hepper 1979; Hepper and Jakobsen 1983). Recent findings also reported that a growth stimulant isolated from the brown alga *Laminaria japonica* increased hyphal growth of *G. margarita* sterile spores germinated in vitro (Kuwada et al. 2006).

3.5 Host/Non Host Plants

Early germination trials showed that AMF are able to germinate in axenic culture in the absence of the host (Godfrey 1957; Mosse 1959; Hepper and Smith 1976; Powell 1976; Koske 1981a). Thus, host-derived signals do not represent essential factors for spore germination of AMF. Accordingly, the presence of growing host roots did not trigger the relief of spore dormancy in different AM fungal species (Tommerup 1983a). Nevertheless, host roots, crude or purified root exudates and compounds derived by their fractioning positively affected spore germination and germling growth in different experimental conditions, depending on both plant and AM fungal species (Graham 1982; Bécard and Piché 1989; Gianinazzi-Pearson et al. 1989; Nair et al. 1991; Tsai and Phillips 1991; Giovannetti et al. 1993a, 1994, 1996; Suriyapperuma and Koske 1995; Tawaraya et al. 1996b; Buée et al. 2000; Nagahashi and Douds 2000; Scervino et al. 2006). The key compounds exuded from host roots able to induce hyphal branching in AMF, strigolactones, stimulated spore germination in *Gigaspora rosea, G. intraradices* and *Glomus claroideum*, and increased mitochondrial density and respiration in *G. intraradices* (Tamasloukht et al. 2003; Besserer et al. 2006). Modulation of AM fungal spore germination was also reported in the presence of exudates of mycorrhizal and non mycorrhizal host roots and of their differential flavonoid components, which showed species-specific effects (Scervino et al. 2005a, b). Detailed information on fungal responses to host-derived signals is given later in this volume (Chapters 2 and 4).

It is interesting to note that transgenic plants may or may not affect AMF life cycle, since the experimental works showed different results depending on the type of genetic modification and gene product expressed. Elfstrand et al. (2005) reported that the constitutive 35S-driven expression of *Mtchit* 3-3, a class III chitinase gene

in *Medicago truncatula* root-organ cultures, was associated with stimulation of spore germination of *G. intraradices* and *Glomus constrictum*, suggesting that the *Mtchit* 3-3 gene product might directly act on the walls of AM fungal spores. Actually, one gene belonging to class III chitinases was specifically induced in mycorrhizal *M. truncatula* (Salzer et al. 2000; Bonanomi et al. 2001).

Root exudates of both non host and ectomycorrhizal plants often showed no effects on spore germination (Daniels and Trappe 1980; Azcón and Ocampo 1984; El-Atrach et al. 1989; Gianinazzi-Pearson et al. 1989). Nevertheless, contradictory fungal behaviours were reported, in in vitro and in vivo experiments (Ocampo et al. 1980; Glenn et al. 1985; Parra-Garcia et al. 1992; Schreiner and Koide 1993a, b; Giovannetti and Sbrana 1998). The release of inhibitory compounds by non-hosts was reported by different authors (Vierheilig et al. 2000; Roberts and Anderson 2001; Oba et al. 2002; Bainard et al. 2009), and a heat-labile factor able to reduce *G. gigantea* and *G. intraradices* germination and growth was detected in root exudates of a non mycorrhizal tomato mutant (David-Schwartz et al. 2001, 2003; Gadkar et al. 2003).

3.6   Microorganisms

Although several species of AMF germinate well in axenic culture, some growth stimulation by soil and rhizosphere microorganisms has been reported (Mosse 1959; Watrud et al. 1978; Daniels and Trappe 1980; Azcón-Aguilar et al. 1986; Azcón 1987, 1989; Gryndler et al. 2000; Scervino et al. 2008; Pivato et al. 2009). The mechanisms of such activity remain unknown. Many laboratory experiments indicated that different bacterial isolates may affect spore germination and hyphal extension. For example, *Streptomyces orientalis* stimulated germination of *G. mosseae* (Mugnier and Mosse 1987), diverse field isolates of *Streptomyces* spp. increased germination of *G. margarita* by production of volatile compounds (Carpenter-Boggs et al. 1995; Tylka et al. 1991), and *Klebsiella pneumoniae* increased hyphal extension in *G. deserticola* germlings (Will and Sylvia 1990).

Differential effects of factors released by *Bacillus subtilis*, *Mesorhizobium mediterraneum* and a PGPR strain on *G. mosseae* and *G. rosea* spore germination and growth was reported by Requena et al. (1999). *G. mosseae* spore germination was not affected by bacteria, whereas a fungistatic effect was evidenced in *G. rosea* when challenged with a strain of *B. subtilis*, although such strain was able to induce hyphal growth enhancement in *G. mosseae*.

Several saprophytic fungi isolated from *G. mosseae* sporocarps decreased or did not affect germination of *G. mosseae* spores on water agar (Fracchia et al. 1998). By contrast, the soil fungus *Trichoderma* spp. enhanced the development of mycelium from germinating spores of *G. mosseae* (Calvet et al. 1992). A recent study reported the increase of *G. rosea* hyphal length in the presence of exudates of *Drechslera* sp., a common fungal endophyte isolated by the inner cortical cells of the grass *Lolium multiflorum* (Scervino et al. 2009).

Gram-positive bacteria (*Paenibacillus* spp. and *Bacillus* spp.) were found associated or attached to fungal hyphae (Artursson and Jansson 2003), and among them *Paenibacillus validus* induced the production of new spores of *G. intraradices* grown in plates in dual culture in the absence of the host (Hildebrandt et al. 2002, 2006).

Different taxa of microbes are associated with spores collected from the field, which may remain contaminated even after surface disinfestation procedures (Mayo et al. 1986; Ames et al. 1989; Walley and Germida 1996). Investigations on the role played by such resident microbial populations are very interesting. For example, spore-associated bacteria, including *Pseudomonas* and *Corynebacterium*, enhanced germination of *Glomus versiforme* spores in vitro, confirming that this fungal species germinates best under non-sterile conditions (Mayo et al. 1986). Other bacteria were intimately associated with the outer spore wall of *G. clarum* (Walley and Germida 1996), or embedded in the electron-dense spore wall of *Glomus* species (Filippi et al. 1998; Maia and Kimbrough 1998), confirming previous reports on the occurrence of chitin-decomposing microorganisms in washed, healthy spores of *G. macrocarpum* (Ames et al. 1989). Recent PCR-DGGE analyses showed that bacterial species associated with spores of *Glomus geosporum* and *G. constrictum* belonged to taxonomic groups known to degrade biopolymers (*Cellvibrio, Chondromyces, Flexibacter, Lysobacter*, and *Pseudomonas*) (Roesti et al. 2005), suggesting that such microbes, being able to digest the outer walls of AMF, mainly composed of chitin, may aid spore germination.

In the family *Gigasporaceae*, spores originating from different geographic areas were shown to harbour intracellular symbionts belonging to β-proteobacteria (Bianciotto et al. 2000, 2003), which could possibly affect germination, since an isolate of *G. margarita*, cured of its endobacteria, showed delayed germling growth (Lumini et al. 2007). Actually, previous results showed that germination frequency of *G. decipiens* spores was significantly enhanced by diverse intracellular strains of *Burkholderia vietnamiensis*, but not by *Burkholderia pseudomallei* (Levy et al. 2003).

4 Modes of Spore Germination

Glomeromycotan fungi germinate in different ways depending on the genus. Spores of most *Glomus* species germinate by regrowth from the end of hyphal attachments (Godfrey 1957; Mosse 1959). Many germ tubes may emerge from the old subtending hypha, as in *G. clarum*, or a single one, as in *G. mosseae* and in *G. caledonium*. Some *Glomus* species, such as *Glomus viscosum*, germinate after forming a balloon-shaped swelling at the broken end of the subtending hypha (Godfrey 1957; Walker et al. 1995). By contrast, germ tubes of *Gigaspora*, *Scutellospora* and *Acaulospora* species emerge directly through the spore wall. Though, different germination structures can be formed depending on the genus. A simple structure is produced by *Gigaspora* spores, which germinate after a papillate layer has formed in the inner

part of the spore wall. Light and electron microscopy studies of this mode of germination were performed in *G. margarita* (Becker and Hall 1976; Sward 1981b, c). In other genera inner (or germinal) walls are involved in germination, with the formation of specialised structures usually on the outer surface of the innermost wall. Species of *Scutellospora* develop germination shields (Walker and Sanders 1986) whose morphology has been recently used to taxonomically revise the family *Gigasporaceae* (Oehl et al. 2008). A different structure, described in some species of *Acaulospora* and *Kuklospora*, was termed "germination orb" (Spain 1992) since it differs morphologically from *Scutellospora* shields, while persisting after germination. The cellular events leading to spore germination in *A. laevis* were monitored at the ultrastructural level, by means of sequential sampling of spores incubated in conditions allowing germination (Mosse 1970a, b). Germination structures were described as dense peripheral compartments, containing cytoplasm and many nuclei, from which germ tubes arose and pushed through the outer layers of the spore wall. Some *Pacispora* species are known to develop *Glomus*-like spores with germination structures morphologically similar to *Scutellospora* shields, but differing from them, since they are delicate, deteriorating over time and therefore difficult to discern (Walker et al. 2004; da Silva et al. 2008). Distinctive simpler germination structures occur in spores of *Archaeospora trappei* and some *Ambispora* species (Spain 2003; Spain et al. 2006; Goto et al. 2008).

Multiple germination can be defined as the abilily of fungal spores to germinate several times by producing successive germ tubes when those formed previously are severed from the parent spores (Koske 1981b). This capacity was described in spores of a *Glomus* sp. (Mosse 1959), and later studied in *G. gigantea*, whose spores were able to germinate up to ten times over a period of 50 days, after their germ tubes had been severed (Koske 1981b). Multiple germination may be considered an additional strategy to increase the probability of successful infection of a host root by germinating spores of AMF.

5 Development of Pre-symbiotic Mycelium

After germination, hyphae generally follow a forward, linear growth, with a strong apical dominance and regular, right-angled branches. Hyphae are thick-walled, aseptate, about 5–10 μm wide, and contain many nuclei (Fig. 1a, b).

Cytoplasm and nuclei can be easily observed migrating along two directions in hyphae originating from spores during germination (Mosse 1959). Ultrastructural studies confirmed these early observations, obtaining clear evidence of a swirling motion of the cytoplasm, and suggested redistribution of spore cytoplasm into the germ tube (Sward 1981c). Two-photon fluorescence microscopy and video-enhanced microscopy allowed the detection of nuclei moving along hyphae originating from germinated spores of *G. rosea* and *G. caledonium*, respectively (Bago et al. 1998; Logi et al. 1998). Such movement could be a microtubules (MT)-dependent process, since in *G. mosseae* germlings nuclei were always detected in close association
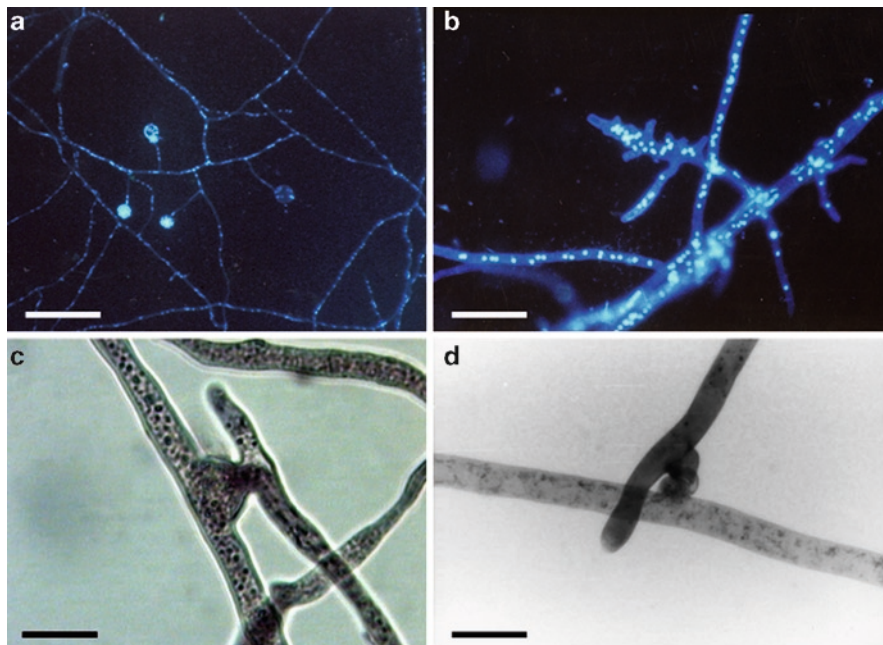
Fig. 1 Micrographs showing differential stainings of mycelium originated by *Glomus mosseae* spores growing in the absence of the host. (**a**, **b**) DAPI-stained mycelium showing nuclear distribution along hyphae and in secondary spores. Scale bars = 130 and 40 μm, respectively; (**c**) Haematoxylin-stained anastomosing hyphae showing protoplasm continuity in the hyphal bridge. Scale bar = 13 μm; (**d**) Succinate dehydrogenase localisation and Trypan blue staining of an incompatible interaction between hyphae belonging to geographically different isolates. Scale bar = 10 μm

with MT, as visualised by indirect immunofluorescence microscopy (Astrom et al. 1994), confirming previous observations on the growth of *G. margarita* germ-tubes (Sward 1981c).

The elongating germ tubes give rise to a mycelial network whose extension is highly variable between individuals. Even when growing in the most suitable media, hyphal growth of AMF is poor. For example, mycelial length in *G. caledonium* reached 30–50 mm after 10–15 days growth on water agar, and the mean growth rate of the mycelium during the early phase was 1.97 ± 0.39 μm/min (Logi et al. 1998). Accordingly, hyphal growth rate in *G. mosseae* growing in the absence of root factors ranged between 1.65 and 2.7 μm/min (Mosse 1959; Giovannetti et al. 1993b). New hyphae of *G. clarum* extended up to 8 mm after 10 days incubation (Louis and Lim 1988). Hyphal length of *G. margarita* after 9 days growth ranged between 18 and 25 mm (Bécard and Piché 1989; Gianinazzi-Pearson et al. 1989), while that of *G. gigantea* reached 54.4 cm after 15 days growth in vitro (Douds et al. 1996).

Studies on transgenic plants designed to constitutively express the insecticidal toxin from *Bacillus thuringiensis* reported diverse effects on hyphal growth of *G. mosseae* germinated sporocarps, which was lower in the presence of *Bt* corn 176

than in the presence of *Bt* 11 or non-transgenic plants. By contrast, hyphal length of *G. mosseae* did not show differences when grown in soil samples containing *Bt* and non-*Bt* plant residues (Turrini et al. 2004a; Castaldini et al. 2005). Root exudates of aubergine plants transformed to express the antimicrobial *Dm*-AMP1 defensin from *Dahlia merckii* did not affect hyphal growth of *G. mosseae*, as compared with non transgenic plants (Turrini et al. 2004b).

Fungal hyphae expanding from the primary mycelium or from branches meet frequently and often fuse, by means of hyphal fusions (anastomoses), when growing on agar or on membranes (Fig. 1c). The occurrence of anastomosis in AMF was mentioned by some authors who did not report any quantitative data on the frequency of hyphal fusions in the different species or on the cytological events involved (Godfrey 1957; Mosse 1959; Tommerup 1988). In 1999 for the first time anastomoses between living hyphae of individually germinated spores of AMF were monitored via a combination of time-lapse and video-enhanced light microscopy, image analysis, and epifluorescence microscopy (Giovannetti et al. 1999) The percentage of contacts leading to anastomosis ranged from 35% to 69% in hyphae from the same germling and from 34% to 90% in hyphae from different germlings of the same isolate of *G. mosseae, G. caledonium, G. intraradices*. By contrast, no anastomoses were detected between hyphae from the same or different germlings of *G. rosea* and *Scutellospora castanea*. Such differential behaviour of AM fungal species belonging to *Glomeraceae* and *Gigasporaceae* families was later confirmed by other authors (de Souza and Declerck 2003; de la Providencia et al. 2005).

Spatiotemporal studies made it possible to monitor anastomosis formation: complete fusion of hyphal walls and the establishment of cytoplasmic flow in the fusion bridge took about 35 min after a hyphal tip showed directed growth towards another hypha, both in *G. caledonium* and in *G. mosseae* mycelia. Protoplasmic continuity, the distinctive mark of true anastomoses, was evidenced by SDH activity in hyphal bridges, where cellular organelles moved at the speed of 1.8 μm/s (Giovannetti et al. 1999). Nuclear migration through fusion bridges suggested that genetic exchange could occur by means of anastomosis between hyphae derived from genetically different individuals. Accordingly, other studies demonstrated that geographically and genetically different *G. mosseae* isolates were unable to fuse (Giovannetti et al. 2003) (Fig. 1d), while genetic exchange occurred, by means of anastomosis, between genetically distinct isolates of one population of *G. intraradices* from the same field (Croll et al. 2009). Such nuclear exchange may represent a fundamental mechanism allowing the maintenance of genetic diversity in AMF, hitherto regarded as ancient asexuals.

6 Biochemical Changes During Germination and Pre-symbiotic Growth

The germination of AM fungal spores is characterized by increased activity of the cytoplasm, involving essential biochemical changes for the switching from a metabolically quiescent state to active metabolism.

Early studies on biochemical events that take place during germination and growth of germlings in *G. caledonium* reported that kinetics of radioactive leucine and uracil incorporation was suggestive of RNA and protein synthesis being operative by 35 min after imbibition (Beilby and Kidby 1982). The response of ungerminated and pregerminated spores to inhibitors of nucleic acid synthesis suggested that the synthesis of mRNA, unnecessary for germination of *G. caledonium* spores, was required for germling growth, and that mitochondrial DNA was synthesized during germination and hyphal growth (Hepper 1979; Beilby 1983). However, production of detectable amounts of ribosomal and mRNAs during imbibition and cold storage was shown in ungerminated spores of *G. rosea* (Franken et al. 1997). Other authors were not able to demonstrate the occurrence of DNA synthesis in vitro, during and after germination of *G. margarita* spores, by using cell cycle inhibitors or direct labelling of nuclear DNA (Burggraaf and Beringer 1989). By contrast, the capability of DNA replication was reported to occur in a small nuclear population of germlings of the same species (Bianciotto and Bonfante 1993). More evidences of DNA replication and transcription during germination and early stage of fungal growth are reported in Section 7.

Protein synthesis was demonstrated to be essential for spore germination and germling growth by studying the effects of the protein synthesis inhibitor cyclohex-imide (Hepper 1979) and later confirmed by using radioactive leucine and the same metabolic inhibitor (Beilby 1983). The early report, based on ^{14}C labeled acetate, that amino acid biosinthetic pathway were operating within 35 min of imbibition in *G. caledonium* (Beilby and Kidby 1982), has been recently confirmed by ^{15}N labeling experiments and gene expression studies, which showed the ability of *G. intraradices* and *G. mosseae* to synthesize aminoacids from endogenous reserves (Breuninger et al. 2004; Gachomo et al. 2009).

A net synthesis of lipids was observed during germination and germ-tube growth of *G. caledonium* spores, with an increase of free fatty acids and polar lipids and a decrease in neutral lipids (Beilby and Kidby 1980). Total lipid content increased from 45% of dry weight in ungerminated spores to 55% and 75% of dry weight in 7 and 14 days old germinated spores, respectively. However, in other experiments, using ^{13}C-labeled substrates and nuclear magnetic resonance spectroscopy, no detectable labeling of lipids was reported (Bago et al. 1999a), suggesting the lack of lipid biosynthesis in *G. intraradices* germinating spores. Later experiments, using ^{13}C labeled glycerol or ^{14}C acetate furtherly supported this hypothesis (Bago et al. 2002b; Trépanier et al. 2005). On the other hand, the occurrence of labeled 18- and 20-carbon fatty acids but not of 16-carbon fatty acids in germinating spores of *G. intraradices* and *G. rosea*, suggested that germlings could elongate and desaturate palmitic acid even in the absence of fatty acid synthase activity (Trépanier et al. 2005).

Other biosynthetic abilities of AMF during spore germination and germling growth have been demonstrated in *G. caledonium* and *G. intraradices*, which were able to synthesize sterols (Beilby and Kidby 1980; Fontaine et al. 2001a, b), as confirmed by the use of sterol biosynthesis inhibitors (Zocco et al. 2008).

The biosynthesis of polyamines, important regulators of fungal growth and differentation (Walters 1995), was studied in *G. mosseae* and *G. rosea* in order to

assess the effects on AMF of polyamine biosynthesis inhibitors used to control plant disease. An increase in polyamines levels was observed after germination in *G. mosseae*, although enhanced germling growth in the presence of exogenous putrescine and spermidine suggested a low, growth limiting level of their endogenous concentrations (El Gachtouli et al. 1996). Interestingly, polyamine biosynthesis seems to occur only via the ornithine decarboxylase in *G. mosseae*, while in *G. rosea* the alternative pathway using arginine decarboxylase was active (Sannazzaro et al. 2004).

As for carbohydrate metabolism, cytochemical studies and isozyme staining performed on spores or germ tubes showed the occurrence of many enzymes of central metabolic pathways such as glycolysis, tricarboxylic acid cycle (TCA), pentose phosphate pathway and gluconeogenesis (Macdonald and Lewis 1978; Hepper et al. 1986; Saito 1995), many of which were operative 35 min after hydration. A rapid increase in spore ATP concentration after 45 min was evidence of the presence of an active respiratory system in *G. caledonium* germlings (Beilby and Kidby 1982).

A thorough survey of biochemical potentiality of germinating spores of *G. intraradices* was performed by using ^{13}C-labeled substrates and nuclear magnetic resonance spectroscopy. The labeling patterns observed were consistent with significant carbon fluxes via various pathways, confirming that gluconeogenesis, TCA, glycolysis, and pentose phosphate pathway are operational in germlings, and supporting the important role played by glyoxylate cycle and non-photosynthetic one-carbon metabolism during germination (Bago et al. 1999a).

Since triacylglycerols (TAG) and free fatty acids may represent a large proportion of AMF spores' weight (Beilby and Kidby 1980; Gaspar et al. 1994), their degradation is central to the process of spores germination and germling growth. Actually, the breakdown of TAG was assessed 5 days after germination in *G. versiforme* spores, probably by an active lipase (Gaspar et al. 1994, 1997).

Ultrastructural data on the movement and disappearance of lipid globules in hyphae originating from germinating spores (Maia and Kimbrough 1998; Bago et al. 2002a, b) support the hypothesis that storage lipids are used to provide precursors for anabolism through glyoxylate pathway and gluconeogenesis, and to fuel respiratory chains by β-oxidation and TCA, as confirmed by labeling experiments (Bago et al. 1999a; Lammers et al. 2001). Such hypothesis was also confirmed by the detection of isocitrate lyase and malate synthase genes involved in the glyoxylate cycle and of an acyl CoA dehydrogenase involved in fatty acid β-oxidation in *G. intraradices* and *G. rosea* spores (Lammers et al. 2001; Bago et al. 2002b).

Interestingly, trehalose was detected in spores of *G. etunicatum* in small quantities, decreasing during the early germination stage, suggesting its role as a source of energy before the start of lipid breakdown (Bécard et al. 1991).

Electron microscope observations of membrane-bound crystals in spores of *G. margarita* and *A. laevis* suggested the occurrence of protein storage material, observed in different stages of apparent breakdown in *G. margarita* germlings (Bonfante et al. 1994; Mosse 1970b; Sward 1981a, b). Native and denatured

protein profiles of *G. mosseae* showed the presence of bands whose intensity decreased during spore germination, supporting the hypothesis of the existence of storage proteins in AM fungal spores (Avio and Giovannetti 1998; Samra et al. 1996). In addition to storage proteins, spores may utilize N stored in the form of aminoacids, especially asparagine, which was present in high concentration in quiescent spores of *G. intraradices* and *G. caledonium* (Beilby and Kidby 1982; Gachomo et al. 2009).

Transmembrane electric potential differences and ion fluxes in AM fungal hyphae showed a generally weak polarization of germ tubes growing in the absence of host derived signals, confirming a basal metabolic activity with low ATP consumption (Berbara et al. 1995; Ayling et al. 2000; Ramos et al. 2008).

In summary, AMF spores possess a large pool of enzymes allowing them to germinate and grow. Though, in the absence of host roots germling growth is arrested, even before depletion of spore reserves (see Section 8), while a boost of metabolism, primarily an increase of respiration (Tamasloukht et al. 2003; Bücking et al. 2008), occurs in the presence of root exudates. Interestingly, analyses of electric potential differences and H^+ ion flux profile in AM fungal hyphae showed a strong influence of host derived signals, which induced ion fluxes enhancement depending on the specific hyphal domains, suggesting a differential activation and distribution of electrogenic H^+-pump isoforms through plasma membrane (Ayling et al. 2000; Ramos et al. 2008).

7 Cytological and Genetic Changes During Germination and Pre-symbiotic Growth

Early evidence of cell cycle activation in AMF growing in the absence of the host was reported by Mosse, who described the development of dense regions containing normal cytoplasm and many dividing nuclei in spores of *A. laevis* prior to germination (Mosse 1970a). Also, Sward (1981b) observed a large number of nuclei with highly condensed chromatin and prominent nucleoli in *G. margarita* spores after 24 h of incubation on water agar. Cytological studies showed that nuclei from quiescent spores of *G. versiforme* were in the GO/G1 phase, whereas nuclei from mycorrhizal roots were in the synthetic and G2/M phases (Bianciotto et al. 1995). Mitotic spindles were also detected in germinated spores of *G. mosseae* by tubulin immunostaining, confirming the occurrence of DNA replication during pre-symbiotic growth (Requena et al. 2000). In the latter work, the gene *Gm*TOR2, encoding a protein with high homology to the C terminus of *Saccharomyces cerevisiae* TOR2 (controlling cell cycle), was characterised. Under treatment with the anti-inflammatory drug rapamycin, which interferes with TOR2 by arresting *S. cerevisiae* cell cycle in G1 phase, *G. mosseae* spore germination was unaffected, whereas hyphal growth decreased, suggesting that nuclear replication in the pre-symbiotic stage is only necessary for hyphal growth (Requena et al. 2000).

EST sequencing from germinated spores of *G. intraradices* and *G. rosea* revealed putative homologues to cell cycle and meiosis-specific genes from other fungi, such as chromatin assembly factor, ubiquitin-encoding genes (Stommel et al. 2001) and *Neurospora crassa* NDT80, known to control exit from pachytene phase of meiosis (Jun et al. 2002). Furthermore, a putative gene involved in the biosynthesis of new nucleotides was detected in germinated spores of *G. intraradices* (Jun et al. 2002).

The occurrence of nuclear division was inferred in non symbiotic mycelium by using image analysis counts of the number of nuclei (Bécard and Pfeffer 1993), which decreased from 2,000 to 800 in individual spores during the early days of germination, suggesting the migration of nuclei from spores to hyphae. This was confirmed by data on the occurrence of cytoskeletal components, both microtubules and microfilaments, in the mycelium originating from germinating spores of *G. mosseae* and *G. caledonium* (Astrom et al. 1994; Logi et al. 1998). The presence of such components is consistent with the role of cytoskeleton in the migration of nuclei and cellular organelles during active growth. Expression of β-tubulins in germinating AM fungal spores (Franken et al. 1997; Butehorn et al. 1999) was confirmed by the detection of sequences putatively encoding other cytoskeletal proteins, such as α-tubulin, β-actin, dynein and actin-related protein, possibly involved in nuclear and nutrient movements, in *G. intraradices* germinated spores (Jun et al. 2002). Recently, full-length β-tubulin gene has been sequenced from *G. gigantea* and *G. clarum*, showing some peculiar traits compared to fungi other than glomeromycota (Msiska and Morton 2009).

Nuclear division in *G. rosea* hyphae was also detected in the presence of host root exudates or of the synthetic strigolactone GR24, which induced an accumulation of nuclei in the apical area of treated hyphae (Buée et al. 2000; Besserer et al. 2008).

Early experiments showed that inhibitors of mRNA translation hindered AM fungal spore germination (Hepper 1979; Beilby 1983). Accordingly, differential display analysis of *G. rosea* did not show changes in RNA accumulation patterns during hyphal development, suggesting that in this phase proteins are produced only by translating transcripts synthesized prior and during spore germination (Franken et al. 2000).

Many expressed genes detected in germinating AM fungal spores showed homology to those encoding for proteins involved in translation, protein processing, primary metabolism and transport processes (Franken et al. 1997; Lammers et al. 2001; Stommel et al. 2001; Jun et al. 2002; Bago et al. 2002a, 2003). The identification of genes putatively codifying for several enzymes involved in carbon metabolism and lipid breakdown often confirmed biochemical data.

An interesting gene, *G. mosseae Gm*GIN1, was highly and specifically expressed in non symbiotic mycelium, whereas it was silenced during the symbiosis, both in the intraradical structures and the extraradical mycelium (Requena et al. 2002). Interestingly, several genes with homology to the N-terminus of *Gm*GIN1, sequenced from *Magnaporthe grisea*, *N. crassa*, *Gibberella zeae* and *Aspergillus nidulans*, encode for a family of proteins playing an essential role in polarized growth, septal formation and hyphal morphological changes in the phytopathogenic

fungus *Ustilago maydis* and in the ectomycorrhizal fungus *Suillus bovinus* (Gorfer et al. 2001; Weinzierl et al. 2002).

A 14-3-3 protein encoding gene, known to be involved in modulation of cell ion pumps and channels, was detected in *G. intraradices* mycelium (Porcel et al. 2006). This finding suggests an important role of this gene in controlling the activity of P-type H^+-ATPases, detected in *G. intraradices* and *G. mosseae* (Requena et al. 2003; Corradi and Sanders 2006), which are responsible of the maintenance of hyphal ionic gradient during polarized growth (Ramos et al. 2008).

Interestingly, a sequence showing strong similarity to an endonuclease involved in lateral transfer of an rDNA intron has been detected in *G. intraradices* germinated spores, suggesting the occurrence of lateral gene transfer during nuclear exchange between anastomosing hyphae belonging to genetically different AMF (Jun et al. 2002; Croll et al. 2009).

Induction of genes encoding for putative pyruvate carboxylase and mitochondrial ADP/ATP translocase, involved in respiration enhancement activity, has been observed in *G. rosea* and *G. intraradices* during early responses to host root factors, before hyphal branching (Tamasloukht et al. 2003, 2007). The expression of the former gene could explain the stimulatory effects exerted by CO_2 on AM fungal growth (Bécard and Piché 1989), whereas the expression of the latter gene could be necessary for the delivery of large quantity of ATP produced at high respiration rates (Requena et al. 2003). Activation of such genes and oxygen consumption were induced by host root exudates after 0.5–3 h, when no morphological change in hyphal growth pattern was detectable yet. On the contrary, no differences in the expression of key metabolic genes during the first 48 h after strigolactone analogue GR24 treatment were observed in *G. rosea*, which showed strong enhancement in transcript levels after 2 days of incubation, independently of GR24 treatment (Besserer et al. 2008). These findings suggest that other unknown signal molecules may be active and that strigolactone-induced mitochondrial activity is due to post-translational regulation of key enzymes (Delano-Frier and Tejeda-Sartorius 2008; Rani et al. 2008). The need of host-derived signals for developmental stages following spore germination can be inferred by results obtained with the *pmi* mutants of *Solanum lycopersicum*, which are regularly colonised by extraradical mycelium and mycorrhizal roots but are not susceptible to colonisation by hyphal germlings (David-Schwartz et al. 2001, 2003).

AM fungal spores germinating in the absence of host-derived factors constitutively release unknown compounds which are perceived as signals by host plants and are able to elicit recognition responses, such as a transient cytoplasmic calcium induction in soybean cells (Navazio et al. 2007) and the accumulation of starch in *Lotus japonicus* roots (Gutjahr et al. 2009). Ca^{2+}-mediated signaling was also suggested by expression of genes involved in Ca^{2+}-mediated signal transduction in *M. truncatula* roots in the presence of a diffusible factor released by *G. mosseae* (Weidmann et al. 2004). Previous studies had reported the release of a diffusible signal by *G. mosseae, G. rosea, G. gigantea, G. margarita* and *G. intraradices* growing in the presence of host plants (Chabaud et al. 2002; Kosuta et al. 2003). The perception of such signals by *M. truncatula* induced root expression of the early nodulin gene

*Mt*ENOD11, which was related, both spatially and temporally, with the appearance of hyphal branching enhancement. Moreover, factors released by *G. margarita* and *G. intraradices* mycelium growing nearby *M. truncatula* plant roots were able to induce lateral root formation (Olah et al. 2005) and those released by *G. intraradices* branching hyphae elicited root calcium-spiking responses (Kosuta et al. 2008). No information is still available on the chemical nature of AM fungal factor(s).

Although many studies reported germling growth improvement by different microorganisms, little is known about the molecular mechanisms of such phenomenon. Changes in AM fungal gene expression in response to the perception of microbial derived factors were detected by Requena et al. (1999) during co-culture of *G. mosseae* with a strain of the rhizobacterium *B. subtilis*, inducing mycelial growth increases. In particular, down-regulation of the putative gene *Gm*FOX2, encoding a protein involved in long-chain fatty acids catabolism, was evidenced. It is not known which is the signaling pattern between bacteria and fungi, although it has been hypothesized that an increase in fungal cAMP, due to the perception of flavonoid/estrogen bacterial signals, could be responsible for the glucose repression stage that down-regulates *Gm*FOX2 (Requena et al. 1999).

8 Growth Arrest in the Absence of the Host

Although spores of AMF are able to germinate in vitro in response to different edaphic and environmental conditions, they are not capable of extensive independent hyphal growth, and, in the absence of the host, germlings cease growth within 8–20 days (Mosse 1959; Daniels and Graham 1976; Beilby and Kidby 1980; Koske 1981a; Hepper 1984b; Bécard and Piché 1989; Giovannetti et al. 1993b; Schreiner and Koide 1993b; Logi et al. 1998) (Fig. 2).

Microchambers allowing continuous observation of living mycelium over a period of several hours, showed that when no host-derived signals from the surrounding environment were perceived by *G. caledonium* and *G. rosea* germlings, hyphae entered a state of developmental arrest. Cytoplasm, nuclei and cellular organelles were retracted from the tips and from peripheral hyphae and retraction septa were produced, separating viable from empty hyphal segments (Logi et al. 1998). In vivo two-photon microscopy, carried out on *G. rosea* germlings, showed differences in the organization and distribution of nuclei between actively growing hyphae and those undergoing septation (Bago et al. 1998, 1999b). Protoplasmic flow rate, measured in actively growing germlings on the basis of the movement of cell particles – nuclei, small vacuoles, mitochondria, fat droplets, tiny organelles – ranged from 2.98 to 4.27 μm/s in living hyphae of *G. caledonium* (Giovannetti et al. 2000). Microchambers and two-photon microscopy studies revealed that neither protoplasm streaming nor nuclear movements occurred in protoplasm-retracting hyphae and that progressively enlarged vacuoles led to the formation of empty areas where a cross wall was eventually formed (Bago et al. 1998, 1999b; Giovannetti et al. 2000) (Fig. 3a, b).
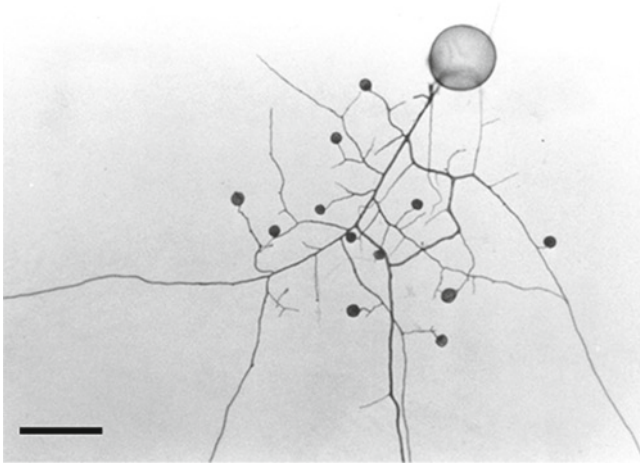
Fig. 2 Micrograph showing the limited growth of a *Glomus mosseae* spore in the absence of host derived signals. Scale bar = 240 μm
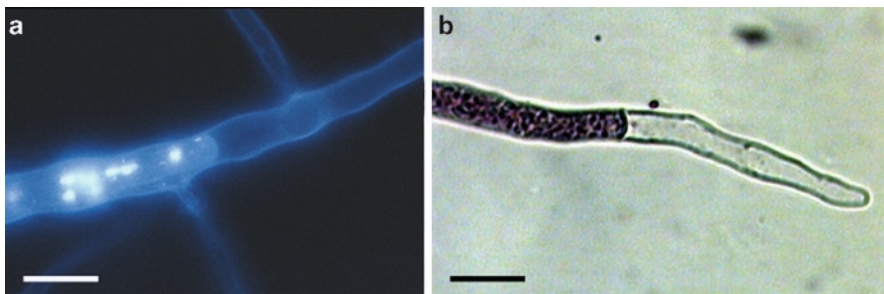
Fig. 3 Micrographs showing protoplasm retraction during growth arrest in hyphae originating from *Glomus mosseae* spores. (**a**) DAPI staining, evidencing nuclar occurrence in retracting protoplasm. Scale bar = 7 μm; (**b**) Haematoxylin staining showing a viable hyphal compartment below a septum isolating the empty hyphal tip. Scale bar = 10 μm

Metabolic activity was still detectable in *G. caledonium* 6-month-old hyphae proximal to the mother spore, which was able to retain infectivity, suggesting that such resource reallocation is functional to long-term maintenance of viability, allowing survival of fungal propagules in the absence of host plants (Logi et al. 1998; Giovannetti et al. 2000).

The reasons for such behaviour have been investigated with the aim of determining whether vital metabolic pathways may be blocked. The main results have been considered earlier in this chapter (see Section 6), and they indicate that germinating spores do possess the metabolic machinery for hyphal growth and that spore reserves are not totally depleted during germling growth (Hepper 1979; Beilby and Kidby 1980; Koske 1981b). Germinating AM fungal spores showed low respiratory

activity and reduced resource utilization, allowing limited biosynthesis, whereas higher respiration rates and use of C sources, sustaining growth and morphogenesis, were detected after the perception of host root factors. Respiratory metabolism seems a suitable control target for non symbiotic growth arrest, which has been suggested to represent a strategic mechanism preventing spore reserves consumption in the absence of host-regulated germination.

9   Concluding Remarks

Several survival strategies are supposed to have affected the evolutionary history of AMF, allowing them to overcome their obligate biotrophic status. The first survival strategy is represented by the wide host range – ~80% of land plant species –, which increases the possibility of individually germinated spores to come into contact and colonise host roots: such strategy, relying wholly on chance, appears a weak explanation for 460 million years continued existence. A second evolutionary mechanism allows the survival of spores germinated in the absence of host roots by mycelial growth arrest, which is accompanied by peripheral protoplasm withdrawal and resource reallocation towards mother spores, functional to retaining long-term colonisation ability. Challenges remain concerning factors triggering the onset of growth arrest and the molecular mechanisms involved. Further energy-saving mechanisms allow the unequivocal discrimination of host from non host roots, since AM fungal hyphae undergo a biochemical switch and a distinctive pattern of hyphal morphogenesis only after perceiving host-derived signals. Recently, we obtained data on the ability of AMF germlings to plug into a compatible mycorrhizal mycelium by means of anastomoses, thus gaining access to plant-derived carbon before undergoing growth arrest, enhancing their survival chances. The ability of AM fungal mycelium to form anastomosis and to discriminate self from nonself may represent a fundamental additional survival strategy. These strategies may compensate for the lack of host-regulated spore germination, an apparently inconsistent behaviour for obligate symbionts, and contribute to the survival of individuals and populations of AMF.

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Chapter 2
Functional Categories of Root Exudate Compounds and their Relevance to AM Fungal Growth

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Abstract It is well established that plants grown under limited phosphorus (Pi) conditions are more readily colonized by arbuscular mycorrhizal (AM) fungi. It is also known that certain components of host root exudates can stimulate hyphal growth and branching of AM fungi and these compounds are elevated when the host is grown under Pi stress. To obtain a more general picture of the types of compounds exuded by host roots that effect the growth of AM fungi, a global analysis was performed on crude exudates of Ri TDNA-transformed carrot roots grown in the presence and absence of Pi. The results show that there is a distinct population of exudate compounds that are elevated in the absence versus the presence of Pi. Putative identifications were made for some of these compounds from data obtained by Fourier Transform Ion Cyclotron Mass Spectrometry (FTMS). The results were then compared to components of biologically active fractions purified by two dimensional thin layer chromatography (2D TLC). The data selection was restricted to compounds that were initially identified to be elevated in the crude – Pi exudate. The categories of compounds, such as plant growth regulators, phenols, flavonoids, and sesquiterpenoids were selected that had the most relevance to AM fungal/host interactions. The previous results with some of these compounds will be discussed in relation to new results obtained from FTMS and their involvement in presymbiotic growth of AM fungi. None of the reported strigolactones which stimulate AM fungi hyphal were found although several sesquiterpene lactones were identified. A number of hydroxy fatty acids were also found, and they were elevated in the – Pi crude exudate and found to be present in 2D TLC fractions. One hydroxy fatty acid, 2-hydroxytetradecanoic acid, stimulated lateral branching of the primary germ tube of *Gigaspora gigantea*, when applied to Petri dishes in amounts as low as 1–10 ng.

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Abbreviations

2 HTDA	2 Hydroxytetradecanoic acid
AMF	Arbuscular mycorrhizal fung
2D-TLC	2 Dimensional thin layer chromatography
FTMS	Fourier Transform Ion Cyclotron Mass Spectroscopy
2 HHDA	2 Hydroxyhexadecanoic acid
Pi	Phosphorus
BF	Branching factor

1   Introduction

We have been studying the life cycle of arbuscular mycorrhizal (AM) fungi which infect about 80% of all land plants. They form a mutualistic symbiosis with host plants whereby the AM fungus extracts mineral nutrients (notably Pi) from the soil and gives them to the plant in exchange for sugar molecules generated from photosynthesis. Metabolites present in the liquid secreted (exudate) by the host plant roots contain constitutive compounds that stimulate the growth/hyphal branching of AM fungi (Elias and Safir 1987; Bécard and Piché 1989; Giovannetti et al. 1993; Nagahashi and Douds 2000). These compounds are elevated in the exudates of plant roots grown under phosphorus (Pi) stress (Nagahashi and Douds 2000; Akiyama et al. 2002; Yoneyama et al. 2007b), and the enhanced exudation of these compounds can increase appressoria formation and therefore enhance the colonization of the host root by AM fungi (Tawaraya et al. 1998).

Some of these exuded compounds stimulate elongation growth (Nair et al. 1991; Becard et al. 1992; Scervino et al. 2005; Nagahashi and Douds 2007) which probably allows the fungus to explore the soil at farther distances from the germinated spore. Other compounds stimulate extensive hyphal branching (Giovannetti et al. 1993; Buee et al. 2000; Nagahashi and Douds 2000) and have been referred to as branching factors (BFs). These compounds are most effective near or at the root surface and increase the chances for the fungus to come in physical contact to form appressoria in the cell wall grooves between epidermal cells. Recently, a BF has been identified in *Lotus japonicus* (Akiyama et al. 2005) as 5-deoxy-strigol. Two other strigolactones, strigol from *Menispermum dauricum* root culture (Yasuda et al. 2003) and sorgolactone from *Sorghum* exudates (Besserer et al. 2006) were also shown to be BFs (Akiyama et al. 2005). Two of these types of compounds, orobanchol and 5-deoxystrigol (Yoneyama et al. 2007a, b) are elevated in the exudates of red clover and sorghum when the host plants were grown under Pi deficiency.

Earlier work has shown that there are multiple compounds or categories of compounds in carrot root exudates that can stimulate the growth and branching of AM fungi (Nagahashi and Douds 2000). Because of the potential for finding other categories of compounds, in addition to strigolactones, the exudates from Ri T-DNA transformed carrot roots grown in liquid culture (Nagahashi and Douds 2000) were examined in greater detail. We were particularly interested in compounds, involved in the presymbiotic events of the AM fungal/host root interactions, that are elevated in exudates of carrot roots grown in the absence of Pi. A list of these compounds could then be used to construct a library which should contain various hyphal growth/branching stimulators. These compounds, if available, could be used for initial testing in a bioassay (Nagahashi and Douds 1999) and will form a basis for future confirmation and identification of any particular compound. Some of the compounds identified here were grouped into functional categories that may have the most relevance to fungal or AM fungal growth and development.

2 Fourier Transform Ion Cyclotron Mass Spectroscopy

Historically, one of the earlier methods used to obtain a metabolic fingerprint was two dimensional thin layer chromatography (2-DTLC) (Tweeddale et al. 1996) and this was followed with a better separation of metabolites by HPLC (Walker et al. 2003b). A recent form of mass spectrometry, Fourier transform-ion cyclotron mass spectroscopy (FTMS), is known to have the greatest resolution (>100,000) and highest mass accuracy (<1 ppm) compared to other forms of separation such as HPLC or gas chromatographic mass spectrometry (Roessner et al. 2001). This was demonstrated by finding over 3,000 metabolites in plant tissues generated during cellular metabolism (Aharoni et al. 2002). The resolving power of FTMS is very high so it was used to further separate components that were initially purified by 2D-TLC. The FTMS analyses and internal calibration for mass accuracy were the same as reported earlier (Mungur et al. 2005).

Components of the metabolome can be detected directly (Tweeddale et al. 1996; Aharoni et al. 2002; Sumner et al. 2003) and then identified. These components can be thought of as the end products of gene expression and help define the biochemical phenotype of a cell or plant tissue (Fiehn 2002). Quantitative and qualitative analyses of large numbers of metabolites can provide a broad view of the biochemical status of an organism that can be used to monitor and assess gene function (Fiehn et al. 2000). Plant responses to environmental conditions may involve altered gene expression which result in qualitative changes in metabolic pools and therefore qualitative identification of the metabolites is critical (Zulak et al. 2007).

A systematic study to determine the chemical composition and complexity of a root exudate has not been performed (Walker et al. 2003a). We have used FTMS to analyze the metabolites present in the exudates from carrot roots grown in the
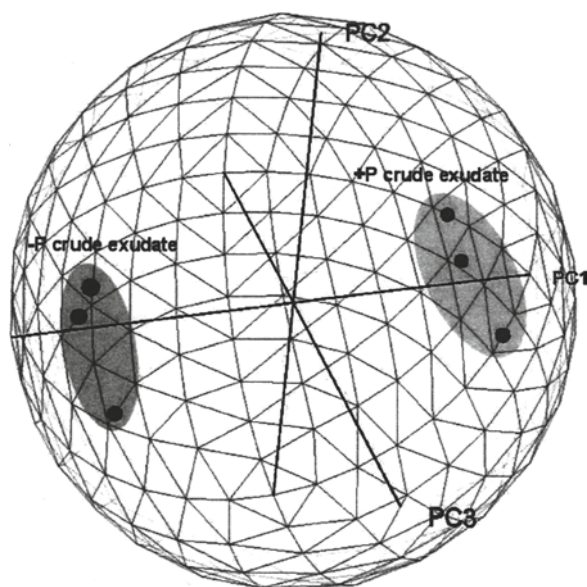
Fig. 1 Global principle component analysis (PCA) of crude exudates from Ri T-DNA transformed carrot roots grown in the presence and absence of 35 μM phosphorus (Pi). PCA is a statistical method for visualizing the variation within the high dimensional data and was performed by using all observed metabolites in the crude exudates. Three separate pairs of samples were analyzed per treatment and the results clearly show global metabolite differences between components in the –Pi versus +Pi exudate

presence and absence of Pi (all details on the initial separation of crude exudate fractions, fungal spore isolation, roots grown in liquid culture, and bioassays were reported earlier by Nagahashi and Douds 1999, 2000). The analyses were performed on three separate isolations, and it was apparent that there was a sub-population of compounds that were elevated during Pi stress (relative to the control) and a cluster of compounds that were higher in the control (compared to Pi stress) as shown in Fig. 1 (principle component analyses). The scatter plot distribution further indicates that the majority of compounds have a molecular mass less than 400 (Fig. 2). Data was selectively mined to build a library of compounds that were elevated under –Pi since this group would likely contain elevated concentrations of hyphal growth/branching stimulators (Nagahashi and Douds 2000). Compounds that were detected in fractions purified by 2D TLC (Table 1) and further separated by FTMS were then compared to those elevated under Pi stress in the crude exudates. The 2D fractions analyzed contained hyphal growth/branching factors as had been reported previously (Nagahashi and Douds 2000).

The molecular formula assigned to an empirical mass in the dataset was based on a mass variance of <3 ppm for target metabolites (most compounds were <0.2 ppm), putative adducts, and the percent natural abundance of elements (C^{13} isotope). The exact masses alone were not sufficient to unequivocally identify a specific
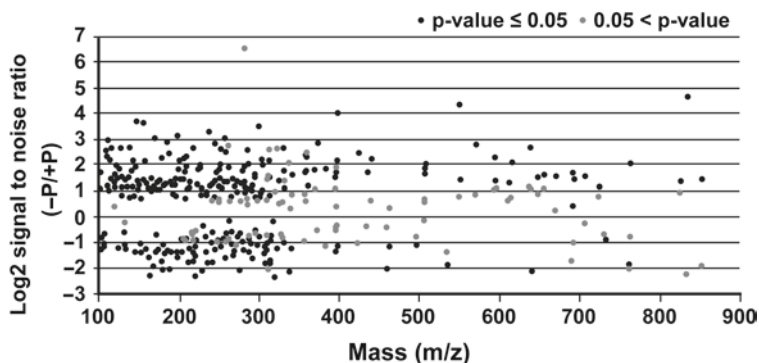
Fig. 2 The scatter plot distribution of metabolites in the crude exudates that are altered in relative abundance between –Pi and +Pi treatments. Comparisons of the average levels of the detected masses based on absolute intensities of –Pi and +Pi crude fractions were conducted using the Student t-test. The average relative intensity log 2 ratios, p-values from absolute intensity comparisons, and detected masses (neutral mass) were plotted. The data also indicates that the majority of compounds have molecular masses less than 400

Table 1 TLC separation and hyphal branching activity of fractions semipurified from carrot root exudate

Fraction #	RF value	Fluorescence	Biological activity
7	0.90	Bright blue	+2
6	0.78	Faint blue	+3/+4
3	0.68	Faint (sits on top of 2)	+3/+4
2	0.63	Faint blue	+2
5	0.57	Bright blue	+3
4	0.48	Faint blue	+2
8	0.35	Faint blue	No activity
1	0.19	Bright blue	+3

The one dimensional TLC fraction numbers, along with the RF values, are given in the order in which they were first isolated and tested for biological activity. The biological activity was scored in a hyphal branching bioassay with *Gigaspora gigantea* (0 = to controls; +4 = most complex) as reported earlier (Nagahashi and Douds 2000). All active fractions fluoresced (Nagahashi and Douds 2000) and this was used to monitor further separation by 2D-TLC where all isolated fractions were each run separately in the second dimension.

compound since there may be several isomers of the same exact mass and molecular formula. Putative identifications were made by searching four databases, ChemFinder, CHEMnetBASE, PubChem Compound, and Kegg Phytochemical Compounds, for known plant associated compounds or intermediates of plant secondary metabolic pathways, likely components of general root functions, or likely root associated compounds that have been reported to be attractants for root parasites or stimulators of fungal growth.

3 Plant Growth Regulators During AM Fungal/Host Interactions

Although plant hormones normally function in plant development, they may also play a significant role in plant symbiotic associations (Frankenberger and Arshad 1995; Hause et al. 2007). Their role in initial signaling events or precolonization events is not known and their precise role during colonization events is still unclear (Ludwig-Muller 2000; Jentschel et al. 2007). Most studies have focused on application experiments or determination of phytohormone levels in infected roots versus uninfected controls during the early and later stages of colonization (Hause et al. 2007; Jentschel et al. 2007). The work with auxins has shown an improved colonization of host roots with the exogenous addition of auxins to host roots (Dutra et al. 1996). The changes in the levels of various auxins occur during early and later stages of colonization (Shaul-Keinan et al. 2002; Jentschel et al. 2007) but the increase or decrease depended on the type of auxin that was analysed.

Exogenously applied gibberellic acid (GA3) at 10^{-7} M to *Pisum sativum* seedlings (El Ghachtouli et al. 1996) showed selective inhibition of arbuscule formation in *G. mosseae* but not of other infection parameters of the root. At 10^{-5} M, there was a complete inhibition of colonization (El Ghachtouli et al. 1996). On the other hand, certain changes in the plant hormonal balance have been related to a mycorrhizal effect. The colonization of roots of *Bouteloua gracilis* resulted in an increase in gibberellic acid (GA)-like activity in the leaves with a significant decrease of levels in roots (Allen et al. 1982). In contrast, infected roots of *Agropyron thrachycaulum* had more GAs than uninfected controls (El Ghachtouli et al. 1996). In addition, it was shown that mycorrhizal plants exude more gibberellins than non-mycorrhizal plants (Mada and Bagyaraj 1993).

The growth difference between a colonized and an uncolonized host plant is largely due to the improved Pi nutrition of the infected plant, but it may also be related to the ability of the fungus to synthesize or induce the synthesis of plant hormones (Ludwig-Muller 2000; Barea and Azcon-Aguilar 1982). Alternatively, phosphorus stress itself may increase the levels of hormones of an uninfected host and consistent with this observation is that root ABA levels are elevated during drought stress and ABA may be necessary for a sustained colonization by AM fungi under this condition (Fester and Hause 2007).

When carrot roots were grown in liquid culture, many of the gibberellins, their metabolic intermediates, and abscisic acid (ABA) were elevated under – Pi conditions and were found in various fractions purified by 2-D TLC (Table 2). Some of these compounds were commercially available and were tested in a bioassay using *Gigaspora gigantea* as the test organism to determine if they had a role in hyphal growth or hyphal branching. Over 120 isomers/derivatives of gibberellins/gibberellic acid have been identified, and in carrot root exudates, GA12 and GA53 were elevated the most under – Pi (Table 2) with the GA synthesis intermediate, ent-kaurenoic acid, elevated the highest. Most of the tentatively identified gibberellins or potential intermediates of gibberellin synthesis were not commercially available and could not be

Table 2 Plant related growth regulators and related metabolites found in carrot root exudate

FTMS (neutral)	TM	Error (ppm)	Adduct	Mode	Predicted formula	Compound	TLC fraction	Crude exudates			P value
								-P	+P	-P/+P	
386.1342	364.1522	0.13	Na	pESI	$C_{19}H_{24}O_7$	Gibberellin 8	2D-7	7.74	24.60	0.3	0.004
384.1549	362.1729	0.03	Na	pESI	$C_{20}H_{26}O_6$	Gibberellin A19	2D-1	19.37	16.97	1.2	0.400
370.1758	348.1937	0.51	Na	pESI	$C_{20}H_{28}O_5$	Gibberellin 53	2D-4	10.21	5.20	1.9	0.390
368.2199	368.2199	0.00	Parent	nESI	$C_{20}H_{32}O_6$	11,15,16,17-Tetrahydro-xy-19-kauranoic acid	2D-1	16.45	6.95	2.4	0.011
364.1886	364.1881	1.34	Parent	nESI	$C_{20}H_{28}O_6$	Gibberellin 44 (diacid)	2D-7	6.37	3.95	1.6	0.410
336.2298	336.2301	0.59	Parent	nESI	$C_{20}H_{32}O_4$	1,19-Dihydroxy-17-kauranoic acid	2D-1	144.30	27.10	5.3	0.005
333.2303	316.2038	0.06	NH_4	pESI	$C_{20}H_{28}O_3$	Gibberellin 12 (aldehyde)	2D-7	21.20	4.62	4.6	0.001
304.2402	304.2402	0.00	Parent	pESI	$C_{20}H_{32}O_2$	ent-Kaurenoic acid, ME	2D-1	11.13	2.98	3.7	0.020
280.1308	280.1311	0.98	Parent	nESI	$C_{15}H_{20}O_5$	Abscisic acid; 13-hydroxy/phaseic acid	2D-6	161.31	27.00	5.9	0.001
264.1362	264.1361	0.22	Parent	nESI	$C_{15}H_{20}O_4$	Abscisate	2D-7	44.28	24.00	1.9	0.072

For this table and all subsequent tables, the following all apply: Exudates were collected from carrot roots grown in liquid culture with or without 30 μM Pi. The average crude exudate intensity ratio (–Pi/+Pi) and the significant difference (ANOVA) between the two variables is indicated by the p value. Each mode of FTMS analysis was calibrated to internal standards to ensure ultra-high mass accuracy prior to the peak selection process. All errors are less than 3 ppm and most are less than 0.2 ppm. To recover enough material in separated fractions to test for hyphal branching activity and perform FTMS analyses, TLC fractions were obtained by combining six separate crude preparations before TLC. The 2D fraction listed for any given compound contained the highest intensity for that compound. Mass detected = FTMS; Theoretical mass = TM; Mode = mode of detection which is either positive (p) or negative (n) electrospray ionization (ESI). The putative identification of each compound is listed.

tested. The two commercially available gibberellins, gibberellin A4 and gibberellic acid (GA3) were tested but neither compound promoted nor inhibited hyphal branching between 1 ng and 1 μg (data not shown). In the bioassay, abscisic acid (1 ng to 1 μg) had no effect on hyphal growth/branching of *Gigaspora gigantea*. Although the hormones tested did not induce a presymbiotic change in the morphology of hyphae of germinated AM fungal spores, they could conceivably trigger some metabolic event that may be necessary for further steps in colonization process.

4 Compounds That Stimulate Elongation Growth of AM Fungi

Since flavonoids can act as signaling molecules during the nodulation of legumes which involves gene induction, chemoattraction, and growth stimulation for *Rhizobium* (Hirsch and Kapulnik 1998) and have been reported to induce germination of basidiospores of an ectomycorrhizal fungus (Kikuchi et al. 2007), their role in AM fungal/root interactions has been of great interest. Recently, flavonoids have been shown to influence root colonization by *Glomus* and *Gigaspora* species. This was determined by correlating the number of entry points and the percentage of the root colonized by using sterilized tomato seedlings grown in culture which were then treated with germinated AM fungal spores in the presence or absence of flavonoids (Scervino et al. 2007).

Some results have indicated that certain flavonoids can stimulate hyphal branching while others have little or no effect on branching (Nagahashi and Douds 2000; Scervino et al. 2005). Where branching has been stimulated, no morphological evidence was shown and the type of branching pattern was not discussed (Scervino et al. 2005). Other experiments have indicated that quercetin did not stimulate lateral hyphal branches off the primary germ tube of *Gigaspora species* (Nagahashi and Douds 2000). However, the most consistent and pronounced effect of flavonoids is the presymbiotic stimulation of hyphal length (Bécard et al. 1992; Douds et al. 1996; Nair et al. 1991; Scervino et al. 2005). An early report showed that quercetin not only increased the elongation growth but also prolonged the duration of presymbiotic growth (Bécard et al. 1992). Depending on the AM fungal species and the flavonoid tested, the stimulation of growth of AM fungal hyphae (Bécard et al. 1992; Scervino et al. 2005) can vary. Flavonoids are found in the exudates of leguminous crops (Isobe et al. 2001) and the isoflavonoids, biochanin A and formononetin, have been reported in clover exudates (Nair et al. 1991) and they stimulated the hyphal growth of *Glomus* sp (INVAM-112). A recent report (Scervino et al. 2005) has looked at the growth of various AM fungi in the presence and absence of six different commercially available flavonoids and they reported that different flavonoids can induce a fungal species effect and even a genus specific effect.

However, the dependence of flavonoids in AM fungus/host interactions has been challenged since one report showed that mutant maize plants, without chalcone synthase activity, can still be colonized even though they couldn't make flavonoids (Bécard et al. 1995). Carrot root exudates were reported to contain various

flavonoids (Bel Rhid et al. 1993) but subsequent work did not confirm this result (Bécard et al. 1995). These results aside, it is still apparent that flavonoids, such as quercetin, have a pronounced effect on the growth of some AM fungi (Bécard et al. 1992) but not others (Bécard et al. 1992; Douds et al. 1996). Whether there is a species specific AM fungal response to a particular compound in a host root exudate (Scervino et al. 2005) or a preferential response to a specific compound(s), when a mixture of chemical signals is present, remains to be determined. The absence of flavonoids, in a particular host root exudate, does not preclude their role in the early stages of hyphal growth since AM fungi may react to them if they are present and react to other compounds when they are not present.

Our analyses of carrot root exudates by FTMS (Table 3) did not detect quercetin or kaempferol, or any of the other reported flavonoids that stimulated growth of AM fungi (Scervino et al. 2005). The flavonol, apiforol and the flavonoid, apigenin 8-C-glucoside were detected, but were not elevated in the –Pi exudate. Only the glycoside form of apigenin was found in TLC fraction 2D-1 (Table 3), although the aglycone was previously identified in exudates of carrot seedlings (Poulin et al. 1993). In the cases where flavonols stimulate hyphal growth, it has been the aglycone form (Bécard et al. 1992; Poulin et al. 1993; Scervino et al. 2006). Commercially available apigenin has been reported to stimulate AM fungal growth (Scervino et al. 2006), but whether or not the apigenin glycoside we detected can be potentially hydrolyzed to apigenin in the exudate is not known. In contrast, the C-glycosylflavonoid (isovitexin 2′-O-β-glucoside) was elevated in melon roots under Pi stress (Akiyama et al. 2002). When the compound was extracted from the roots, added to a nutrient medium, and then applied to germinated melon seeds in the presence of *Glomus caledonium* spores, an increase in colonization was observed (Akiyama et al. 2002). In this case, it was not determined if the glycosylated form was also found in the exudate since the preparation was a root extract, and it was not determined whether it was involved in precolonization events or after appressoria formation.

Flavonoids come under the general category of phenolics, which in addition to flavonoids, also include terpenoids, and stilbenoids. Some of these compounds were detected in this study (Table 3) and of the compounds tested so far, scopolin, chlorogenic acid (Table 3), and scopoletin (Table 4) did not stimulate hyphal elongation growth or branching of *Gigaspora gigantea* between 10 ng and 1 µg in the microinjection assay. A previous report indicated that abietic acid (1–100 µM) did not stimulate hyphal branching when tested in the microinjection assay with *G. gigantea* (Buee et al. 2000).

Like the flavonoids, another potential category of compounds may also act as a hyphal growth stimulator. A recent report has indicated that a nitrogenous base, 5′-deoxy-5′-methylamino-adenosine, mainly stimulated the elongation growth of the AM fungus *Gigaspora margarita* (Kuwada et al. 2006). The compound was isolated and purified from extracts of the brown alga, *Laminaria japonica*, but it was not detected in the crude extracts or 2D TLC purified fractions that were analyzed in this study. Future confirmation work that shows the presence of this compound in a host exudate, along with its biological activity, will be necessary to determine if this compound or other nitrogenous bases have a role AM fungi/host interactions.

Table 3 Metabolites found in carrot root exudate that were identified and predicted to be involved in the biosynthesis of terpenes, flavonoids, stilbenes, and/or lignin. In some cases, compounds were not detected in either the +Pi crude exudate or both −Pi and +Pi exudates but were detected in 2D fractions. This occurred because many more crude exudate isolations were combined before TLC separation

FTMS (neutral)	TM	Adduct	Error (ppm)	Mode	Predicted formula	Compound	TLC fraction	Crude exudate			
								−P	+P	−P/+P	P value
472.1239	432.1056	C_2H_3N	1.08	pESI	$C_{21}H_{20}O_{10}$	Apigenin 8-C-glucoside/7-O-beta-D-Glucosyl-5,7,4-trihydroxyflavone	2D-1	ND	ND	–	–
360.1920	338.2100	Na	0.96	pESI	$C_{19}H_{30}O_5$	1,6,10-Farnesatriene-3,5-8 triol	2D-7	5.71	ND	–	–
354.0948	354.0951	Parent	0.80	nESI	$C_{16}H_{18}O_9$	Chlorogenate/Scopolin	2D-7	24.84	ND	–	–
352.2250	352.22497	Parent	1.35	nESI	$C_{20}H_{32}O_5$	2β,6α,17-Trihydroxy-ent-kauran-19-oic acid	2D-7	32.64	10.57	3.09	0.070
334.2139	334.2144	0.985	1.52	pESI	$C_{20}H_{30}O_4$	9,15-Dihydroxy-16-kauren-19-oic acid	2D-4	19.79	15.10	1.31	0.019
333.2303	316.2038	NH_4	0.62	pESI	$C_{20}H_{28}O_3$	Helioscopinolide A/ent-15-oxokaur-16-en-19-oic acid	2D-7	21.20	4.62	4.59	0.022
322.1909	300.2089	Na	0.07	pESI	$C_{20}H_{28}O_2$	Dehydroabietic acid	2D-5	5.92	1.89	3.13	0.062
302.2247	302.2246	Parent	0.40	pESI	$C_{20}H_{30}O_2$	Abietic acid	2D-1	15.85	5.35	2.93	0.001
274.0841	274.0841	Parent	0.09	pESI	$C_{15}H_{14}O_5$	Apiforol	2D-5	13.11	12.31	1.06	0.059
272.214	272.214	Parent	0.06	pESI	$C_{19}H_{28}O$	4,19-Epoxy-18-nor-16-kaurene	2D-1	16.32	5.33	3.06	0.051
254.2040	254.2034	Parent	0.19	pESI	$C_{19}H_{26}$	18-Nor-4(19),8,11,13-abietatraene	2D-1	8.82	3.35	2.63	0.018
192.1126	170.1307	Na	0.18	pESI	$C_{10}H_{18}O_2$	6-Endo-Hydroxycineole/2-isoprepenyl-5methyl-hex-5-ene-1,4-diol	2D-1	5.74	2.27	2.53	0.106
172.0501	150.0681	Na	0.47	pESI	$C_9H_{10}O_2$	4-Coumaryl alcohol/prenylpropanoate	2D-7	4.29	ND	–	–

ND = not detected so a −Pi/+Pi ratio could not be determined. Exudates were collected from carrot roots grown in liquid culture with or without 30 μM Pi. The average crude exudate intensity ratio (−Pi/+Pi) and the significant difference (ANOVA) between the two variables is indicated by the p value. Each mode of FTMS analysis was calibrated to internal standards to ensure ultra-high mass accuracy prior to the peak selection process. All errors are less than 3 ppm and most are less than 0.2 ppm. To recover enough material in separated fractions to test for hyphal branching activity and perform FTMS analyses, TLC fractions were obtained by combining six separate crude exudate preparations before TLC. The 2D fraction listed for any given compound contained the highest intensity for that compound. Mass detected = FTMS; Theoretical mass = TM; Mode = mode of detection which is either positive (p) or negative (n) electrospray ionization (ESI). The putative identification of each compound is listed.

Table 4 Compounds found in carrot root exudates that are known to stimulate biochemical or morphological changes in fungi or have a role in plant/parasitic interactions

FTMS (neutral)	TM	Adduct	Error (ppm)	Mode	Predicted formula	Compound	TLC fraction	Crude exudate			P value
								$-P$	$+P$	$-P/+P$	
328.2013	306.2195	Na	0.47	pESI	$C_{19}H_{30}O_3$	Dictyopyrone A	2D-4	45.25	13.13	3.45	0.001
282.0532	282.0532	Parent	1.34	pESI	$C_{16}H_{10}O_5$	Cochilophilin	2D-4	3.36	1.11	3.03	0.346
214.0240	192.0423	Na	1.09	pESI	$C_{10}H_8O_4$	Scopoletin	2D-4	5.27	3.79	1.39	0.155

Exudates were collected from carrot roots grown in liquid culture with or without 30 μM Pi. The average crude exudate intensity ratio $(-Pi/+Pi)$ and the significant difference (ANOVA) between the two variables is indicated by the p value. Each mode of FTMS analysis was calibrated to internal standards to ensure ultra-high mass accuracy prior to the peak selection process. All errors are less than 3 ppm and most are less than 0.2 ppm. To recover enough material in separated fractions to test for hyphal branching activity and perform FTMS analyses, TLC fractions were obtained by combining six separate crude preparations before TLC. The 2D fraction listed for any given compound contained the highest intensity for that compound. Mass detected = FTMS; Theoretical mass = TM; Mode = mode of detection which is either positive (p) or negative (n) electrospray ionization (ESI). The putative identification of each compound is listed.

5   Compounds That Promote Morphological Changes, Biochemical Changes, or Inhibit Plant or Fungal Growth

Other compounds of particular interest were those chemicals that were known to be signals, attractants, stimulants, or inhibitors of parasitic organisms of roots. Although these compounds have a defined role in other systems, they may also have a significant role in AM fungal host interactions as is the case for strigolactones (Akiyama et al. 2005) which will be discussed in further detail in the next section. Compounds that induce the formation of haustoria may also be of interest since the parasitic root haustoria would be analogous to the development of penetration hyphae after appressoria formation. The haustoria inducing factors include flavonoids (xenognosin and formononetin), p-hydroxy acids (ferulic acid), quinones (2,6-dimethoxy-ρ-benzoquinone, benzoquinone, dihydroquinone) and cytokinins such as zeatin (Estabrook and Yoder 1998).

Under the general category of phenols (which include flavonoids), many have been identified as allelopathic chemicals and include salicylic acid, ρ-hydroxybenzoic acid (p-HBA), vanillic acid (VA), syringic acid, ferulic acid (FA), caffeic acid (CA), and coumaric acid (Chung et al. 2002; Yamane et al. 1992). Of these allelopathic compounds, FA, p-HBA, VA and CA were either found associated with carrot root cell walls or in carrot root cytosol (Nagahashi et al. 1996). Caffeic acid and FA inhibited the growth of germinated spores of both *G. gigantea* and *G. margarita* (this isolate, DAOM 194757, was reclassified as *Gigaspora rosea* (Bago et al. 1998) after genetic analyses) while VA stimulated the length of the primary germ tube and hyphal branching of the primary germ tube of *G. rosea* and the total hyphal length and number of branches off the primary germ tube of *G. gigantea*. Para-hydroxybenzoic acid had no effect on *G. gigantea* but stimulated the length of the primary germ term tube, overall hyphal growth and auxiliary cell production of *G. rosea* (Douds et al. 1996).

Other types of compounds, such as dictyopyrone A, induce a morphological change in fungi. Dictyopyrone A promoted stalk formation in *Dictyostelium discoideum* (Arai et al. 2005) and also had remarkable effects on the growth and differentiation of *Dictyostelium* cell cultures (Maeda et al. 2003). Chemicals that attract parasites have also been reported. Cochliophilin A is a potent attractant for zoospores of the fungus *Aphanomyces cochliophilin* which causes root rot of spinach (Horio et al. 1992). Scopoletin stimulated the germination of seeds of the parasitic weed, *Striga asiatica*, and at low concentrations (2.5 ppm), it stimulated germination and elongation of *Striga* seedlings more than a natural stimulant (Worsham et al. 1962). Dictyopyrone A, cochliophilin A and scopoletin were all elevated in the –Pi exudates of carrot roots (Table 4) but only scopoletin was commercially available. When it was tested in the bioassay from 1 ng to 10 μg, it did not induce branching or stimulate the growth of *G. gigantea*.

Compounds like dictyopyrones, cochliophilin, scopoletin (or its derivatives), allelochemicals, and haustoria inducing factors may have properties which may be of potential relevance in AM fungal-host interactions in that they may serve more than one purpose. This reasoning is based on recent work where naturally occurring compounds in host root exudates that stimulated seed germination of parasitic plants

(see next section) also stimulated hyphal branching of an AM fungus (Akiyama et al. 2005). The work with phenolic compounds further supports this contention since p-hydroxy acids can be phytotoxic, stimulate haustoria formation and stimulate AM fungal growth. Secondary metabolites that serve multiple functions may be a way to achieve many benefits for a plant at a low metabolic expenditure (Perry et al. 2006).

6 Sesquiterpenoids and Their Role in AM Fungal/Plant Host Interactions

Some germination stimulants for parasitic weeds found in the exudates of host plants (Ayman et al. 2006; Yasuda et al. 2003) have been identified as strigolactones (called strigolactones because they were lactones found in *Striga* plants). Strigolactones have been regarded as sesquiterpenoids which are biosynthesized by either the cytosolic mevalonic acid pathway or the plastid associated nonmevalonic, methylerythritol phosphate (MEP) pathway (Akiyama 2007). Recent work showed that sesquiterpenoids are derived from C_{40} carotenoid (Matusova et al. 2005) originating from the MEP.

These seed germination stimulants have also been shown to stimulate hyphal branching of AM fungi during the presymbiotic growth phase (Akiyama et al. 2005). The strigolactone, 5-deoxy-strigol, was the first hyphal branching factor (BF) to be identified (Akiyama et al. 2005). Two other strigolactones, strigol and sorgolactone, were also shown to be BFs (Akiyama et al. 2005) and although a fourth strigolactone, orobanchol, was reported to be a BF, no evidence for stimulation of hyphal branching was presented (Yoneyama et al. 2007b). 5-deoxystrigol was isolated and purified from the exudate of *Lotus japonicus* and found to stimulate hyphal branching of *G. margarita* within the range of 30 pg to100 ng (Akiyama et al. 2005). However, a concentration dependence versus the number of branches was not shown quantitatively or morphologically. The greatest branching that was observed was induced by a semipurified lipophilic fraction isolated from the root exudate of *L. japonicus* (compare Figs. 1 and 4 of Akiyama et al. 2005). Other results showed that the hyphal branching pattern stimulated with semipurified fractions of carrot root exudates was very complex and resembled a bush. This branching pattern was the same observed when a primary germ tube of *G. gigantea* was at or very near the surface of a carrot root (Nagahashi and Douds 2000). Although "novel" strigolactones were reported to be found in carrot seedling exudates, no data was shown that would indicate what they might be or why they were novel (Yoneyama et al. 2006). A recent investigation with exudates of transformed carrot roots obtained a fraction purified by HPLC that not only contained a BF but also stimulated seed germination of the parasite Orobanche (Besserer et al. 2006). However, the active fraction was not confirmed as a strigolactone.

None of the strigolactones, that have been identified as BFs, were found in the crude exudates or concentrated TLC purified exudate components analyzed with FTMS (Table 5). Several sesquiterpene lactones, tentatively identified, were elevated in exudates of carrot roots grown under Pi stress. Costunolide and artemisinin (Table 5) were reported stimulants for seed germination of parasitic plants

Table 5 Sesquiterpenoids or sesquiterpene lactones found in carrot root exudates

FTMS (neutral)	TM	Adduct	Error (ppm)	Mode		Compound	TLC fraction	Crude exudate			
								−P	+P	−P/+P	P value
376.1525	376.1522	Parent	0.79	pESI	$C_{20}H_{24}O_7$	Eupatundin	2D-6	5.75	2.57	2.24	0.001
304.1284	282.1467	Na	0.97	pESI	$C_{15}H_{22}O_5$	Artemisinin	2D-4	8.04	7.52	1.07	0.095
250.1567	250.1569	Parent	0.78	pESI	$C_{15}H_{22}O_3$	Arbusculin A	2D-3	43.60	27.60	1.58	0.031
232.1463	232.1463	Parent	0.13	pESI	$C_{15}H_{20}O_2$	Alantolactone/costumolide	2D-7	38.00	39.70	0.96	0.081
230.1307	230.1305	Parent	0.78	pESI	$C_{15}H_{18}O_2$	Dehydrocostus lactone	2D-3	11.72	8.85	1.32	0.310

Exudates were collected from carrot roots grown in liquid culture with or without 30 μM Pi. The average crude exudate intensity ratio (−Pi/+Pi) and the significant difference (ANOVA) between the two variables is indicated by the p value. Each mode of FTMS analysis was calibrated to internal standards to ensure ultra-high mass accuracy prior to the peak selection process. All errors are less than 3 ppm and most are less than 0.2 ppm. To recover enough material in separated fractions to test for hyphal branching activity and perform FTMS analyses, TLC fractions were obtained by combining six separate crude preparations before TLC. The 2D fraction listed for any given compound contained the highest intensity for that compound. Mass detected = FTMS; Theoretical mass = TM; Mode = mode of detection which is either positive (p) or negative (n) electrospray ionization (ESI). The putative identification of each compound is listed.

(Besserer et al. 2006), but future work with AM fungi will be necessary to determine if they play a role in AM fungal/host interactions. The presence of artemisinin needs to be confirmed in plant roots and/or transformed hairy root cultures other than those of the genus *Artemisia.* Although the commercially available artemisinin had no effect on the hyphal branching of the AM fungus *Gigaspora rosea* (Besserer et al. 2006), it should be tested on other AM fungi. Sclerosporin ($C_{15}H_{22}O_2$; mass = 234.1619) was found in several of the 2D fractions but it was not enriched in the –Pi exudate. Arbusculin A and eupatundin were enriched in the –Pi crude exudate and were found with highest intensity in 2D-3 and 2D-6 (Table 5). If any of the aforementioned sesquiterpenoids are available (via a vendor or private source), only future work will determine if they have a role in AM fungal/host interactions.

7 Hydroxy Fatty Acids Which May Affect Fungal Growth

An earlier analysis of exudate components of several grasses showed a number of various length fatty acids (Dormarr et al. 2002). Although 16C fatty acids (palmitic acid) cannot be synthesized by germinating spores or extraradical hyphae of AM fungi (Trépanier et al. 2005), they are required for fungal growth. The effects of 16C fatty acids, or their derivatives, on AM fungi could be expressed as a morphological change and/or a physiological or biochemical change in metabolism. The fatty acids are obtained from the host during the colonization process but their role in presymbiotic events has not been defined.

A number of long chain fatty acids were found in grass exudates (Dormarr et al. 2002) and *Lupinus* cultivar exudates (Lucas Garcia et al. 2000) and furthermore, a recent analysis has found decanoic acid, hexadecanoic acid, tetradecanoic acid, octadecenoic acid some of their methyl esters in root extracts of transgenic tobacco plants (Mungur et al. 2005). Also found were some hydroperoxy derivatives and hydroxy stearic acid. A number of hydroxylated forms of these fatty acids were found in carrot root exudates and they were elevated in exudates of roots grown under Pi stress (Table 6). This is the first evidence that a variety of hydroxy fatty acids may be found in root exudates. Virtually nothing is known about hydroxy fatty acids in plant exudates but a hydroxylated form of palmitic acid, 2-hydroxyhexadecanoic acid (2-HHDA) is a functionally active component of sphingolipids which stimulate the production of fruiting bodies of the fungus *Schizophyllum commune* (Kawai et al. 1986).

The complex hydroxy fatty acids (Table 6) are similar to plant and fungal oxylipins (Tsitsigiannis and Keller 2007). The compound identified as 9,12,13-trihydroxyoctadeca-10-enoic acid, and enriched in – Pi exudate, is likely to be a reduced form of a plant oxylipin. Plant oxylipins are synthesized from polyunsaturated fatty acids (18:2 or 18:3 fatty acids) which are oxygenated at the C9 and/or C13 position and these hydroperoxides can then be reduced to hydroxy fatty acids (Tsitsigiannis and Keller 2007). Oxylipins represent a highly diverse family of secondary metabolites that have recently been implicated as a novel class of host-microbe signaling molecules (Tsitsigiannis and Keller 2007).

Table 6 Hydroxy fatty acids found in carrot root exudates

FTMS (neutral)	TM	Adduct	Error (ppm)	Mode	Compound	Compound	TLC fraction	Crude exudate			
								-P	+P	-P/+P	P value
430.2251	354.3134	K - K	0.21	pESI	$C_{22}H_{42}O_3$	2-Hydroxy-13-docosenic acid	2D-1	2.79	2.00	1.40	0.080
352.2221	330.2406	Na	1.44	pESI	$C_{18}H_{34}O_5$	5,8,12-Trihydroxy-9-octadecenoic	2D-1	10.91	6.41	1.70	0.070
346.2119	324.2301	Na	0.34	pESI	$C_{19}H_{32}O_4$	Octadecadienoic acid; Me ester	2D-7	8.04	3.82	2.11	0.040
342.1808	304.2250	K	0.21	pESI	$C_{16}H_{32}O_5$	9,10,16-Trihydroxyhexadecanoic acid	2D-4	9.91	6.65	1.50	0.013
330.2406	330.2406	Parent	0.00	nESI	$C_{18}H_{34}O_5$	9,12,13-Trihydroxy-octadec-10-enoic acid	2D-1	11.42	8.80	1.30	0.039
326.1857	288.2301	K	0.87	pESI	$C_{16}H_{32}O_4$	10,16-Dihydroxyhexadecanoic acid	2D-4	23.91	13.10	1.80	0.027
310.1912	272.2351	K	0.61	pESI	$C_{16}H_{32}O_3$	2-Hydroxyhexadecanoic acid	2D-6	11.89	4.10	2.90	0.017
298.1546	260.1988	K	0.19	pESI	$C_{14}H_{28}O_4$	3,11-Dihydroxytetradecanoic acid	2D-7	5.97	4.02	1.50	0.066
282.1592	244.2038	K	2.19	pESI	$C_{14}H_{28}O_3$	2-Hydroxytetradecanoic acid	2D-6	3.06	1.21	2.53	0.034
280.1648	258.1831	Na	0.23	pESI	$C_{14}H_{26}O_4$	3,5-dihydroxy-7-tetra-decenoic acid	2D-3	141.00	ND	–	–
214.0240	176.0685	K	2.06	pESI	$C_7H_{12}O_5$	2-Hydroxyheptanedioic acid	2D-5	4.93	4.20	1.17	0.155
210.1231	188.1412	Na	0.50	pESI	$C_{10}H_{20}O_3$	3-Hydroxydecanoic acid	2D-1	5.23	1.66	3.15	0.065

Exudates were collected from carrot roots grown in liquid culture with or without 30 µM Pi. The average crude exudate intensity ratio (−Pi/+Pi) and the significant difference (ANOVA) between the two variables is indicated by the p value. Each mode of FTMS analysis was calibrated to internal standards to ensure ultra-high mass accuracy prior to the peak selection process. All errors are less than 3 ppm and most are less than 0.2 ppm. To recover enough material in separated fractions to test for hyphal branching activity and perform FTMS analyses, TLC fractions were obtained by combining six separate crude preparations before TLC. The 2D fraction listed for any given compound contained the highest intensity for that compound. Mass detected = FTMS; Theoretical mass = TM; Mode = mode of detection which is either positive (p) or negative (n) electrospray ionization (ESI). The putative identification of each compound is listed.

Although 2- and 3- (or α- and β-) hydroxy fatty acids are minor constituents of fatty acids, they are synthesized by plants, animals and microorganisms. They are essential components of triacylglycerides, sphingolipids, waxes, and other lipids in plants (Jenske and Vetter 2009). Other hydroxy fatty acids, in particular the 3-hydroxy fatty acids, have antifungal properties (Sjögren et al. 2003). Some of the identified hydroxy fatty acids in Table 6 were commercially available and were tested in the bioassay for their effect on germinated spores of *G. gigantea.* Three-hydroxy fatty acids, including 3-hydroxydecanoic acid (3-HDA), have previously exhibited antifungal properties between 10 and 100 μg (Sjögren et al. 2003). At 5 μg/5 μL, 3-HDA inhibited AM hyphal tip growth with no recovery, at 0.5 μg/5 μL hyphal tips were inhibited but recovery branches were formed, and at 50 ng/5 μL or less, no inhibition or stimulation of growth occurred. Recovery branches induced by hyphal growth inhibitors are formed right behind the tip of a germ tube that has stopped growing and they have a distinct morphology compared to normal lateral branch formation (Nagahashi and Douds 2000). Recovery branches of an inhibited germ tube of *Gigaspora s*pecies always retain the negative geotropic response, compared to lateral branches which do not. They allow the germ tube to continue growing away from an inhibitor and this pattern resembles a candelabra (Nagahashi and Douds 2000; Nagahashi and Douds 2007).

Two-hydroxyhexadecanoic acid (2-HHDA) had no effect between 0.1 ng and 1.0 μg/5 μL on AM fungal growth or branching. However, 2- hydroxytetradecanoic acid (2-HTDA) at 1 μg/5 μL, inhibited hyphal tip growth but a recovery branch formed and growth continued so the initial inhibition was overcome. At 1 ng to 10 ng/5 μL, 2-HTDA stimulated the development of lateral branches (Fig. 3) of *G. gigantea*. Two fractions purified by 2D TLC (2D-6 and 2D-7) contained 2-HTDA and this compound was elevated in the – Pi exudate (Table 6). The extra branches stimulated with 2-HTDA provided many growing tips which could be further
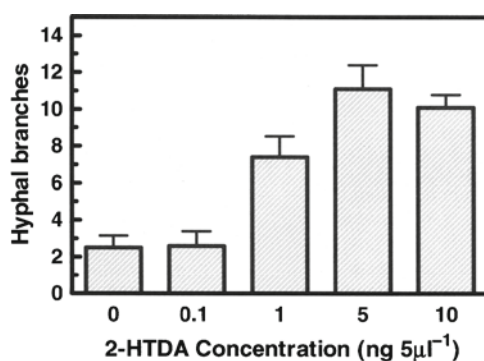

Fig. 3 Stimulation of hyphal branching of *Gigaspora gigantea* with low concentrations of 2-hydroxytetradecanoic acid (2 HTDA). 2 HTDA was found to be elevated in the exudate of carrot roots grown in the absence of phosphorus and in fractions further purified by two dimensional thin layer chromatography (see Table 6). Five μL of each dilution was applied in the microinjection bioassay. Means of 15 observations ± SEM
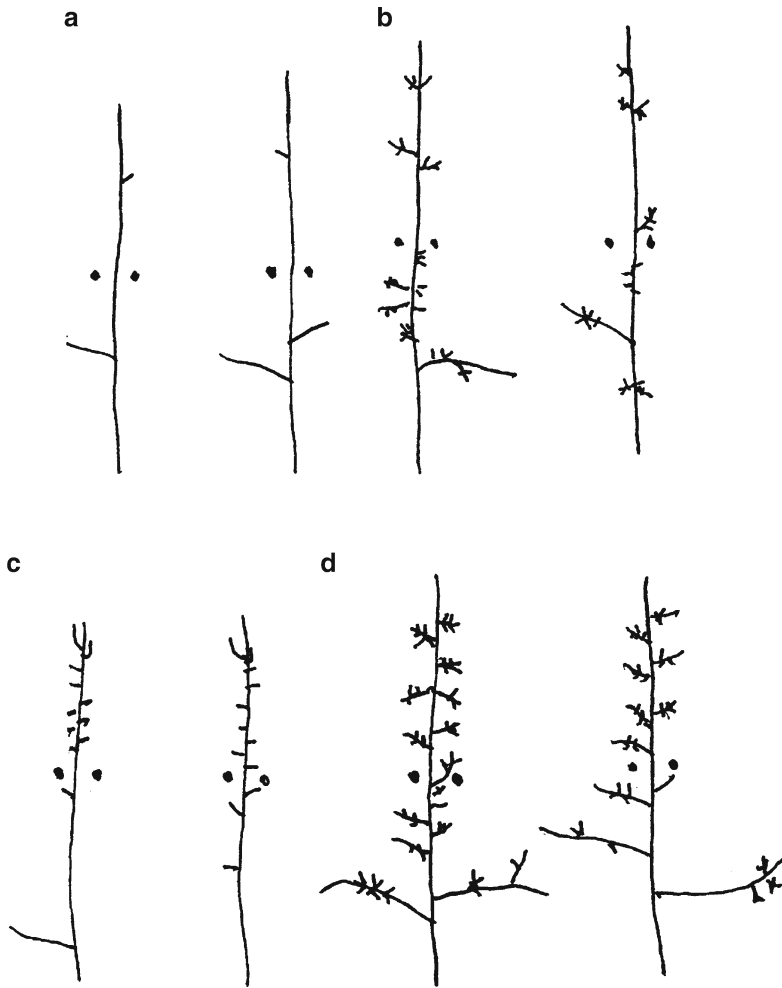
Fig. 4 Tracings made right on the Petri plates show the synergistic hyphal branching response of Gigaspora *gigantea* to 2-HTDA and crude exudate of carrot roots. (**a**) Germinated spores received 5 μL injections of methanol at positions marked by the dots (controls). (**b**) Germinated spores treated with exudate only (5 μL of a semifpurified fraction that was diluted 250 fold with methanol to reduce the branching on the primary germ tube to allow for counting hyphal tips more accurately). (**c**) Germinated spores treated with 5 ng/5 μL of 2-HTDA. (**d**) Germinated spores were treated with a mixture of 2HTDA and exudate. The results in (d) showed that the small branches stimulated by 2-HTDA provided starting points for further branching on the germ tubes

stimulated by other BFs present in an exudate fraction (semipurified exudate concentrated in a C18 SEPAK cartridge and eluted between 50% and 70% acetonitrile). This was demonstrated when the semipurified exudate treatment alone (Fig. 4b) and 2-HTDA treatment alone (Fig. 4c) were compared to a mixture of the two samples. It was apparent that a synergistic response was induced (Fig. 4d) where

the total number of branches with both treatments together was greater than the sum of each individual treatments (sum of individual treatments = 44.0 ± 1.4 compared to treatments together = 55.2 ± 4.4 were statistically different by ANOVA, p< 0.0154). Similarly, under the same bioassay conditions, virtually identical stimulation of lateral branch formation was obtained for *Gigaspora rosea* but only when quercetin was added to the growth medium (data not shown). Quercetin was also required to induce hyphal branch formation of *Gigaspora rosea* (initially identified as *G. margarita* but was reclassified) by a crude exudate of carrot roots (Becard et al. 1992; Nagahashi and Douds 2000).

The presence of an AM fungal stimulator and inhibitor in the same exudate or same fraction (Table 6) may sound contradictory, but it should be kept in mind that the inhibitors are only effective at much higher concentrations than the stimulators, which are active at 1/1,000 that of an inhibitor. The inhibitors are likely to be present at low levels at the surface of unchallenged roots so that inhibition does not occur, but the stimulators would still be effective.

8 Conclusions

The effect of 2-HTDA on germinated AM fungal spores indicated that different types of hyphal growth/branching stimulators exist. The various hyphal growth/branching stimulators also appear to induce different morphological patterns of hyphal growth. Some flavonoids stimulate hyphal elongation, strigolactones stimulate branching (BFs) and elongation of secondary hyphae, and 2-HTDA appears to mainly stimulate lateral branch formation. These morphological responses indicate that several types of chemical compounds of hyphal growth stimulators exist. It has been demonstrated that several chemical compounds in one chemical category (strigolactones, Akiyama et al. 2005) can stimulate hyphal branching while several types of flavonoids can stimulate hyphal elongation (Scervino et al. 2005). Similar to the lack of a universal germination stimulant of broomrape species (Fernandez-Aparicio et al. 2009), there may not be a universal stimulant for AM hyphal branching or hyphal elongation. This is consistent with the previous report that indicated multiple hyphal growth/branching stimulators are present in carrot root exudates (Nagahashi and Douds 2000; Nagahashi and Douds 2007), and furthermore, it is likely they will act synergistically (Fig. 4). Evidence for the synergistic interaction has already been reported. One *Gigaspora* species, *G. rosea*, only has maximized hyphal branching when the germinated spores are grown on media containing quercetin and then either treated with semipurified exudate (Nagahashi and Douds 2000) or GR24, a strigolactone analogue (Besserer et al. 2006). This may explain why the branching induced by purified strigolactones (Akiyama et al. 2005) was not nearly as extensive as that achieved with crude or semipurified exudate preparations (Nagahashi and Douds 2000; Buee et al. 2000) or like that observed when hyphae grow near or at the surface of a host root (Nagahashi and Douds 2000). Many possible synergistic interactions could exist between different chemical

categories of BFs, BFs from the same chemical category (strigolactones for example), and BFs with compounds that are hyphal elongators or lateral branch stimulators. Other than the overall morphological branching patterns induced by these various stimulators, the metabolic events triggered by these compounds during the presymbiotic phase may indeed directly stimulate preinfection as well as further colonization events and this can only be determined by future research.

Finally, this is the first FTMS metabolic profile performed on a plant exudate although a recent metabolic fingerprint of a transgenic tobacco root extract was achieved with this instrumentation (Mungur et al. 2005). Low molecular mass compounds have been assumed to predominate root exudates (Walker et al. 2003a) and results from FTMS analyses confirmed this belief (Fig. 2). Earlier information indicated that AM fungal growth stimulators/branching factors have a MW less than 500 (Giovannetti et al. 1996) and the BFs identified so far have a MW around 330 (Akiyama et al. 2005) and the elongators about 250–300 MW (Scervino et al. 2007). Furthermore, it was shown that Pi stress on plants can cause a substantial change in the distribution of low MW compounds in root exudates with an increase in some components and a decrease in others (Figs. 1 and 2). Putative identification of these compounds can lead to future hypotheses and functional studies involving the fungal mechanisms used and/or metabolic pathways influenced by plants grown under Pi stress. Tentative identification of compounds will allow for the systematic screening of various types of potential elongators and branch stimulators and if positive results are found, then chemical confirmation of the putative compound will have to be achieved. FTMS analyses can be used to study other environmental stresses or conditions in relation to the changes induced in the biochemical composition and metabolism of exudates and/or root extracts in general.

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Chapter 3
The Making of Symbiotic Cells in Arbuscular Mycorrhizal Roots

Andrea Genre and Paola Bonfante

Abstract The establishment of arbuscular mycorrhizae (AM) requires the host root cells to undergo significant structural and functional modifications leading eventually to reciprocal beneficial effects. We here discuss some of the responses that are induced in root cells during the different steps of AM colonization. A combination of genetics, molecular and cellular approaches reveals in fact that the establishment of a functional symbiosis appears to be at the end of a series of plant-controlled checkpoints, each of which is required but non-sufficient to achieve the next one. The recognition of AM fungi as symbionts takes place in the rhizosphere, and switches the host plant to an alert status, by activating specific signalling pathways and responses on a cell to organism level. Direct cellular contact on the root surface is a central event in the interaction that mainly impacts on the contacted cell, where prepenetration responses open the way to the intracellular accommodation of the fungal symbiont. This constructive phase extends to epidermal and cortical cells and culminates with the establishment of functional arbuscules, which accomplish the key symbiotic functions related to nutrient exchanges. The modulation of arbuscule development and functioning represent the core process of the symbiosis and its study keeps bringing novel information on the molecular, cellular and metabolic mechanisms that rule this ancient interaction.

Abbreviations

AM   Arbuscular mycorrhiza
GFP   Green fluorescent protein

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LMD   Laser microdissection
PPA   Prepenetration apparatus

1 Introduction

Arbuscular mycorrhizae (AM) are symbioses that are established between two
eukaryotes – a fungus and a plant – and lead to an overall improvement on the fit-
ness of both partners. AM enhance plant nutrient uptake thanks to the fine explora-
tion of the rhizosphere by the fungus, which in return receives plant carbohydrates
that are essential for the completion of its life cycle. All AM fungi belong to the
Glomeromycota, an ancient phylum that has coevolved with plants for at least 400
million years, and has probably assisted the colonisation of dry lands by higher
plants (Bonfante and Genre 2008). Together with root nodules, AM are considered
to be the most important symbioses 'that help feed the world' (Marx 2004). AM
fungi supply the majority of land plants with nutrients, thus increasing biomass
production, and confer resistance against biotic and abiotic stresses. These fungi
have a few peculiar traits. They are obligate plant biotrophs, having so far been
unculturable in the absence of their host (Declerck et al. 2005). Their syncytial
hyphae and spores possess thousands of nuclei, making classic genetics approaches
not practical (Pawlowska 2005; Croll et al. 2009) and stable transformation proto-
cols not available at present (Helber and Requena 2008). Due to such characteris-
tics, and to the open question about the capacity of these fungi to have sexual
reproduction, the concept of species is poorly defined among Glomeromycota. This
also reflects a high degree of variability in their functional traits and ecological
impact (van der Heijden and Scheublin 2007), and is probably one of the reasons
why the genome sequencing of *Glomus intraradices* has not yet been accomplished
(Martin et al. 2008). Lastly, their process of root colonization is non-synchronous
and early structures such as hyphopodia coexist with mature symbiotic organs such
as arbuscules, the branched intracellular structures where most nutrient exchanges
are thought to occur. This clearly complicates the study of the symbiotic interac-
tion, due to the impossibility of obtaining homogeneous samples corresponding to
a single phase of the colonization.

Notwithstanding these experimental constraints, AM are currently inside the
mainstream of biology: on one hand, their application in sustainable agriculture
represents a constant stimulus to extend our knowledge of this unique symbiosis.
On the other hand, a combination of genetics, DNA technologies, genomics and
cell biology, mostly applied to the plant side, is now offering new opportunities to
reveal the secrets that allow the establishment and functioning of this ubiquitous
beneficial interaction.

The widespread distribution of AM, together with their conserved structural
(e.g. interface compartment morphology) and functional features (e.g. phosphate
uptake), strongly suggests the existence of common molecular and genetic determi-
nants across different plant taxa, ranging from liverworts and ferns to angiosperms.

The development of microarray-based transcriptome profiling projects is on the way to confirm this hypothesis: exploring AM-triggered gene expression in legumes and non-legumes (Küster et al. 2007; Hohnjec et al. 2003; Guimil et al. 2005; Guether et al. 2009) is revealing the presence of a largely common molecular background underlying AM functioning, in particular Pi uptake (Harrison et al. 2002; Javot et al. 2007; Gómez-Ariza et al. 2009), irrespectively of fungal identity (Liu et al. 2007).

Another fundamental support to the concept of the existence of common genetic determinants came from the identification of plant mutants that are impaired in their ability to establish a functional symbiosis. At least seven genes that are required for both the AM symbiosis and the root-nodule symbiosis with rhizobia have been identified in legumes by this approach. These so-called common symbiosis (*SYM*) genes encode for proteins that are involved in a signal transduction network that mediates the development of intracellular accommodation structures for fungal and bacterial symbionts by the host cell (Oldroyd and Downie 2006; Parniske 2008). Mutants in any of the common SYM genes are characterized by an early block of AM fungal infection in the outer cell layers. Transcriptome analysis showed that common SYM gene mutations also affect the expression of most AM-induced genes (Liu et al. 2003; Kistner et al. 2005; Siciliano et al. 2007), while phenotypic analyses showed that *DMI2* and *DMI3* are required for the induction of prepenetration responses in *M. truncatula* (Genre et al. 2005).

Among the common SYM genes that have been characterized in *Medicago truncatula*, DMI2 encodes for a receptor-like kinase (Endre et al. 2002) that is considered as the entry point into the common symbiotic signalling pathway, due to its potential to transmit a perception event through its intracellular kinase domain. DMI1 is a trans-membrane potassium channel with nuclear localization (Parniske 2008) that is proposed to have a role in compensating for the charge imbalance produced during calcium spiking. oscillations in calcium concentration, in fact, are known to be a key element in the signalling pathway that leads to rhizobial infection, and have also recently been demonstrated in response to AM fungal diffusible signals (Kosuta et al. 2008). The calcium-calmodulin-dependent protein kinase DMI3 is essential for AM establishment (Lévy et al. 2004). Its potential in sensing calcium concentration makes it a perfect candidate for the response to calcium oscillations that are induced by AM fungi or the Nod factor. Lastly, the nuclear protein IPD3 has been identified as the target of DMI3 kinasic activity (Messinese et al. 2007) and is considered to be among the final actuators of gene regulation at the terminus of the common SYM signalling pathway (Parniske 2008).

By contrast, our knowledge of the molecular basis of the interaction establishment and signal exchange between the partners is more limited (Smith et al. 2006; Paszkowski 2006). The establishment and functioning of AM involve different scenarios. Reciprocal recognition of the plant and fungus is initiated in the rhizosphere, while the first direct cellular contact on the root surface is a central event in the interaction. Lastly, the colonization of inner root tissues and its modulation represent the functional core of the symbiosis and still have several unclear aspects, although it has focussed the attention of researchers since the very first studies of AM.

2   Rhizospheric Communication

Diffusible molecules released by the fungus and plant in the rhizosphere are perceived
by the reciprocal partners. This molecular dialogue is supposed to take place during
the pre-symbiotic phase of the interaction between the plant and the fungus, keeping
both partners timely informed about their reciprocal proximity. With the use of a
direct bioassay, Akiyama et al. (2005) identified a plant molecule (a strigolactone)
present in root exudates and eliciting the hyphal branching in AM fungi (Fig. 1).
Mycelial proliferation, as an alternative or in combination with chemiotropic responses
(Sbrana and Giovannetti 2005), is a successful strategy to achieve root colonization,
since it increases the possibility of a contact with the host surface. Strigolactones are
very unstable molecules in the soil aqueous solution. This makes them ideally suit-
able for the establishment of a steep concentration gradient around the roots, which
has an obvious impact on the pattern of AM fungal development. Besserer et al.
(2006) found that a *Sorghum* strigolactone strongly and rapidly stimulated the
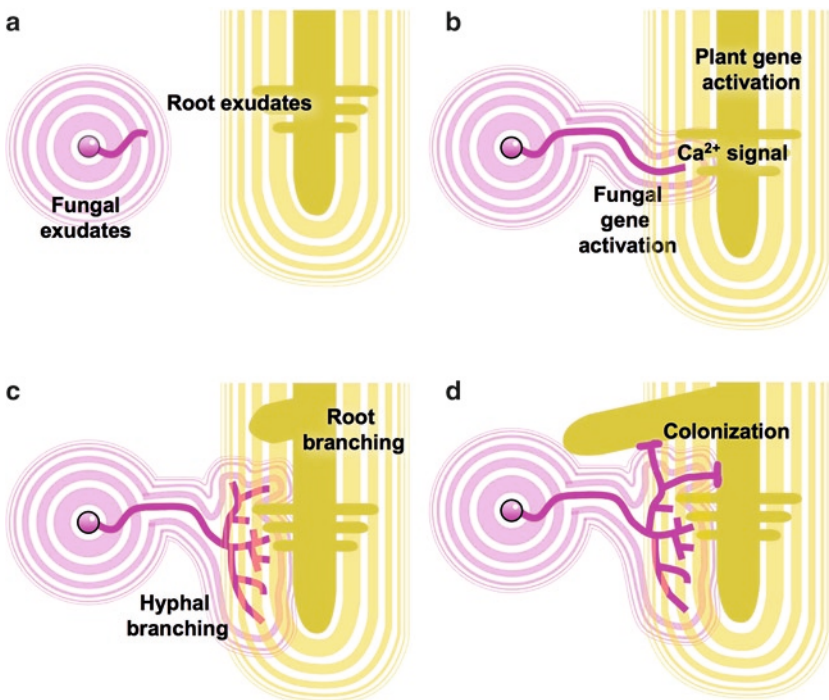metabolism of the AM fungus *Gigaspora rosea*, at concentrations as low as 10^{-13} M.

Fig. 1 Schematic summary of the molecular dialogue between an AM fungus and its host plant.
Both symbionts release active molecules (**a**). When these are perceived by the reciprocal partner,
specific molecular, cellular and morphogenetical responses are triggered (**b**, **c**), which eventually
lead to successful colonization (**d**)

Within one hour of treatment, the density of mitochondria in the fungal cells increased, and their shape and movement changed dramatically.

Interestingly, strigolactones were first described as molecules that stimulate seed germination in parasitic plants such as Striga (Cook et al. 1966). The dualistic role of these molecules initially opened the question on what was their evolutionary history. Interestingly, *Physcomitrella patens*, a basal plant with a gametophytic dominant cycle, and *Arabidopsis thaliana*, a non-host-plant for AM, both release strigolactones (Delaux et al. 2009), suggesting that the involved metabolic pathways may have preceeded the divergence between bryophytes and vascular plants and evolved independently of AM symbiosis.

A recent work by Gomez-Roldan et al. (2008) has demonstrated that strigolactones are in fact plant hormones, involved in shoot growth regulation by inhibiting the development of lateral buds. This adds a new and possibly more basic role for these molecules, suggesting that their leakage from the roots into the soil may have turned useful to very different root-interacting organisms (symbiotic fungi and parasitic plants) as they co-evolved with their hosts.

As the AM fungus starts to branch in the vicinity of the root, plants perceive diffusible fungal signals, even in the absence of a physical contact. The chemical nature of such a fungal factor, often referred to as 'Myc factor' in analogy to the Nod factor of nitrogen fixing rhizobia (Kosuta et al. 2003), is still unknown. Nonetheless, the Myc factor was demonstrated to activate plant responses, in experiments where a membrane prevented physical contacts between the two partners, while allowing the diffusion of soluble molecules. *MtEnod11* and other genes involved in signal transduction are up-regulated (Kosuta et al. 2003; Weidmann et al. 2004), showing that the plant is perceiving AM fungal presence in the rhizosphere. The development of lateral roots is also stimulated (Olah et al. 2005), suggesting that the plant is also adopting a 'branching strategy' to increase the chances of contacting its symbiont. These presymbiotic responses have been described as being part of an "anticipation program", which prepares the symbionts for a successful association (Paszkowski 2006). How plant and fungus perceive and transduce these symbiotic signals is not fully understood at the moment. In analogy with the rhizobium-legume symbiosis (Oldroyd and Downie 2006), calcium has long been hypothesized to be involved in the AM signal transduction. Ca^{2+} is the most widespread intracellular messenger and couples a wide array of extracellular stimuli to specific physiological responses (Hetherington and Brownlee 2004). Transient elevations of cytosolic free Ca^{2+} concentrations can be recorded in cells undergoing biotic or abiotic stresses, and encode information which is subsequently transduced in a cascade of cellular events (Sanders et al. 2002).

Ca^{2+} oscillations were recorded in root hairs of *Medicago truncatula* plants expressing the calcium reporter cameleon (Kosuta et al. 2008) in the presence, but not in the contact of AM hyphae. These results confirmed the involvement of calcium in AM symbiosis. A role for this cellular messenger had been previously suggested by the work of Navazio et al. (2007), who recorded a rapid and significant increase in cytosolic Ca^{2+} level (which was dissipated within 30 min)

in aequorin-transformed soybean cells treated with the exudate of germinated spores of different AM fungi. The medium did not elicit the accumulation of reactive oxygen species nor programmed cell death, and induced no such Ca^{2+} transient in *Arabidopsis* cells expressing the same construct. These data suggest that fungal signals are released since spore germination, even in the absence of any plant-derived stimulus to the fungus.

A long term response of host plants to diffusable fungal signals was also detected using starch content as a marker for root carbohydrate status. Dependence on the plant's carbon supply most probably makes the fungus an obligate symbiont (Smith and Read 2008) and the fact that light-limitation results in decreased photo-assimilate production by the plant and in limited mycorrhizal colonization supports this view. This strict relationship was confirmed by a decrease in starch levels during mycorrhizal colonization of *L. japonicus* roots, and in particular by the disappearance of amyloplasts in arbsculated cells. However, after one week in the presence of AM fungal exudates or in the presence of a Millipore membrane preventing contact between the symbionts but allowing solute diffusion, *Lotus japonicus* accumulated starch in the root cortical cells, and this status persisted for at least four weeks (Gutjahr et al. 2009).

Such a change in carbohydrate metabolism seems therefore to be yet another element of the anticipation program that prepares the plant to AM colonization. Fungal diffusible signals released in the rhizosphere may therefore deeply influence the host plant development, metabolism, physiology and gene regulation along a time scale of seconds, minutes, hours and days.

3   The Root Epidermis Checkpoint

Plant and fungus come into physical contact for the first time on the root surface. Hyphae, originating from a spore or another colonized root, usually grow along the epidermis, often following the groove between epidermal cells with an evident contact between the two partner walls, but in the absence of an effective adhesion, as these hyphae can be easily peeled off the root. Plant cell responses clearly associated with this stage have not been described yet. By contrast, the development of a swollen hyphopodium, which closely adheres to the epidermal cell wall, is the unambiguous hallmarks of the initiation of the symbiotic phase. Predicting the site and time when a hypha will start differentiating into a hyphopodium is still impossible, although the live observation of fungus-root interactions in vitro suggests that only part of the root system is competent for AM colonization. Although the precise definition of such an area of competence is still missing, indications will possibly come from the study of early plant cell responses to fungal signals.

The first visible response of the epidermal cell to hyphopodium formation, is the migration of the nucleus, which positions directly underneath the contact point. During this process, an aggregation of cytoplasm also develops under the hyphopodium, including cytoskeletal bundles that radiate from the nucleus towards the

fungus and large patches of endoplasmic reticulum. Nuclear migration is generally completed within less than 2 h, with a variability depending on the initial nucleus position. The response is normally triggered in more than one epidermal cell in contact with the hyphopodium. Once the nucleus is positioned below the hyphopodium, additional cytoplasm re-structuring takes place, associated with a second phase of nuclear movement across the cell lumen and away from the hyphopodium. This movement is accompanied by the creation of a broad cytoplasmic column linking the nucleus to its initial position below the hyphopodium. This novel cellular structure has been called the prepenetration apparatus (PPA). A longitudinal array of microtubules and microfilaments is organized within this column and the endoplasmic reticulum proliferates all along the PPA (Genre et al. 2005).

These observations indicate that a strict check–in procedure is associated with direct cell-to-cell contact and hyphopodium development (Genre and Bonfante 2007), even if a variety of patterns, partly mirroring the anatomical diversity of the host, has been described. For example, epidermal cell penetration is known to take mainly place through a radial wall in *Lotus japonicus* (Bonfante et al. 2000), while tangential walls are the main entry site in another legume such as *Medicago truncatula* (Genre et al. 2005).

Further studies implementing live confocal microscopy with transmission electron microscopy have revealed that the PPA is extremely rich in Golgi stacks, secretory vesicles, trans-Golgi network and endosomes, strongly suggesting this structure to have a direct role in the production of the perifungal membrane (Genre et al. 2008). The first clue suggesting the PPA involvement in such membrane proliferation processes comes in fact from the observation that hyphal penetration – at the site of initial cytoplasmic aggregation – occurs only after the PPA has completely formed, and precisely follows its track across the cell. Furthermore, complete hyphal growth across the epidermal cell associates with the dismantling of the PPA and displacement of the nucleus from the vicinity of the hypha. The PPA mechanism is also known to be replicated in the inner root tissues (Genre et al. 2008).

These observations suggest that the PPA is likely to be involved in the synthesis of the perifungal membrane. Direct evidence of membrane proliferation inside the PPA is nonetheless still missing, and is expected to come from the use of fluorescent protein markers for the plasma membrane to label the growing perifungal membrane ahead of the penetration hypha.

The AM-specificity of the PPA response is supported by the absence of comparable structures in root epidermal cells upon the contact with fungal pathogens such as compatible strains of *Phoma medicaginis* or *Colletotrichum trifolii*. Such pathogens exclusively induce a localized aggregation of cytoplasm and organelles at the contact site, never developing into the transcellular column typical of the AM-triggered response (Genre et al. 2009). The second interesting outcome of these experiments was the demonstration that nuclear repositioning is a non-specific response, induced by both symbiotic and pathogenic fungi, and even by physical stimulation through a micromanipulator. Similarly, the interaction between an AM fungus and *Arabidopsis thaliana* may occasionally lead, under strong inoculation

pressure, to direct contact between germination hyphae and roots. Detailed *in vitro* observations of plant lines expressing GFP-HDEL (a tag for the endoplasmic reticulum) show that this contact with the AM fungus does not anyway lead to any significant response in the contacted cells of the non-host-plant (P. Bonfante, unpublished results). This further indicates the prepenetration responses to be AM-specific.

4 The PPA-Associated Transcriptomic Response

The transcriptome profile of AM roots has been investigated in several plants, including *Medicago truncatula*, where a large number of EST collections and micro-array data is available (e.g. Küster et al. 2007, Liu et al. 2003; 2007). However, information on the early stages of the symbiosis is still limited (Weidmann et al. 2004; Seddas and Arias 2009). The main reasons are that a strict timing control and a morphological marker are required for a precise sampling, in order to reduce transcript dilution. In addition, root colonization is a recursive process that is currently impossible to synchronize. This implies that different stages of colonization (presymbiotic signalling, hyphopodium adhesion, inner tissue colonization, senescent arbuscules) may coexist in the same sample. To focus the investigation on the transcriptome profile of host cells during the initial contact with AM fungi, Siciliano et al. (2007) have used in vitro root cultures of *M. truncatula* expressing GFP-HDEL, inoculated with *Gigaspora margarita*. Since this fluorescent protein construct allows the localization of PPAs, their position was used as a hallmark to target the sampling of short root pieces containing the responding cells. Gene expression in such root segments was compared with non-inoculated roots and *dmi3–1* mutant roots. In this mutant, PPA formation and fungal penetration do not occur due to the loss of function of DMI3, a calcium and calmodulin-dependent protein kinase, member of the common SYM pathway (Lévy et al. 2004). The experiments suggest that at least two genes are required for PPA formation: an *expansin-like* and a *nodulin-like protein*. Both are significantly up-regulated in the root fragments containing these structures, when compared with the control and the *dmi3–1* mutant. In situ hybridization confirmed the preferential accumulation of *Exp-like* mRNA in epidermal cells after fungal contact. In addition, an *Avr9/Cf-9 rapidly elicited protein 264*, was found to be up-regulated in the mutant, suggesting its negative regulation by DMI3 activity, and opening new vistas on the mechanisms that control compatibility in AM. Another indication of a potential crosstalk between the plant responses to symbiotic and pathogenic interactions comes from cell biological studies: the same *dmi3–1* mutant, in fact, also shows severe alterations in its response to pathogenic fungi. Whereas wild type *M. truncatula* organises a strong cytoplasmic aggregation and nuclear repositioning in the root epidermal cells contacted by *Phoma medicaginis* or

Colletotrichum trifolii, *dmi3–1* only shows nuclear repositioning, indicating that DMI3 activity is also required in the organization of defense responses (Genre et al. 2009).

5 Inside the Root: Symbiosis at Work

An intriguing question opened by the specialized cellular and molecular responses of epidermal cells is whether epidermal and cortical cells accomplish different functions during the building up of a functioning symbiotic root. Can we hypothesize that cortical cells are mostly involved in the symbiosis functioning and epidermal cells in recognition mechanisms? The best known functional marker of AM is a plant phosphate transporter (MtPT4), which is located in arbusculated cells and more precisely in the periarbuscular membrane (Harrison et al. 2002). Recent data based on the expression analysis of tomato root laser-dissected cells demonstrate that phosphate transporters (PT) are differentially expressed in three cortical cell populations (Balestrini et al. 2007). In particular, five plant and one fungal PT genes are highly expressed in the arbusculated cells, suggesting that plants ensure Pi uptake through the functional redundancy of a gene family. Furthermore, harvesting of epidermal, cortical and vascular cylinder cells gives an even clearer picture, suggesting that functions like nutrient transport are a cell-specific activity (Gómez-Ariza et al. 2009).

The evolutionary success of AM symbiosis is attributed to the improved plant mineral nutrition. Molecular data have given sound confirmations to this view: in addition to phosphorus, nitrogen is the other important element taken up by most mycorrhizal fungi. Genes involved in organic and inorganic N uptake have been identified in AM and ectomycorrhizal fungi (Bonfante and Anca 2009). In the transcriptome analysis of *L. japonicus*, 47 putative transporters were identified; out of them, 24 might be important for nutrient acquisition processes, including P, N, S, K, Zn transporters (Bonfante and Genre 2008, 2009). Nitrogen can be found in two major forms in soils, organic and inorganic compounds, the latter being represented by NO_3^- and NH_4^+: both forms are taken up by AM fungi. There is evidence that the subsequent N transfer from the fungus to the plant cells occurs in form of NH_4^+ (Guether et al. 2009). Because the putative NH_4^+ transporter resulted to be the strongest regulated gene in our array experiment (31,000 fold), the full coding sequence was isolated and characterized in detail. The localization of the *LjAMT2;2* transcripts via LM revealed their accumulation in arbusculated cells. Heterologous complementation of a yeast mutant demonstrated that the LjAMT2;2 protein is a functional NH_4^+ transporter, which is active at acidic pH, binds charged NH_4^+ in the apoplastic interface compartment and releases the uncharged NH_3 into the plant cytoplasm: these findings offer new scenarios to the nutritional exchanges occurring in the interface space.

6   The PPA and the Evolutionary Origin of the Interface Compartment

Concerning the plant cell responses to AM colonization of the root inner tissues, the PPA mechanism appears to be conserved with specific modulations related to the different colonization patterns. Outer and inner cortical cells in fact organize broad cytoplasmic columns that anticipate their colonization by transcellular hyphae. Furthermore, arbuscule development also appears to depend on the same cellular mechanism, since inner cortical cells where arbuscules are about to form show local accumulations of cytoplasm along the trunk hypha (PPA-like aggregations) that anticipate the appearance of fungal branches (Genre et al. 2008).

PPAs have been observed in both legumes (*Medicago truncatula* and *Lotus japonicus*) and non-legumes (*Daucus carota*), in very divergent patterns of root colonization such as Arum- and Paris-type mycorrhizae (Dickson 2004), suggesting that this response is widespread and has an ancient evolutionary origin. These observations indicate the PPA as a conserved cell mechanism, which is present in phylogenetically distant plants, where it is at the same time modulated according to the resulting morphogenetic process (e.g. in epidermal or cortical cells, and upon the development of a terminal or an intercalary arbuscule).

An evo-devo approach to this subject opens the stimulating question about the evolutionary origin of prepenetration responses. The ultrastructural details of PPA aggregations, and especially the abundance of cytoskeleton elements and secretion-related membranes, closely recall the patterns observed during cell plate deposition at the end of cell division. Several other analogies can be drawn between PPA assembly and cell division events: both processes involve intense exocytosis; in both cases, de novo cell-wall deposition occurs within the cell lumen rather than along the pre-existing wall; the thin apoplastic space separating the fungal cell wall from the host perifungal membrane is full of cell wall components of plant origin and is very similar in composition to the cell plate. In addition, several cell wall synthesis-related plant genes, such as *MtCel1* (Liu et al. 2003), which has been associated with cellulose synthesis, and *Mt-XTH1* (Maldonado-Mendoza et al. 2005), which encodes a xyloglucan endotransglucosylase-hydrolase involved in the construction of xyloglucan polymers in plant cell walls, as well as several genes encoding hydroxyproline-rich glycoproteins, expansins and arabinogalactan-proteins, which are important wall components are known to be upregulated in mycorrhizal roots (Balestrini and Bonfante 2005; Harrison 2005).

On the other hand, the focal proliferation of the perifungal membrane, associated with the secretion of cell wall components, shares several traits with polar growth, the main alternative mechanism of plant cell development and differentiation. Considering perifungal membrane proliferation as a 'reversed' process of polar growth, where a cell wall protrusion is produced inside the cell lumen rather than extending outwards (Fig. 2), is also extremely intriguing and an analogous hypothesis has already been made to explain the origin of infection thread formation inside the root hairs contacted by nitrogen-fixing rhizobia (Arrighi et al. 2008).
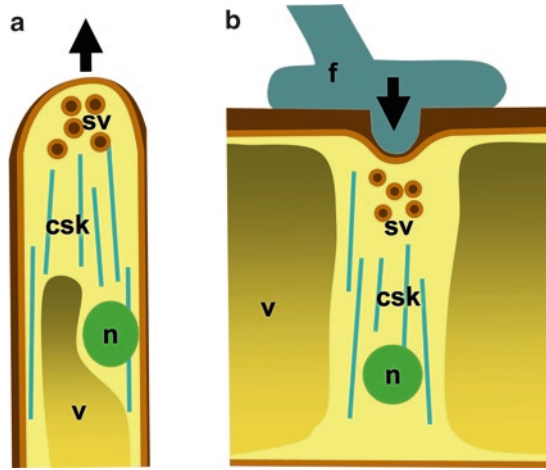
Fig. 2 Simplified model highlighting the possible similarities between the polar growth of a root hair tip (**a**) and the formation of the interface compartment during the prepenetration responses to AM colonization (**b**). While one process leads to an outgrowth of the cell wall whereas the other produces a deposition of cell wall materials inside the cell lumen, several analogies can be envisaged, which suggest the possibility that the AM-induced prepenetration apparatus has derived from the introversion of polar growth. In both cases the nucleus (n), cytoskeleton (csk) and elements of the secretory pathway (sv) are involved. Both processes lead to a proliferation of the cell membrane and wall, which is directed outwards in root hair tips, inwards in the prepenetration apparatus (*arrows*). f: fungus; v: vacuole

Altogether, cellular and molecular hints suggest that the cells involved in the perifungal interface construction can co-opt pre-existing molecular mechanisms (e.g. gene networks) to perform similar functions in a new context, as often suggested for evo-devo events in higher plants (Friedman et al. 2008). In particular, primordial, basic mechanisms, such as cell plate deposition or tip growth, might have been recruited and modulated when the necessity for assembling an intracellular niche for the AM symbiont arose. This hypothesis might explain the existence of common molecular and genetic determinants for AM interactions over the plant taxa. Such mechanisms, well established upon AM colonization, might have been further co-opted to install nitrogen-fixing symbiosis, a hypothesis that is soundly supported by the set of SYM genes required for the colonization by both AM fungi and rhizobia (Markmann and Parniske 2009).

7   Conclusions

This short summary of the recent progresses in our comprehension of the AM establishment and functioning reveals the active role of the host plant in preparing and directing fungal penetration across the root. The plant seems to switch to an

alert status as soon as it perceives fungal presence. This can be visualized in terms of calcium-mediated signalling, gene regulation, and even morphogenetic responses such as the proliferation of lateral roots. A "constructive" phase follows hyphopodium contact, leading to the creation of the intracellular niche where the fungus is hosted in both epidermal and cortical cells. This constructive phase culminates with the establishment of functional arbuscules, which accomplish the key symbiotic functions related to nutrient exchanges.

Altogether, our current knowledge of the AM interaction suggests that the plant is deploying a series of checkpoints along the way to a functional symbiosis. Remarkably, each of these steps is associated with a high probability of failure. Long-distance rhizospheric signalling may be uneffective and the plant and fungus may simply not meet, or the contact may not be anticipated by the required 'pre-alert' status (Genre and Bonfante 2007), and thus result in an arrest of the interaction. Furthermore, hyphopodium adhesion does not guarantee epidermal cell penetration: many of the contacted cells start, but do not complete, PPA development, and even those that do may die upon fungal penetration, possibly due to the loss of the necessary synchrony between the developmental programs of either partner. The dead of colonised cells has often been observed in *in vitro* systems, where the progress of the interaction can be precisely followed over time. Although we cannot exclude that plant cell death may derive from the artificial conditions in which the interaction is being observed, the programmed death of single cells could also be a basic – and very well known in plant pathology – mechanism for limiting the invasiveness of the fungus. Very little is known in fact on the processes by which the extension of the colonization is controlled. Similarly, the arrest of fungal growth through an interruption of the accommodation responses can also occur in the inner tissues. An analogous mechanism, involving the arrest of perifungal membrane proliferation, could be acting to control arbuscule morphogenesis. Javot et al. (2007) described stunned arbuscule morphology in the *M. truncatula* mutants where the arbuscule-specific phosphate transporter *MtPT4* is knocked out, suggesting that the plant can block fungal branching in the presence of an insufficient phosphate supply. Lastly, the maintainance of arbuscule activity in nutrient exchanges is limited in time and ends with arbuscule senescence and collapse (Toth and Miller 1984; Alexander et al. 1988; Javot et al. 2007) but its duration is one more obscure aspect of AM. The few data currently available do not allow the inference of general parameters concerning arbuscule life span in different plant/fungus combinations.

In brief, the establishment of a functional symbiosis appears to be at the end of a series of plant-controlled preconditions, each of which is required but non-sufficient to achieve the next one. We can question whether this dominant position played by the green host is genuine or we are underestimating the role of the fungus because of the limited tools currently available to investigate these troublesome organisms. Each of the above mentioned steps in root colonisation is in fact involving specific fungal morphogenetical programs: hyphal branching in the rhizospere, hyphal swelling and adhesion during hyphopodium formation, controlled growth within the host cells, extensive development in the intercellular spaces, production of frequent and short branches during arbuscule formation. Although we still know very little

about the fungal transcriptional and cellular modifications associated to each of these events, it is easy to speculate that hyphae react to root colonization at least as intensively as the plant cells do, not to mention the need for synchronizing each other's responses and developmental processes. The stable genetic transformation of AM fungi and the identification of the elusive Myc factor and its receptors can today be envisaged as the most urgent steps towards a better definition of each partner's reciprocal role during the establishment of the AM symbiosis.

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Chapter 4
Strigolactones and Their Role in Arbuscular Mycorrhizal Symbiosis

Soizic Rochange

Abstract Molecular signals are exchanged between arbuscular mycorrhizal (AM) fungi and their host plants during the pre-symbiotic stage, and help mutual recognition prior to any contact between the two partners. In particular, root exudates from host plants are known to trigger a switch in fungal development and metabolism, committing the fungus towards the establishment of the symbiosis. Strigolactones, a group of carotenoid-derived metabolites exuded by plant roots, are major contributors to this effect. Their importance in the normal development of mycorrhizae has been established through the analysis of strigolactone-deficient mutants. Interestingly, strigolactones were previously known as germination stimulants of the parasitic plants *Striga* and *Orobanche*. In addition, our group and others recently proposed that strigolactones or related compounds are novel hormones regulating shoot branching in monocots and dicots. The activity of strigolactones on three different types of organisms has stimulated progress in the understanding of their functions. This chapter reviews the current knowledge of strigolactone structural features, and recent advances and prospects in the elucidation of their biosynthetic pathway and of their mode of action.

Abbreviations

AM   Arbuscular mycorrhizal
CCD   Carotenoid cleavage dioxygenase
RNAi   RNA interference
SMS   Shoot multiplication signal

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NCED   Nice-Cis-Epoxycarotenoid dioxygenase
ABA   Abscisic acid
GA   Gibberellic acid

The existence of pre-symbiotic molecular signals exchanged between the plant and fungal partners during arbuscular mycorrhizal (AM) interaction was demonstrated long ago (Mosse and Hepper 1975). These signalling events probably contribute to mutual recognition and to the preparation of symbiotic stages. Most conspicuous is the developmental switch triggered in AM fungi by root-derived signals. As obligate biotrophs, AM fungi do not exhibit sustained growth in the absence of a host root. Rather, hyphae produced by germinated spores grow for a limited period of time, then undergo cytoplasmic retraction and septation (Mosse 1988). Host roots have the ability to stimulate spore germination as well as subsequent hyphal growth and to trigger a morphological change in hyphae described as branching (Giovannetti et al. 1993; Buée et al. 2000). This easily visible morphological change has been used as a marker of plant perception by the fungus. This effect does not require contact between the root and the fungus, seems to involve diffusible signals of less than 500 Da (Giovannetti et al. 1996), and can be reproduced with root exudates alone. Root-exuded stimulatory compounds were called "branching factors", and their identity remained unknown for years.

Reciprocally the fungus emits diffusible signals that affect root gene expression (Kosuta et al. 2003; Weidmann et al. 2004), intracellular signalling events (Navazio et al. 2007; Kosuta et al. 2008) and lateral root development (Oláh et al. 2005). These compounds, called "Myc factors" by analogy with the well-known Nod factors in Rhizobium/legume symbioses, are currently being characterized.

1   The Elusive "Branching Factors"

Given the known contribution of flavonoids as signals in Rhizobium/legume interactions, the activity of these compounds on pre-symbiotic fungal development was first examined. Indeed, several flavonoids can enhance growth and/or branching of AM fungal hyphae (Requena et al. 2007). However, effects of individual flavonoids seem to depend on AM fungal species (Scervino et al. 2005). Moreover, flavonoid-deficient chalcone synthase mutants of *Zea mays* exhibit normal colonization rates by AM fungi (Bécard et al. 1995), and their root exudates display a branching activity comparable to those of wild-type plants (Buée et al. 2000). These genetic arguments rule out the possibility that flavonoids are essential components of the branching-stimulatory activity of root exudates.

An *in vitro* bioassay on *Gigaspora* spp. was designed to develop non-targeted, purification-based approaches towards identification of the branching factors (Nagahashi and Douds 1999). Buée et al. (2000) semi-purified an active fraction from carrot root exudates and demonstrated the production of branching factors by roots of diverse plant species and mutants. It took several years until Akiyama et al. (2005)

finally succeeded in purifying and identifying a branching factor from *Lotus japonicus*, 5-deoxy-strigol. This compound belongs to the strigolactone family of secondary metabolites and is exuded in minute amounts, explaining the difficulties to purify this molecule in sufficient quantities for chemical identification. These findings were confirmed and extended by Besserer et al. (2006), who detected a strigolactone in an active root exudate fraction from a monocot, sorghum. Natural strigolactones as well as synthetic analogues like GR24 were found to be active on a range of diverse AM fungi at subnanomolar concentrations. In some AM fungal species for which hyphal branching is difficult to observe like *Glomus* spp., strigolactones were shown to enhance spore germination rates. The production of strigolactones by a wide range of plant species and their activity on phylogenetically distant fungi are consistent with the broad distribution of AM symbiosis and the low degree of specificity in this interaction, pointing towards a "universal" mode of communication between plants and AM fungi.
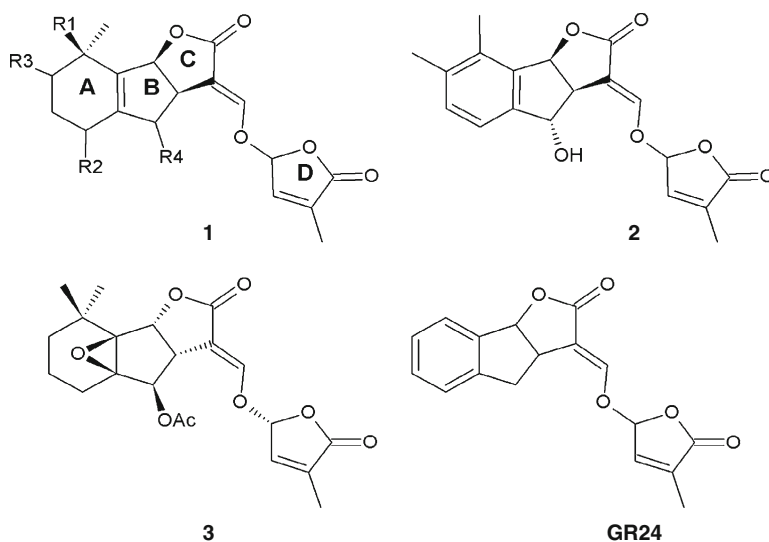
2   Structure, Occurrence and Metabolic Origin of Strigolactones

2.1   Structural Features

Strigolactones had been known since the 1970s as root-released compounds able to trigger germination of parasitic plant seeds. Parasitic plants of the genera *Striga* and *Orobanche* are, like AM fungi, obligate biotrophs. They produce high numbers of tiny seeds, practically devoid of reserves, that germinate only in the close vicinity of host (or false host) plant roots. The considerable damage caused to crops by these parasites in many regions of the world led researchers to identify the germination stimulants produced by host plants. Cook et al. (1966, 1972) completed the daunting task of characterizing the major germination stimulants produced by cotton, strigol and strigyl acetate (Fig. 1). These molecules comprise four carbon rings, two of which (C and D) carry a lactone function. The D ring is linked to the ABC moiety via an enol ether bond. The collective name strigolactones was given to compounds sharing the same overall structure (Butler 1995). Strigolactones, which stimulate beneficial AM fungi and parasitic weeds, seem to have both positive and deleterious effects on the plant that produces them (Paszkowski 2006). Since AM fungi have a far more ancient evolutionary origin than parasitic angiosperms, it seems likely that rhizosphere signalling mediated by strigolactones was first used for AM symbiosis then exploited by parasitic weeds to detect their hosts.

2.2   Diversity and Distribution

In the past few years strigolactones have been characterized in root exudates of several plant species including dicots and monocots (for a recent review see

Fig. 1 Chemical structures of known natural strigolactones and the synthetic analogue GR24. (1) General structure of strigolactones. The substitutions found in different strigolactones are listed in the table. Two strigolactones bear differences with this general structure on the A ring: solanacol (2) and fabacyl acetate (3). This revised structure for solanacol has recently been proposed by Takikawa et al. (2009)

	R1	R2	R3	R4
strigol	CH$_3$	OH	H	H
strigyl acetate	CH$_3$	OAc	H	H
sorgolactone	H	H	H	H
orobanchol and 2'-epiorobanchol	CH$_3$	H	H	OH
orobanchyl acetate	CH$_3$	H	H	OAc
5-deoxystrigol	CH$_3$	H	H	H
sorgomol	CH$_2$-OH	H	H	H
7-oxoorobanchol	CH$_3$	H	=O	OH
7-oxoorobanchyl acetate	CH$_3$	H	=O	OAc
7-hydroxyorobanchyl acetate	CH$_3$	H	OH	OAc

Yoneyama et al. 2009). To date, 13 different strigolactones have been characterized and another 5 structures have been tentatively proposed. It can be anticipated that more remain to be identified given the different possibilities of substitution at several sites on the strigolactone backbone (Fig. 1). Stereochemistry contributes to the diversity of strigolactones and affects bioactivity on *Striga* and *Orobanche* seeds, although the importance of this parameter depends on the parasitic plant species (Zwanenburg et al. 2009).

Although strigolactones have been analysed in a limited number of taxa, the broad picture is that a given species produces a mixture of two to five different strigolactones, which overlap between species. For example the different species of Fabaceae produce a combination of three to five out of six strigolactones present in the family (Yoneyama et al. 2008; Xie et al. 2009). The amounts and ratios of different strigolactones can vary depending on the cultivar, age and growth conditions (Yoneyama et al. 2009). Strigolactones have been detected in a variety of plant species, and there is no report of a plant that does not produce them. Of particular interest is the case of non-mycotrophic plants. *Arabidopsis thaliana* and white lupin, which are non-hosts for AM fungi, produce strigolactones (Goldwasser et al. 2008; Yoneyama et al. 2008), in spite of the potential deleterious effects of these compounds through parasitic interactions. A possible explanation has emerged with the discovery of strigolactone hormonal function *in planta* (see Section 4).

Strigolactone detection remains difficult in most species and requires very sensitive mass spectrometers, as well as a knowledge of candidate molecular structures. It will hopefully become easier in the coming years with the improvement of mass spectrometers and the continuing progress in the analysis of novel strigolactones. Studying the distribution of strigolactones within and outside the plant kingdom should shed light on the evolution of these compounds, their synthesis and possible functions in addition to those already known.

2.3 Biosynthetic Origin

Strigolactones have been extensively studied, originally because of the germination stimulant activity of these compounds towards parasitic weeds, which represent an important nuisance in agriculture. Early studies assumed that they were sesquiterpene lactones, i.e. derived from farnesyl diphosphate. On the basis of structural similarities with other compounds, Bouwmeester et al. (2003) proposed that alternatively, strigolactones may derive from the carotenoid pathway. This hypothesis was investigated through pharmacological and genetic approaches (Matusova et al. 2005). Maize plants were treated with inhibitors targeting the synthesis of carotenoids or their precursors. Notably, exudates of plants treated with fluridone, which inhibits phytoene desaturase, the second dedicated enzyme in the carotenoid pathway, exhibited reduced ability to stimulate *Striga* germination. The same phenotype was observed in mutants with reduced amounts of carotenoids. Although strigolactone production was not measured directly in this study (it was assessed indirectly via *Striga* seed germination tests), these two lines of evidence strongly supported the hypothesis that strigolactones derived from carotenoids. In the same article, Matusova et al. (2005) proposed a series of reactions that could yield strigolactones starting from a C_{40} carotenoid such as β-carotene. In this tentative pathway, the precursor carotenoid is first cleaved to yield a C_{15}-aldehyde intermediate. The terminal cycle of β-carotene is left intact by this cleavage, providing the A ring of strigolactones. Further hydroxylation, hydrogenation, oxidation, epoxidation,

decarboxylation and cyclization reactions would then afford the ABC moiety. The D ring is speculated to be produced by another pathway, and coupled to the ABC moiety to produce 5-deoxy-strigol, which would subsequently be converted to the different forms of strigolactones.

Candidate enzymes in the strigolactone biosynthetic pathway were proposed only for the initial cleavage step. Other catalytic steps were attributed to enzymes belonging to large multigene families. Nonetheless, this paper proved extremely valuable to the community, not only by placing strigolactones in the right metabolic pathway, but also by opening new possibilities to find strigolactone-deficient mutants. This eventually led to decisive clues about a new, unexpected function of strigolactones (see Section 4).

3   Importance in AM Symbiosis

Whether hyphal branching is relevant in itself (for example by enhancing the chances of root contact) or is a manifestation of other fungal events is still a matter of debate. Section 6 will review evidence that strigolactones trigger important changes in fungal energy metabolism, which could be the really important event committing the fungus towards symbiosis. In any case, because strigolactones are not the only compounds known to stimulate hyphal growth and branching, we wanted to determine their importance in pre-symbiotic fungal development and in AM interactions more generally. To address this question, we tried to obtain plants unable to produce strigolactones. A first step involved an approach similar to that developed by Matusova et al. (2005). Maize plants were treated with the carotenoid synthesis inhibitor fluridone, and their ability to develop AM symbiosis was examined (Gomez-Roldan et al. 2007). Treated plants were significantly less colonized than the controls, and this effect could be partially reverted by exogenous treatment with the strigolactone synthetic analogue GR24. Similar results were obtained with carotenoid-deficient mutants. Nevertheless, this study did not provide definite conclusions on the importance of strigolactones, because (a) strigolactone production in the different plants was not measured, and (b) inhibition of the general carotenoid pathway induces pleiotropic effects including photobleaching, and prevents growth under normal conditions for a long period of time. We thus decided to search for plant mutants affected further down the pathway, to avoid any effects unrelated to strigolactones. Given the difficulty to detect and quantify these compounds in root exudates, a direct screen for strigolactone-deficient plants would have been extremely difficult. Instead, we took a reverse genetic approach based on the tentative biosynthetic pathway proposed by Matusova et al. (2005). As explained in the previous section, this pathway postulates the cleavage of a carotenoid substrate (possibly a C_{40} carotenoid like β-carotene). In contrast with later catalytic steps, carotenoid cleavage is potentially carried out by a limited number of enzymes. These Carotenoid Cleavage Dioxygenases (CCDs) comprise a small multigene family of around ten

members (Schwartz et al. 2004), making them amenable to a reverse genetic approach. Before launching a systematic RNAi strategy for all members, we decided to analyse already characterized mutants. Such mutants were available for CCD7 and CCD8 in several plant species. We decided to focus on pea mutants *rms1* (*ccd8*) and *rms5* (*ccd7*). Wild-type pea plants produce two major strigolactones, orobanchyl acetate and fabacyl acetate (Gomez-Roldan et al. 2008; Yoneyama et al. 2008; Xie et al. 2009). We showed that both *rms1* and *rms5* mutants were unable to produce these two compounds at detectable levels (Gomez-Roldan et al. 2008). The ability of their root exudates to stimulate *Gigaspora rosea* hyphal branching was significantly reduced. In addition, root colonization by AM fungi was strongly reduced in these mutants compared to the wild-type, although they were not completely unable to develop symbiotic structures. Exogenous treatment with GR24 restored root colonization almost back to normal rates (Gomez-Roldan et al. 2008). Together, these results indicate that strigolactones are necessary for a normal level of root colonization. It must be noted, however, that the strigolactone-deficient mutants are still able to form mycorrhizal structures. Whether residual mycorrhization is due to low amounts of strigolactones in the mutants or to other stimulatory compounds is still unclear. This question will be difficult to answer because the fungal response to strigolactones is several orders of magnitude more sensitive than their detection by mass spectrometry. Interestingly, the severity of the mycorrhizal phenotype seems to depend on inoculum density (V. Gomez-Roldan and S. Rochange, unpublished results). It is possible that a high inoculum density enhances the probability for fungal spores to be in the close vicinity of roots, thereby decreasing the need for stimulation by strigolactones.

The same mutants were used to determine the importance of strigolactones in the stimulation of parasitic plant seed germination. As in the case of AM fungi, different classes of compounds including dihydrosorgoleone, sesquiterpene lactones and strigolactones are known to be active on *Striga* and/or *Orobanche* seeds (Bouwmeester et al. 2003). The ability of root exudates of strigolactone-deficient pea mutants to trigger germination of *Orobanche crenata* seeds was dramatically reduced (Gomez-Roldan et al. 2008), indicating that strigolactones are major germination stimulants in this species. Strigolactone-deficient mutants of rice also induced less germination of *Striga hermonthica* seeds, and exhibited enhanced resistance to infection by this parasite (Umehara et al. 2008).

4 Hormonal Function in Plants

The *ccd* mutants mentioned above had been extensively studied previously for their developmental phenotype. These mutants and others in the same signalling pathway exhibit enhanced shoot branching. Genetic and physiological studies – including an important series of graft experiments – led to the conclusion that

ccd7 and *ccd8* mutants lacked a novel hormone inhibiting lateral bud outgrowth (see for example Johnson et al. 2006). This hormone, clearly distinct from other signals controlling apical dominance and shoot branching like auxin and cytokinin, was temporarily called SMS for Shoot Multiplication Signal. Despite the efforts of several groups to characterize the chemical structure of the SMS, its identity remained elusive.

Having shown that both *rms1* and *rms5* pea mutants were strigolactone-deficient, and knowing that these mutants also lacked the SMS, we hypothesized that strigolactones could be the long sought-after signal. In a collaborative study, we demonstrated that strigolactones exhibit the expected properties of the novel hormone: they can inhibit lateral bud outgrowth whether applied locally on buds or fed to the shoot vascular system, and can act at low concentrations and in a specific manner: no effect was observed on SMS-insentive *rms4* mutants (Gomez-Roldan et al. 2008). Simultaneously Umehara et al. (2008) reached the same conclusion using an equivalent series of high tillering mutants in rice: *d10* (*ccd8*) and *d17* (*ccd7*) mutants are unable to produce strigolactones, and their tillering phenotype can be rescued by supplying strigolactones in the watering solution. Furthermore, *d3* mutants (orthologous to *rms4*) remain insensitive to strigolactone application. The effect of strigolactones on shoot branching was extended to *Arabidopsis thaliana* (*max3*, *max4* and *max2* mutants) in both studies. Together, these results strongly support the hypothesis that the novel hormone is a strigolactone or related compound. This discovery has raised considerable interest from the plant development community. Recent advances in the understanding of this new strigolactone function include the proposition that they act downstream of auxin in the control of shoot branching (Brewer et al. 2009), and that auxin and strigolactones control each other's levels in a regulatory feedback loop (Hayward et al. 2009). Meeting reports have also suggested additional functions for strigolactones in plant development, making this new field of investigation very likely to expand in the coming years.

Coming back to AM fungi, it is noteworthy that they not only perceive plant hormones released in soil, but also seem to interfere with the production of these signals: indirect evidence suggests that strigolactone synthesis is reduced in mycorrhized plants (Lendzemo et al. 2007). There are many reports of manipulation of plant hormonal signalling by micro-organisms, including symbiotic fungi. A recent example is the release of auxin and ethylene by truffle, *Tuber melanosporum* (Splivallo et al. 2009). In this respect, it would be very interesting to investigate whether strigolactones also play a role in root development, since AM fungi are known to modulate lateral root initiation (Oláh et al. 2005). The fact that our group contributed to the identification of a new plant hormone while studying signals in AM symbiosis illustrates how powerful AM fungi can be to reveal important features of plant physiology. This probably relates to the ancient origin of these organisms and their co-evolution with plants for several hundred million years. In the future, AM fungi may again help discovering important signals and mechanisms in plants.

5 Strigolactone Synthesis and Its Regulation

5.1 Biosynthetic Pathway

Despite the interest of different communities for strigolactones, their biosynthetic pathway is still largely unknown. The demonstration that strigolactones are apocarotenoids rather than sesquiterpene lactones (Matusova et al. 2005) was a major step forward and the biosynthetic pathway proposed in the same article, although still speculative, serves as a useful framework for further studies.

This pathway postulates as a first step the cleavage of a carotenoid substrate. Rani et al. (2008) proposed 9(Z)-β-carotene as the most likely candidate, but also remarked that (apo)carotenoids with a γ-ring, although not yet reported in higher plants, could be excellent intermediates in strigolactone synthesis.

Carotenoid cleavage is a common reaction in plant secondary metabolism and can be carried out by CCDs. A sub-group of CCDs, 9-cis-epoxycarotenoid dioxygenase (NCEDs), act on epoxy substrates and are involved in abscisic acid (ABA) synthesis. Because NCED mutants (maize *vp14*, Matusova et al. 2005 and tomato *notabilis*, López-Ráez et al. 2008) are partly impaired in *Striga* and *Orobanche* germination stimulant activity, it has been suggested that NCEDs could participate in strigolactone synthesis. However, the strigolactone content in root exudates of these mutants was not determined by biochemical methods, and a reduced production of germination stimulants could be an indirect consequence of the phenotype of the ABA-deficient mutants rather than a direct effect of the mutations on the strigolactone biosynthetic pathway. In contrast, a link between pea *ccd7* and *ccd8* mutations and strigolactone deficiency has been firmly established: strigolactones are undetectable in root exudates and root extracts of both mutants, while the *rms4* mutant with the same developmental phenotype produces strigolactones normally (Gomez-Roldan et al. 2008). Similar observations in rice (Umehara et al. 2008) reinforce this finding.

The involvement of two non-redundant CCDs in strigolactone synthesis came as a surprise since only one cleavage step was expected. Based on the catalytic properties of recombinant AtCCD7 and AtCCD8 in *E. coli*, Schwartz et al. (2004) proposed that these two enzymes act sequentially. AtCCD7 would cleave a C_{40} substrate, and its product would be cleaved again by AtCCD8 to yield 13-apo-β-carotenone (C_{18}). In this hypothesis, the role of CCD7 would thus be to provide an appropriate substrate for CCD8 to cleave. Whether this series of reactions is relevant *in planta* for strigolactone synthesis is still unclear. The carotenoid substrate, CCD7 and CCD8 cleavage sites and their order of intervention remain to be clarified. According to Matusova et al. (2005), a dozen further steps may be necessary to produce the ABC moiety of strigolactones. Rani et al. (2008) examined them in more detail and proposed various combinations to yield the different strigolactone structures. The possible involvement of cytochrome P450 proteins is supported by the fact that Arabidopsis *max1* mutants, which carry a mutation in a cytochrome P450 family member, are impaired in SMS

synthesis (Booker et al. 2005). How the D-ring is produced and coupled to the ABC moiety remains totally unknown, although hypotheses involving a cytosolic origin have been put forward (Matusova et al. 2005).

The newly discovered hormonal function of strigolactones in the regulation of shoot branching will certainly accelerate deciphering the pathway, because it is a lot easier to screen mutants on a developmental phenotype than on the ability of their root exudates to trigger *Striga* germination or AM hyphal branching. Rice seems to emerge as a very powerful model for such studies: in addition to large mutant collections and genomic tools, it offers the possibility to screen for enhanced tillering at the seedling stage. Since the discovery of the hormonal function of strigolactones, three additional mutations conferring this phenotype have been identified in rice by map-based cloning (Lin et al. 2009; Arite et al. 2009; Liu et al. 2009). One of these may affect an enzyme in the strigolactone biosynthetic pathway. DWARF27 (D27) is a novel iron-containing protein localized in the chloroplast. Corresponding mutants are unable to produce the major rice strigolactone, 5-deoxy-strigol, and their tillering phenotype can be rescued by exogenous GR24 application. It is therefore proposed that D27 takes part in strigolactone synthesis, possibly through a redox reaction (Lin et al. 2009). Such genetic approaches seem promising to identify new steps in the strigolactone biosynthetic pathway. Genetic redundancy may hinder the analysis of some of the steps, but the combination of various plant models may help circumvent this problem.

5.2   Regulatory Factors

How strigolactone synthesis is regulated at the molecular level has not been investigated yet, the elucidation of the pathway being still in its infancy. Nonetheless, several physiological conditions are known or suspected to affect strigolactone production.

It has been known for a long time that parasitic plant damage is lower in fields that are highly fertilized (Bouwmeester et al. 2007; Yoneyama et al. 2007b). Biochemical analyses demonstrated that strigolactone production could indeed be reduced by high nutrient availability (Yoneyama et al. 2007a, b; López-Ráez et al. 2008). Analysis of root exudates or root extracts gave similar results, indicating that synthesis itself was targeted, rather than just exudation (Yoneyama et al. 2007a; López-Ráez et al. 2008). This strong regulation makes sense in the context of AM symbiosis, whose main benefit to the plant is the supply of phosphorus (P). AM symbiosis is itself strongly down-regulated by high P fertilization. A reasonable hypothesis is that high P supply inhibits AM symbiosis via a reduced release of strigolactones, which would hamper pre-symbiotic fungal development (Bouwmeester et al. 2007; Yoneyama et al. 2007b). In agreement with this idea, down-regulation of strigolactone production by high P seems to be restricted to mycotrophic species. Strigolactone production is unaffected by P in white lupin, a non-mycotrophic member of the Fabaceae, whereas high P strongly reduces strigolactone content in

root exudates of red clover, a mycotrophic species in the same family (Yoneyama et al. 2007b, 2008). It remains to be investigated, however, whether strigolactone modulation is the only means by which plant limit AM colonization under high P.

Regulation of strigolactone production by other nutrients, especially nitrogen, is less clear. While N deficiency stimulates 5-deoxy-strigol production in sorghum to the same extent as P deficiency (Yoneyama et al. 2007a), orobanchol content is unaffected by N in red clover (Yoneyama et al. 2007b). These discrepancies may relate to the differential abilities of plant species to obtain nitrogen from AM fungi and other sources, but analysis of additional species from different families will be necessary to confirm this hypothesis.

Mycorrhization itself has also been postulated to down-regulate strigolactone production. This assumption is currently based on indirect evidence, like the reduced ability of root exudates from mycorrhizal maize plants to induce *Striga* germination (Lendzemo et al. 2007). This hypothesis, if confirmed biochemically, may account for the decreased capacity of mycorrhizal plants to undergo further AM colonization (Vierheilig 2004). It is supported by the fact that exudates from mycorrhizal and non-mycorrhizal roots have different impacts on the colonization of another root system (Vierheilig et al. 2003), indicating that the so-called autoregulation of AM symbiosis relies at least in part on exudate-mediated mechanisms.

It is tempting to speculate on the potential consequences of nutrient- or AM-controlled strigolactone production on plant development, and more particularly shoot branching. While hairy roots grown *in vitro* are able to produce strigolactones, demonstrating that the complete pathway is active in root cells, it is not known yet whether strigolactones in shoots are produced *in situ* or transferred from roots. Strigolactones seem to be even more difficult to detect in shoots than in roots, and data on nutrient effects on shoot strigolactone content is still scarce. This limited amount of information tends to argue against a similar regulation in roots and shoots (Yoneyama et al. 2007a). In any case, it will be difficult to uncouple nutritional from hormonal effects when studying the impact of nutrient supply on growth and development.

6 Strigolactone Mode of Action

6.1 Cellular Effects

Among the three target organisms of strigolactones, AM fungal hyphae are unique unicellular systems that are most amenable to cell biology and microscopy studies. Increases in phosphate uptake, plasmalemma ATPase activity (Lei et al. 1991), cytosolic pH (Jolicoeur et al. 1998) and transmembrane electric potential difference (Ayling et al. 2000) have been reported in *Gigaspora* spp. growing in the presence of host roots or root exudates. More recently, gene expression studies pointed towards an effect of branching factors on mitochondrial activity and respiration

(Tamasloukht et al. 2003). This prompted Besserer et al. (2006) to examine in detail mitochondrial responses to strigolactone stimulation. Within 1 h, GR24 treatment increased O_2 consumption and mitochondrial density (number of mitochondria per unit of area). Changes in shape and motility were also observed in mitochondria of stimulated hyphae. As shown by treatment with an inhibitor, this increased mitochondrial biogenesis was essential for the hyphal branching response (Besserer et al. 2009). The observed increase in mitochondrial activity reflected enhanced oxidative metabolism as demonstrated by increased NADH and ATP production (Besserer et al. 2008). Different branches of the respiratory chain seemed to be required for hyphal branching and spore germination responses (Besserer et al. 2009). Enhanced respiration paralleled an increased mitosis rate, which allowed to maintain the number of nuclei per unit of hyphal length despite increased growth rates (Besserer et al. 2008). The overall picture seems to be that increased mitochondrial biogenesis and catabolic activity function to meet an increased energy demand in stimulated hyphae and accompany cell cycle progression.

Interestingly, these very rapid cellular responses seem to precede over-expression of genes involved in respiratory metabolism, suggesting that the short-term response relies on post-transcriptional or post-translational modifications. This observation related to the fungal response to GR24 alone contrasts with the results of Tamasloukht et al. (2003) who reported increased expression of several genes involved in mitochondrial function within 1 h in response to semi-purified root exudates. This discrepancy indicates a complex response pattern, possibly related to the activity of other exuded compounds in addition to strigolactones.

The switch in metabolic activity induced by strigolactones may well be the real trigger committing AM fungi towards growth and symbiotic development, and hyphal branching could be a secondary effect of this boost in energy metabolism. What is commonly described as branching is in fact a complex morphological response that can be attributed to a mixture of compounds (Nagahashi and Douds 2007). The chapter by G. Nagahashi et al. in this book covers root-exuded metabolites other than strigolactones that are able to stimulate hyphal branching. Whether these compounds also act by enhancing respiration remains to be investigated to establish a tight link between mitochondrial activity and morphological observations.

It is not known whether strigolactone perception leads to similar signalling and cellular events in the three target biological systems: AM fungi, parasitic weed seeds and plant buds. Cellular response to strigolactones in the latter system has not been investigated yet, but it is difficult to picture how a reaction similar to that of AM fungi (enhanced respiration) could contribute to keep buds dormant. In contrast, stimulation of mitochondrial activity seems a very plausible mode of action in the case of parasitic plant seeds, since seed germination in general is associated with increased respiration and mitochondrial biogenesis (Logan et al. 2001). In addition, strigolactones are known to stimulate ethylene production by *Striga* seeds, and ethylene can in turn enhance mitochondrial metabolism and trigger seed germination. It has therefore been postulated that ethylene acts as a signal mediating strigolactone-induced germination (Logan and Stewart 1991). Whether this also applies to AM fungi and to lateral buds deserves further investigation.

6.2 Perception Mechanisms

The activity of some strigolactones on AM fungi at subpicomolar concentrations (10^{-13} M, Besserer et al. 2006) suggests the existence of a strigolactone receptor and amplification mechanism. Identifying a putative receptor will be a difficult task in AM fungi, because their genetic structure prevents mutant screening and stable genetic transformation methods are not available yet, despite recent progress in transient gene transfer (Helber and Requena 2008). Nevertheless, the *Glomus intraradices* genome sequencing project (Martin et al. 2008) may help finding candidate genes in this species once receptors are identified in other systems. Structure-activity relationships (SAR) studies will probably be very useful to determine the degree of similarity between strigolactone perception systems in the different organisms that respond to these compounds. SAR have been extensively investigated for the parasitic seed germination response and recently reviewed (Yoneyama et al. 2009; Zwanenburg et al. 2009). The C–D part of the molecule seems essential for bioactivity on *Striga* and *Orobanche* seeds, and a model for the interaction of strigolactones with their receptor has been proposed (Mangnus and Zwanenburg 1992). Attempts to identify a strigolactone receptor in *Striga* seeds using biochemical techniques are under way (e.g. affinity chromatography of biotin-labelled strigolactone analogues: Reizelman et al. 2003). Although not yet analyzed in equivalent detail, broad structural requirements appear to be similar for AM fungi (Yoneyama et al. 2009).

The search for a receptor mediating the shoot branching inhibiting activity of strigolactones may well benefit from genetic studies. Mutants such as pea *rms4*, Arabidopsis *max2* and rice *d3* are insensitive to strigolactone application. The mutated genes encode an F-box protein with a leucine-rich repeat domain (Stirnberg et al. 2007). Such proteins function as substrate-recruiting subunits of E3 ubiquitin ligase complexes and trigger the degradation of their targets by the 26S proteasome. They are often involved in signal transduction, and sometimes even in direct signal perception, as in the case of the well-known auxin soluble receptor TIR1 (Tan et al. 2007).

More recently, another mutant in the strigolactone perception pathway was identified in rice (Arite et al. 2009). High-tillering *dwarf14* (*d14*) mutants produce strigolactones but are insensitive to strigolactone application. Positional cloning identified D14 as a protein of the α/β-fold hydrolase superfamily (also annotated as esterase/lipase/thioesterase). Independently, Liu et al. (2009) reported on another high tillering rice mutant, *htd2*. Although not analysed in as much detail and for some reason given a different locus number, *htd2* is on the basis of sequence data allelic to *d14*. D14/HTD2 shares similarities with *Regulator of Sigma B* (*RsbQ*) from *Bacillus subtilis* (Arite et al. 2009), a protein with a hydrophobic cavity that can accomodate a small compound. Some members of the α/β-fold hydrolase superfamily of proteins do not possess catalytic activities but have been identified as receptors, like the gibberellin receptor GID1 (Hirano et al. 2008). When bound to gibberellic acid (GA), GID1 interacts with DELLA transcriptional regulators. This results in binding of the DELLA proteins to SLY1 (an F-box subunit of E3 ubiquitin ligase) and in their degradation by the 26S proteasome.

It is tempting, although totally speculative at this stage, to imagine a strigolactone perception system similar to the described gibberellin pathway. Strigolactones would bind D14/HTD2, which would then interact with a target protein (possibly a DELLA). This protein would then be targeted for degradation in the proteasome by RMS4/MAX2/D3. This speculation is supported by the observation that GA-deficient *ga1* mutants display greatly enhanced shoot branching (Silverstone et al. 1997), while a DELLA mutant in tomato, *procera*, exhibits decreased outgrowth of basal lateral buds (Bassel et al. 2008). In addition, gibberellins are known to stimulate seed germination, as strigolactones do in *Striga* and *Orobanche*. Some degree of cross-talk between strigolactone and gibberellin signalling is therefore a reasonable hypothesis to investigate. For the meantime, receptor candidates RMS4/MAX2/D3 and D14/HTD2 identified by genetic studies shall be analysed biochemically to determine their ability to bind strigolactones. Finally, additional information on strigolactone perception should come from cellular and subcellular localisation of strigolactones in the different biological systems using labelled compounds, such as the recently reported fluorescent strigolactone analogues (Bhattacharya et al. 2009).

In conclusion, strigolactones have important functions in the rhizosphere and within the plant that produces them. Their triple action has driven interest of a large community of plant scientists to this class of compounds, leading to an important acceleration of research in this field. As an illustration, the number of articles retrieved by a "strigolactone" search in PubMed has more than doubled in the last 12 months. Each of the target organisms has its advantages, allowing progress in one of the aspects of strigolactone research and benefiting the whole community. For example, parasitic plants provide a simple and reliable bioassay for strigolactone detection, which helped their identification and led to the important discovery that these compounds are carotenoid-derived. AM fungi, with their unicellular hyphae, allowed to identify mitochondria as a cellular target of strigolactones. Finally, genetic studies carried out in pea, *Arabidopsis* and rice have fueled new candidate genes in the strigolactone biosynthetic and perception pathways. This "synergistic" context offers a stimulating framework to study strigolactones and promises exciting discoveries in the near future.

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Part II
Host-fungal Interactions:
Post-penetration

Chapter 5
Molecular–Physiological Aspects of the AM Symbiosis Post Penetration

Philipp Franken

Abstract The establishment of an arbuscular mycorrhizal symbiosis is characterised by several stages until the fungus has penetrated the root epidermis. The present chapter deals with the subsequent phase where the fungus colonises the root cortex and, when the different functions of the symbiosis are realised. After summarising the morphological and cytological characteristics of this symbiotic phase, different strategies will be described which have been followed to understand the molecular–physiological basis of the symbiosis. Non-targeted approaches resulted in new hypotheses concerning mycorrhizal functioning based on the identification of differentially expressed transcripts or proteins. Some of these hypotheses were further proofed by various biological techniques as localisation of the gene products or down-regulation of the genes in transgenic plants. In contrast targeted approaches were directed to particular plant and fungal functions. Among those functions, the present chapter will concentrate on the assimilation, the metabolism and the distribution of carbohydrates in the plant and the fungus.

Keywords Arbuscule • Array hybridisation • C transfer • Carbohydrate • Expressed sequence tag • Metabolomics • Photosynthesis • Proteomics • Transcriptomics

Abbreviations

AM	Arbuscular mycorrhizal
BAS	Branched absorbing structures
EST	Expressed sequences tags
PPA	Prepenetration apparatus
SUT	Sucrose transporter
TGA	Triacylglyceride

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1 Morphological Features and Cytological Aspects

Arbuscular mycorrhizal (AM) fungi colonise plant host tissues in a unique way. After entering the root from a hyphopodium, they first grow intercellularly or cross outer cells with linear or simple coiled hyphae (Gianinazzi-Pearson and Gianinazzi 1988). Reaching the inner cortex the fungal symbionts switch to a different mode of colonisation. They penetrate the plant cell wall and enter the apoplast. Between cell wall and plant plasma membrane fungal hyphae extensively ramify to form a highly branched haustorium, called arbuscule. This hyphal tree is totally surrounded by the plant periarbuscular membrane. This Arum type of development is the one which is most studied, although the Paris type seems to occur more frequently (Smith and Smith 1997). In the Paris type, hyphae grow from cell to cell without any intercellular phase. In the cells of the whole cortex they form large coils with small intercalated arbuscules. The type of mycorrhiza which is formed seems to be determined by the genomes of both partners, and is not under environmental control (Smith and Smith 1997; Cavagnaro et al. 2001). Moreover, many intermediate types can be observed leading to the term 'Arum-Paris-continuum' (Dickson 2004). Other typical structures of AM fungi are vesicles, which are only formed by members of the Glomineae, and as being lipid-rich are considered to be storage organs (Walker 1995). At the same time, the extraradical mycelium of the fungus is growing into the surrounding soil exploring the environment for mineral nutrients and the roots of other plants. The fact that AM hyphal networks interconnect mycotrophic plants of different species in a given ecosystem has led to the term "Plants on the Web" (Read 1998). How much this contributes to the exchange of nutrients and carbohydrates between plants is, however, a matter of debate (Smith and Read 2008). Extraradical symbiotic hyphae differ from the presymbiotic mycelium in that they produce new spores and form so called "branched absorbing structures" (BAS). The latter are small groups of dichotomous hyphae, which cannot be observed in monoculture, but are formed shortly after the establishment of the symbiosis (Bago and Azcon-Aguilar 1998). Their branching pattern recalls arbuscules and there are suggestions that they may be involved in nutrient uptake from the soil. Sometimes these structures subtend developing spores so that new spores are formed at the tip of the branches of the BAS.

The fungal arbuscule and the surrounding plant cell as the central and name-giving organ of the AM symbiosis has been the subject of many cytological investigations. A break-through in the knowledge about arbuscule establishment has been recently achieved (Genre et al. 2008). In vivo confocal microscopy showed that similar to the initial penetration of the root epidermis (Genre et al. 2005), also during colonisation of the cortex, the plant cell assembles a so called Prepenetration Apparatus (PPA). The pattern is different in Arum- and Paris-type mycorrhiza, but the cytological sequence is similar. At first the plant nucleus migrates to the site, where the fungal hyphae contact the cell, and increases its size (Genre et al. 2008). This enlargement is probably accompanied by an increase in ploidy and in transcriptional activity as it has been reported for AM since a long time (Balestrini et al. 1992; Fusconi et al. 2005). At next, an apoplastic tunnel is formed through fusion of membranous components produced by the Golgi apparatus (Genre et al. 2008). This PPA is entered by the hyphal trunk which traverses

the cell with small intercalated arbuscules (Paris-type) or ramifies to one large terminal arbuscule (Arum-type). Small aggregates rich in endoplasmatic reticulum surround the sites of later ramification during this process similar to the initial PPA. Once the arbuscule has been established, the enlarged nucleus is positioned in the centre of the cell, the morphology of the large vacuole is changing and the amount of cytoplasm and the number of organelles is increased (Bonfante and Perotto 1995). Labelling the organelles with green fluorescent protein revealed a network like structure for plastids, mitochondria and the ER surrounding the arbuscules (Fester et al. 2001; Hause and Fester 2005). Recently, the application of fluorescent protein fusions in living cells indicated two distinct domains of the periarbuscular membranes, one surrounding the arbuscule branch and the other around the fine branches (Pumplin and Harrison 2009). This work showed with organelle-specific dyes in addition that the large vacuole is not fragmented in numerous small ones as it was thought before, but that a continues vacuolar system is remained during arbuscule development.

These cytological observations all indicate an increased metabolic activity of the arbusculated plant cell compared to non-colonised cells of the root cortex. In parallel to these changes inside the cell, a new compartment has been developed in the apoplast: the plant–fungal interface which consists of the fungal cell wall, the plant periarbuscular membrane and a matrix between the two organisms (Gianinazzi-Pearson 1996). Analysis of the interface revealed that the fungal wall in the fine branches of the arbuscules contained in contrast to the mycelium inside and outside of the roots an amorphous form of chitin (Bonfante-Fasolo et al. 1990). The matrix is composed of elements from the primary cell wall of the plant, but fungal components could not be detected (Bonfante and Perotto 1995). These findings indicate a facilitated diffusion of substances and together with the detection of high ATPase activity at the plant periarbuscular membrane (Gianinazzi-Pearson et al. 1991) led to the suggestion that the arbuscule is the place of nutrient exchange. As described in other chapters of the book, this could be confirmed for the mineral nutrient transfer from the fungus to the plant. Where, however, carbohydrates are transferred towards the fungus is currently not clear (see below). Interestingly, the presence of fully developed arbuscules is also a prerequisite for mycorrhiza-induced resistance (see other chapter of the book on host response to biotic stress). Arbuscules are not a permanent structure, but have a life span between four and twelve days (e.g. Alexander et al. 1988). AM fungi are coenocytic organisms, but loss of metabolic activity of the fine branches is accompanied by the formation of cross walls between the active and the inactive region of the arbuscule (Dickson and Smith 2001). Finally, arbuscules get fully degraded and the plant cell gets back to its stage before fungal colonisation (Jacquelinet-Jeanmougin et al. 1987).

2 Non-targeted Approaches

In order to understand the molecular basis of the AM symbiosis, the development of techniques to follow non-targeted approaches opened the way to study the transcriptome, the proteome and the metabolome during the symbiotic interaction

and to compare it with the situation of the single partner under non-symbiotic conditions. The outcomes of such comparisons are new hypotheses concerning the involvement of particular genes, proteins or metabolic pathways of the plant and the fungus during symbiotic functioning. Further experiments subsequently tried to confirm these hypotheses by other methodologies.

2.1 Transcriptomics

2.1.1 Differential Expression of Plant Genes

The first non-targeted attempt to identify mycorrhiza-regulated plant genes was the differential screening of a tomato mycorrhiza cDNA library (Tahiri-Alaoui and Antoniw 1996). Several genes were identified and one putatively encoded a cullin. Cullins are involved in cell cycle control which indicates a role of the gene in the ploidisation of the nuclei in arbusculated cells mentioned above (Tahiri-Alaoui et al. 2002). Differential cDNA screening resulted further in the identification of a mycorrhiza-induced H^+-ATPase gene in barley probably involved in nutrient transfer (Murphy et al. 1997) and the P_i-repressed gene *Mt4* in *Medicago truncatula* (Burleigh and Harrison 1997), a suitable marker for mycorrhization (Burleigh and Harrison 1999).

Another technique, the differential RNA display, was used for the analysis of mycorrhiza in pea (Martin-Laurent et al. 1997). A number of regulated genes were identified and were further analysed for their expression patterns, cellular localisation or for their structure (Krajinski et al. 1998; Martin-Laurent et al. 1998, 2001). Among those *Psam2* belonging to a gene family putatively encodes a secretory carrier membrane protein (Krajinski et al. 1998). It is repressed in mycorrhiza, but induced in the interaction with fungal pathogens and might be involved in the changes which can be observed in exudate composition after colonisation of the roots with symbiotic or pathogenic microorganisms. For the others, no homology to known genes could be revealed, but the protein PSAM1 is specifically located around young developing arbuscules (Martin-Laurent et al. 1998). A modification of the differential display technique, the cDNA-amplified fragment length polymorphism, was applied to the analysis of mycorrhiza mutants in *Lotus japonicum* (Kistner et al. 2005). This analysis indicated the presence of two independent pathways of signalling, because the mycorrhizal specific induction of genes was abolished by the mutations, while the repression was not.

The third technique used for the early non-targeted transcriptomic analyses was the construction of subtractive cDNA libraries. In a first attempt three mycorrhiza induced genes were identified and two encoded enzymes for cell wall modifications putatively being involved in the formation of the matrix between the plant periarbuscular membrane and the fungal arbuscule (van Buuren et al. 1999). Especially the modification of xyloglucans seems to play an important role, because genes encoding enzymes which modify this cell wall constituents were among those rare ones which are commonly regulated in different plant–fungal combinations

(Maldonado-Mendoza et al. 2005; Grunwald et al. 2009). Refining the construction of subtractive cDNA libraries (subtractive suppressive hybridisation) opened the door to the identification of many more genes and the combination with the reverse Northern Blot technique facilitated the confirmation of their differential expression (Wulf et al. 2003; Brechenmacher et al. 2004).

A further increase in the number of genes which could be analysed was the application of large scale sequencing in plant models used for AM research. These models were at the beginning *Lotus japonicum* and *Medicago truncatula*, where the mycorrhiza can be compared with the root nodule symbiosis on the same plants (Cook et al. 1997). A French consortium started the analysis of the *M. truncatula* transcriptome by sequencing cDNA clones derived from control, mycorrhizal and nodulated roots and clustering of the sequences revealed 6,359 identified genes (Journet et al. 2002). The analysis of EST redundancy in distinct cDNA libraries (electronic Northern blots) resulted in predictions of expression patterns for many putative genes. Further EST collections from *M. truncatula* were established from a mixed cDNA library from mycorrhizal roots at different stages of colonisation (Liu et al. 2003) or from a combination of random and subtractive cDNA libraries (Frenzel et al. 2005). *In silico* and experimental analyses of the latter collection revealed a large set of novel, not previously listed mycorrhiza-specific genes of *M. truncatula* including a number of transporters, genes involved in signalling processes and a family of AM-specific lectins (Frenzel et al. 2005).

The first array analysis was carried out with 2,268 clones spotted on nylon membranes (Liu et al. 2003). Hybridisation with cDNA probes from five time points after inoculation with *Glomus versiforme* or fertilization with low and high amounts of phosphate resulted in a number of genes which were regulated by mycorrhiza at late stages, but not by phosphate. In a subsequent experiment, 4,702 plasmid clones of the same mycorrhizal cDNA library instead of the corresponding inserts were used for hybridisation (Liu et al. 2004). Low abundance transcripts could not be detected with this method but, nevertheless, a number of genes were identified which are probably involved in plant-fungus signalling during the interaction. An array representing a part of the *M. truncatula* root transcriptome not directly targeted to the mycorrhizal symbiosis was produced by spotting the sequences from the French consortium (see above) on nylon membranes and glass slides (Küster et al. 2004). This so called Mt6k-RIT array was validated by hybridisations to roots inoculated with an AM fungus or with the root nodule-forming *Sinorhizobium meliloti*. Several well known marker genes for both symbioses were identified, but new genes could also be detected. A detailed hybridisation analysis of the array was carried out and several hundred genes that are regulated by root symbioses were identified; 75 of these showed an overlapping expression pattern between mycorrhiza and root nodules (Manthey et al. 2004). The 6k array was further used for analysing gene regulation in AM (Grunwald et al. 2009). Hybridisation with probes derived from mycorrhizal, P_i-fertilised and phytohormone-treated roots revealed that only a low number of genes are indirectly regulated in AM by increasing abscisic acid or jasmonate levels, but a high number of genes were detected which were commonly induced or repressed by P_i and the AM fungus *Gigaspora rosea*.

This was probably due to the elevated phosphate concentration in the mycorrhizal plants, because P_i concentration were not increased in mycorrhiza with Glomus species, where the increased P_i uptake was translated into growth (Grunwald et al. 2009) and the overlap of gene expression was very small (Liu et al. 2003; Hohnjec et al. 2005). The most comprehensive array was produced by spotting more than 16,000 oligonucleotides on glass slides representing all identified genes from *M. truncatula* deposited in the TIGR database (http://compbio.dfci.harvard.edu/tgi). These arrays were hybridized with cDNA probes from roots colonized with different AM fungi: *G. mosseae* and *G. intraradices* (Hohnjec et al. 2005) or *G. intraradices* and *Gigaspora gigantea* (Liu et al. 2007). In the latter case, the mycorrhiza-regulation of genes in the shoots was also analysed providing a basis for understanding the systemically induced resistance against a bacterial pathogen (Liu et al. 2007). Recently, an even larger number of genes were screened using the Affymetrix Medicago GeneChip carrying 61,278 sequences (Gomez et al. 2009). Six hundred and fifty-two genes showed regulated expression in *G. intraradices*-colonised roots, which enormously extended the number of 110 genes which were detected under similar conditions on the 16k array (Liu et al. 2007).

Arrays were also produced for the second model legume, *Lotus japonicum* and the first one was based on 18,432 non-redundant clones (Kouchi et al. 2004). The array was hybridised with the AM fungi *G. mosseae* and *Gigaspora margarita* (Deguchi et al. 2007) and numerous genes were identified being regulated during early and late stages of the symbiosis. A second generation of arrays were based on 50,000 genes and produced by the Affymetrix technology (Genechip Lotus1a520343). Hybridisation with probes from *Gigaspora margarita*-colonised roots revealed many genes involved in cell reorganisation which indicates their role in the plant response to intracellular arbuscule accommodation (Guether et al. 2009). The most comprehensive array was screened for rice, because the whole genome is known and was spotted on chips (sySYNG003a). Hybridisation with probes derived from mycorrhizal and roots and roots infected with a biotrophic or a necrotrophic fungus revealed genes being regulated by fungal colonisation (Guimil et al. 2005). Moreover, 34% of the mycorrhizal-regulated genes were also detected in the expression analyses with dicotyledonous plants.

One problem with all the gene expression analyses is the simultaneous occurrence of different developmental stages of the mycorrhizal symbiosis. Even when the root is full of arbuscules, still hyphopodia are formed from runner hyphae along the root and early infection stages can be observed. It was therefore necessary to analyse the spatial expression of the identified genes. This was done by *in situ* hybridisation or in transgenic plants harbouring promoter-GUS constructs (for review: Hohnjec et al. 2006). A different approach was based on the availability of mutants at early and late stages of the symbiosis. Using the late-stage pea mutant RisNod24, it was possible to identify directly genes which were regulated during the post-penetration phase (Lapopin et al. 1999; Grunwald et al. 2004). Recently, the technique of laser-dissection was applied to mycorrhizal research and used to confirm the expression of particular fungal and plant genes in arbuscules and in the surrounding cells (Balestrini et al. 2007; Guether et al. 2009; Gomez et al. 2009).

2.1.2 Post-transcriptomic Research

Since the application of the subtractive hybridisation for mycorrhizal research long lists of mycorrhiza-regulated genes are produced. Comparing these lists revealed a surprisingly low number of genes which were commonly detected in the different plant–fungal combinations (Grunwald et al. 2009). A common trend could be however observed, if one looks at the functional categories being affected by mycorrhization. Beside membrane transport, signalling and response to biotic stress, many genes involved in carbohydrate metabolism are induced or repressed in roots colonised by AM fungi. This is very probably based on the impact of the symbiosis on carbon assimilation and translocation in mycorrhizal plants and will be discussed in detail in the second part of this chapter.

Differential expression of a gene and localisation of the gene products are only good indicators for a particular role in symbiotic functioning. In order to confirm such a gene, it is necessary to have a mutant or to down-regulate its expression. Such analyses has been carried out for an allene oxide cyclase involved in jasmonate biosynthesis (Isayenkov et al. 2005) and for mycorrhiza-specific P_i transporters (Maeda et al. 2006; Javot et al. 2007). In addition to this, transgenic lines produced for other purposes were subsequently analysed concerning their response to mycorrhizal colonisation (e.g. Vierheilig et al. 1992, 1995). The results will be discussed in other chapters of the book concerning P_i transporters, the role of hormones or the control of the fungus by the plant. Beside those reports, only one publication exists about a functional analysis of mycorrhiza-regulated genes. Among a gene family encoding subtilases, mycorrhiza-regulated members were identified in *L. japonicum* (Kistner et al. 2005), in *M. truncatula* (Liu et al. 2003) and in rice (Guimil et al. 2005). Suppression of *SbtM1* and *SbtM3* in *L. japonicum* resulted in reduced colonisation of roots with intraradical hyphae and arbuscules (Takeda et al. 2009). This shows that both genes play a crucial role in post-penetration development of the symbiosis, but the function is not clear, because the substrates of both proteases are not known. It is clear that functional analyses have to be carried out for many more mycorrhiza-regulated genes. Lines with insertions spread over the genome of *M. truncatula* (Tadege et al. 2008), *L. japonicum* (Imaizumi et al. 2005) or rice (Kumar et al. 2005) in combination with advanced screening tools will probably help to understand the role of numerous genes in a relatively short time.

2.1.3 Differential Expression of Fungal Genes

One problem in AM research is the difficult accessibility of the fungal site of the symbiosis. These microorganisms cannot be grown in pure culture, they have very complex genomes distributed among hundreds of nuclei and a system for stable transformation does not exist. Only transient expression of introduced constructs was achieved up to now (Forbes et al. 1998; Helber and Requena 2008). Because it has been estimated that only 1–5% of the mRNA from a

mycorrhiza contains fungal transcripts (Maldonado-Mendoza et al. 2002), it was not surprising to rarely find fungal sequences among the ESTs obtained from a symbiotic root system (Harrison and van Buuren 1995; Harrier et al. 1998; Franken et al. 2000; Delp et al. 2000; Ruiz-Lozano et al. 2002; Brechenmacher et al. 2004). After applying RNA extraction methods to pure fungal material (Franken et al. 1997), it was however possible to establish EST collections from AM fungi (Stommel et al. 2001; Lammers et al. 2001; Jun et al. 2002; Lanfranco et al. 2002; Sawaki and Saito 2001; Aono et al. 2004; Breuninger and Requena 2004). Most non-targeted approaches were used to analyse asymbiotic and pre-symbiotic stages or symbiotic stages under particular environmental conditions like mineral nutrient deficiency or heavy metal contamination. These investigations will be discussed in other chapters of the book. Only one subtractive hybridization was aimed for identifying genes specifically expressed in the post-penetration symbiotic extraradical hyphae (Requena et al. 2002). The international efforts for achieving a genomic sequence from *G. intraradices* will however lead to the identification of many more AM fungal genes (Martin et al. 2008). cDNA probes from laser-dissected regions of roots heavily colonized as mentioned above could be used for screening of genome-wide arrays in order to identify all genes expressed during symbiotic stages. Direct fluorescent *in situ* PCR can be subsequently used to verify and to localize the stage-specific expression of AM fungal genes (Seddas et al. 2008) (Table 1).

2.2   Proteomics

There are numerous early descriptions of protein expression patterns in mycor-rhizal roots by 2D gel electrophoresis (Garcia-Garrido et al. 1993; for review see: Bestel-Corre et al. 2004a). Sometimes single peptides were sequenced, but mostly just patterns could be compared. The establishment of large cDNA collections of mycorrhizal or related species (mentioned above) and the application of mass spectroscopy of small amounts enabled researcher to identify the differential occurring peptides. All investigation were carried out with *M. truncatula* and numerous mycorrhiza expressed proteins could be identified (Bestel-Corre et al. 2002; Valot et al. 2005, 2006). A number of analyses were targeted to particular function of the AM symbiosis as the increased tolerance of mycorrhizal plants to heavy metals (Repetto et al. 2003; Bestel-Corre et al. 2004b; Aloui et al. 2009) or the mycorrhiza-induced resistance against root pathogens (Colditz et al. 2005). Using the combination of 2D gel electrophoresis and mass spectroscopy it was also possible to identify fungal proteins in extraradical hyphae during the symbi-otic phase (Dumas-Gaudot et al. 2004; Recorbet et al. 2009). More advanced technologies using different methods than 2D gel electrophoresis or targeted to the secretome of cells in mycorrhizal roots or of fungal hyphae will enlarge the list of symbiosis-regulated proteins in the plant and the fungus known up to now (Mathesius 2009).

Table 1 History of non-targeted approaches for analysing arbuscular mycorrhiza development and function

93	94	95	96	97	98	99	00	01	02	03	04	05	06	07
			Transcriptomics (plant)											
			Differential screening											
				Differential RNA display										
						Subtractive hybridisation								
						Transcriptomics (fungus)								
							Differential RNA display[a]							
								EST collections						
									Large scale sequencing					
										Array hybridisation				
										Subtractive hybridisation				
											2D PAGE, mass spectroscopy			
													Metabolomics	
Proteomics														
2D PAGE														

[a] Analysis of asymbiotic stages (Requena et al. 1999).

2.3   Metabolomics

Although many studies on the transcriptome and the proteome of the AM symbiosis has been carried out, most investigations of metabolites have been targeted to particular compounds, e.g. to isoflavonoids (Harrison and Dixon 1993), sugars (Amijee et al., 1993), fatty acids (Olsson 1999) or apocarotenoids (Strack et al. 2003). Most important were the analyses of the carbohydrate metabolism of the AM fungi, which will be discussed below. An extensive analysis of metabolites of the symbiotic interaction showed especially changes in the mitochondrial and the plastidial pathways (Lohse et al. 2005). A real non-targeted approach was carried out to investigate time-dependent changes in the metabolome of *M. truncatula* during colonization of the root by an AM fungus (Schliemann et al. 2008). This revealed particular clusters of primary and secondary compounds being specific for the AM symbiosis. More analyses in different plants in combination with transcriptomics and proteomics are now necessary to deepen our understanding in the metabolism of the plant and the fungus. These are prerequisites to understand the diversity in the outcomes of AM interactions.

3   Targeted Approaches

All functions of the mycorrhizal symbiosis are realised post penetration when the interaction is fully established. These are the exchange of nutrients (biofertiliser function of AM fungi), the changes in the hormonal balance of the plant (biocontrol function) and the increased resistance and tolerance against pathogens and/or abiotic stressors (bioprotector function) (Smith and Read 2008). Many attempts have been made to understand the molecular and physiological basis for these functions using targeted approaches. The contents of mineral nutrients, heavy metals, phytohormones and secondary metabolites were measured or particular genes, proteins and enzymatic activities were analysed which are known to be involved in nutrient uptake, in the response of plants to pathogens, drought or soil contamination or, in the synthesis and production of phytohormones. All these attempts will be summarised in other book chapters. The following paragraphs will therefore concentrate on carbohydrates which are transferred from the plant to the fungus in exchange for mineral nutrients. As an obligate biotroph, the fungus totally depends on the plant and receives up to 20% of the plant assimilates (e.g. Jakobsen and Rosendahl 1990). In order to understand how the plant deals with this additional carbon sink, C assimilation as the process for the production of carbohydrates was investigated.

3.1   Photosynthesis in Mycorrhizal Plants

In terms of mutualistic exchange of resources, one could assume that the benefit of obtaining mineral nutrients is paid by carbon giving from the plant to the fungus and that this carbon represents a considerable cost. It has been shown in numerous reports

that in mycorrhizal plants CO_2 uptake is enhanced compared to the corresponding controls (e.g. Mathur and Vyas 1995; Caravaca et al. 2003). The amount of phosphate in leaves is a limiting factor for C assimilation (Jacob and Lawlor 1992). The better supply of mycorrhizal plants could be therefore the reason for increased photosynthetic activity compared to control plants. Indeed it has been shown that the application of phosphate to the controls in comparable amounts abolished the effect (Peng et al. 1993; Black et al. 2000). Other papers however reported that even at similar phosphate contents, mycorrhizal plants showed a significant enhanced CO_2 uptake (Fay et al. 1996; Wright et al. 1998a). Reasons for increased assimilation could be also higher chlorophyll contents or a better electron transport activity of the photosystem II. Results concerning these factors were however also contradictionary. Chlorophyll contents or chlorophyll fluorescence were enhanced (Tsimilli-Michael et al. 2000; Estrada-Luna et al. 2000; Sheng et al. 2008 or showed no differences between mycorrhizal and non-mycorrhizal plants (Paradi et al. 2003; Rai et al. 2008). C assimilation is negatively influenced by high sugar concentrations in the leaves (von Caemmerer 2000). One could assume that the increased sink strength in mycorrhizal roots due to the presence of the fungus and the higher metabolic activity of arbusculated cells lead to increased removal of sugars from shoots which would enable increased photosynthetic activity (Kaschuk et al. 2009). Measurements of sugar concentrations in leaves of mycorrhizal and control plants showed, however, no significant differences or even higher concentrations (see below). An important limiting factor for photosynthesis is the amount of CO_2 and water the plant is able to take up. In this context, it is interesting to note that in all reports, where stomatal conductance was measured, this parameter was increased (e.g. Khalvati et al. 2005; Sheng et al. 2008). Even under well-watered conditions, stomata of mycorrhizal plants seemed to be wider opened allowing more CO_2 entering leaf tissues (e.g. Wu and Xia 2006; Auge et al. 2008). If a better water supply of mycorrhizal plants is responsible for this phenomenon is not clear. It might be also the higher content of cytokinin which has been detected in flax and which causes improved stomatal conductance and higher CO_2 assimilation rates (Drüge and Schonbeck 1993).

In summary, many factors could be reponsible for increased CO_2 uptake of mycorrhizal plants and some might play a larger role than others dependent on the plant-fungus combination and the environmental condition. Anyhow, the outcome seems to be in case of beneficial mycorrhizal interactions an increased photosynthetic activity. This can be also seen from experiments under reduced photon fluxes or CO_2 concentrations, where the costs of mycorrhizal colonisation cannot be balanced by increased assimilation and negative effect of the fungus on plant growth become evident (Louche-Tessandier et al. 1999).

3.2 Carbon Distribution and Transfer to the Fungus

If more CO_2 is assimilated as it has been pointed out, the Calvin cycle should be more active and photochemical reactions result in higher levels of energy and reduction equivalents. This should increase the amounts of leaf carbohydrates,
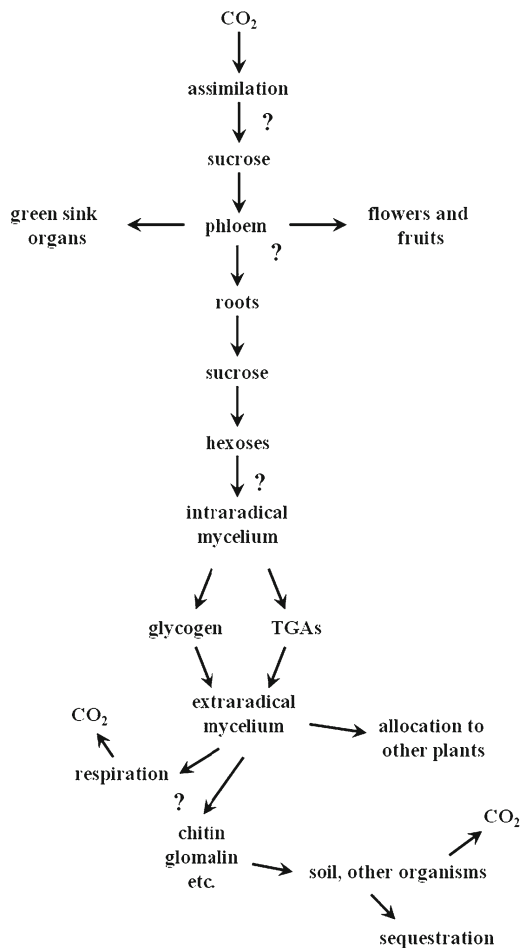
Fig. 1 Carbon assimilation, distribution and metabolism. The graphs show the way of carbon from the athmosphere through a mycorrizal plant and the AM fungus to the soil. Question marks indicate steps where significant gaps in the knowledge about the molecular–physiological background exist: carbohydrate metabolism from assimilation to sucrose, distribution of sucrose in the plant, the way of carbon transfer from the plant to the fungus, the final branches of carbohydrate metabolism and distribution in the extraradical mycelium

especially, when dark respiration is not altered (Valentine et al. 2001). In principle, more glucose phosphate and fructose phosphate should be produced which would be converted into sucrose. Sucrose in turn would be loaded into the phloem for its transport into the sink organs like young leaves, flowers, fruits and roots. Measurement of sugars showed no differences (Rapparini et al. 1996; Perner et al. 2007) or increased concentrations (Wright et al. 1998b; Wu and Xia 2006) in source leaves. Boucher et al. (1999) showed that this was dependent on the AM fungal isolate. A hint for increased carbohydrate metabolism in source leaves

suggested the mycorrhization-induced expression of a gene encoding a hexose transporter (Garcia-Rodriguez et al. 2005). The exact role of the transporter is not clear, because it is not known where it is localised and from which to which compartment it transports the hexoses. Reports on enzyme activities in leaves of mycorrhizal plants are rare. In only one paper, sucrose phosphate synthase was analysed, but the activity was not significantly affected (Black et al. 2000). Up regulation of invertase activity in leaf apoplasts, however, had a strong negative effect on mycorrhization indirectly indicating the important role of sucrose production (Schaarschmidt et al. 2007a). Up-loading of the phloem by sucrose is mediated by sucrose transporters (Kühn 2003) and a mycorrhiza-induced RNA accumulation of all three SUT-encoding genes could be detected in tomato leaves and roots (P. Franken, unpublished). Another analysis however showed no differences in the roots and the leaves for *LeSUT2* and *LeSUT4*, but down regulation of *LeSUT1* (Ge et al. 2008). The opposite results could be based on the fact that all three genes are down-regulated by phosphate (K. Boldt, personal communication). Phosphate concentrations were not affected in the first experiment, because all increases in P uptake were translated into growth. A direct influenec of the AM fungus *Glomus mosseae* on SUT RNA accumulation could be therefore observed. In contrast, the AM fungus *G. caledonium* significantly increase the P concentrations of roots and leaves (Ge et al 2008). This higher P concentrations interfered with the effect of the AM fungus and finally resulted in a balanced transcription of *SUT2* and *SUT4* and in even a down-regulation of *SUT1*. In any case, all analyses of the roots suggest increased transport of carbohydrates to this sink organ. It has been reported in a number of papers, that invertases and sucrose synthases being involved in sucrose turnover to hexoses show increased activity or that the corresponding genes are higher expressed in mycorrhzal roots (Wright et al. 1998b; Blee and Anderson 1998; 2002; Hohnjec et al. 2003; Ravnskov et al. 2003; Schubert et al. 2004; Schaarschmidt et al. 2006; Garcia-Rodriguez et al. 2007; Tejeda-Sartorius et al. 2008). Analyses of sugar concentrations in roots of different plant-fungus interactions showed as in leaves also here opposite results (e.g. Wright et al. 1998b; Garcia-Rodriguez et al. 2007). The amount of available hexoses seems anyway not to be the bottleneck for fungal development: increasing the ratio of hexose to sucrose amounts by overexpressiong an invertase in the roots did not lead to higher colonisation rate (Schaarschmidt et al. 2007b). The form of and the place where carbohydrates are taken up by the fungus inside the root is a matter of debate (Sun and Xu 2009). Feeding hyphae with different hexoses indicated that glucose is the compound which is taken up by the AM fungus (Solaiman and Saito 1997). This is in aggreement with the finding that the amounts of fructose increased in mycorrhizal roots of tomato and *M. truncatula*, while glucose concentrations stayed constant (P. Franken, unpublished) indicating that it is used and icorporated into the metabolism of the fungus. The remaining fructose could be transported back into the plant cytoplasm by a hexose transporter. Such a mycorrhiza-induced plant hexose transporter was cloned, characterised and localised in regions of *M. truncatula* roots colonised by an AM fungus (Harrison 1996). A hexose transporter from the glomalean fungus *Geosiphon pyriformis* however transported galactose, mannose,

xylose and fructose more efficiently than glucose (Schüssler et al. 2006). This fungus lives in symbiosis with cyanobacteria, but is closely related to AM fungi. If the AM fungi are also able to take up other sugars than fructose and glucose, they could use degradation products or non-polymerised residues of plant cell walls (Sun et al. 2009). As mentioned above, such compounds can be found in the matrix between the periarbuscular membrane and the fungal cell wall in arbusculated cells (Bonfante and Perotto 1995). This would point to the arbuscules which take up the carbohydrates from the plant, but other reports indicate that this is not the case. Most hexose transporters are dependent on proton gradients and therefore need the activity of H^+-ATPases. One of the corresponding gene was shown to be expressed during the intraradical phase of the fungus (Requena et al. 2003). The corresponding activity was however not localised on the arbuscules but at the membranes of the intercellular hyphae (Gianinazzi-Pearson et al. 1991). One could however argue that the hexose transporter uses the gradient produced by the proton pumps expressed in the arbusculated plant cell (Gianinazzi-Pearson et al. 2000; Krajinski et al. 2002). Another argument for carbohydrate transfer at sites different than the arbuscules comes from the finding that in plant mutants not allowing arbuscule development, AM fungi can still develop considerable amounts of mycelium (Manjarrez et al. 2008).

All together, the data indicate that carbohydrates are distributed in mycorrhizal plants in a different way than in the corresponding controls. There are however conflicting results and many gaps in our knowledge how carbohydrates are turned over and transported for ensuring the metabolic chain from the assimilation up to the incorporation into the fungal metabolism. Variations in different fungus-plant combinations are evident as such combinations show variable carbon sink strengths (Lerat et al. 2003a, b). Moreover it depends on environmental conditions: reduced light availability affects fungal development (Tester et al. 1986; Franken and Gnädinger 1994; Heinemeyer et al. 2006) and it has been shown in monoxenic cultures that P availability and external nitrogen concentrations influence carbon transfer to the fungus (Olsson et al. 2005, 2006). More data about metabolites and enzymatic activities in the whole plant have to be surveyed in order to fill the gaps in the chain from assimilation to carbon transfer. Most importantly, flux studies have to be carried out (Fernie et al. 2005) to understand this important part of mycorrhizal functioning. Such analyses have been already carried out on the fungal site (see below).

3.3   Carbohydrate Metabolism in AM Fungi

Rather little was known about the carbon metabolism in AM fungi until the introduction of monoxenic cultures as experimental system with the separation of fungal and host compartments (St-Arnaud et al. 1996; for review see: Fortin et al. 2002). This allowed combining isotopic labelling (Pfeffer et al. 2001) and gene expression analyses (see above) to get insights into the route of the carbon taken up by the

AM fungal mycelium: the hexoses entering the intraradical mycelium is rapidly transformed into trehalose (Becard et al. 1991) and glycogen (Shachar-Hill et al. 1995). Most importantly triacylglycerides (TGAs) are produced (Pfeffer et al. 1999) which are the main form of storage lipids in AM fungi (Beilby and Kidby 1980). This rapid turnover ensures a constant hexose gradient and is probably the driving force for the transfer of carbon across the plant–fungal interface. TGAs are transported together with glycogen from the intraradical to the extraradical hyphae (Pfeffer et al. 1999; Bago et al. 2002; Bago et al. 2003). Via beta-oxidation and glyoxalate cycle TGAs are broken down to hexoses again (Lammers et al. 2001) which are necessary for the production of all further compounds and for the retrieval of energy. Metabolic pathways working in both compartments are the pentose phosphate pathway (Pfeffer et al. 1999) necessary for the production of nucleotides and reducing equivalents and the tricarboxylic acid cycle (Bago et al. 2000) as the source for amino acids.

Interestingly, it could be shown that the hyphae from asymbiotic germinating spores can take up hexoses and acetate and are capable of dark fixation of CO_2 (Bago et al. 1999). The reason for the obligate biotrophic nature of the AM fungi seems therefore not to be that they absolutely depend on the carbon transfer from the host. All biosynthetic pathways can be carried out during asymbiosis and in extraradical hyphae with one exception: AM fungi are not able to synthesise lipids without their host (Bago et al. 1999; Pfeffer et al. 1999). In order to find the crucial step in the biosynthesis of lipids, Trépanier et al. (2005) incorporated labelled acetate and sucrose to germinating spores, to extraradical hyphae and to mycorrhiza. This experiment revealed that all tissues were able to elongate or desaturate fatty acids, but only intraradical hyphae could synthesise palmitic acid. The inability for the biosynthesis of 16-carbon fatty acids seems therefore to be the reason for the biotrophy of AM fungi.

The carbon in the extraradical hyphae is finally used e.g. for the production of the chitinious cell wall (Lanfranco et al. 1999), for storage lipids and glycogen in the developing spores (Bonfante et al. 1994) and for long-lasting proteins like the glomalin which is discussed to be important for soil aggregation (Purin and Rillig 2007). These processes are less well investigated, but nevertheless important for understanding mycorrhizal functioning.

4 Final Remarks

More research combing molecular–physiological with other methods is necessary to understand the carbohydrate metabolism of plants and AM fungi living in symbiosis. Such knowledge is necessary to answer important questions in the application and the ecology of mycorrhizal interactions. Finding out how carbon is distributed between different organs of the plant is a prerequisite for predicting the outcome of the application of AM fungi in agri- and horticulture. Will it lead under changing climate conditions to higher yield of food and feed of optimal quality and

to higher biomass for alternative energy production? On the other hand, the fate of carbon in AM fungi determines how carbohydrates can be allocated in plant communities via networks of mycelia and how AM fungi contribute to carbon sequestering in the soil and therefore to the global C cycle. The influence on carbon fluxes in organisms and between them as well as in ecosystems is a mycorrhizal function at least as important as the improved nutrient supply of the plant or the induced resistance and tolerance against biotic and abiotic stresses.

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Chapter 6
Phosphate Transporters in Arbuscular Mycorrhizal Symbiosis

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Abstract In the arbuscular mycorrhizal (AM) symbiosis the reciprocal exchange of nutrients results in a nutritional benefit for both symbionts. The fungus acquires carbon from plant and the plant obtains mineral nutrients from the fungus. While there is evidence for the transfer of phosphorus (P), nitrogen, zinc and copper, current data suggest that P is transferred in the highest quantities and that symbiotic P transfer occurs in the vast majority of AM symbioses. Symbiotic phosphate Pi transfer requires transport proteins to move Pi across the membranes of the AM fungus and plant. In recent years, there has been tremendous progress in the identification of plant and fungal Pi transporter proteins involved in symbiotic Pi transport. Coupled with the physiological data a greater understanding of symbiotic Pi transfer has emerged. Here we summarize the current data about Pi transporters and their expression patterns and roles in AM symbiosis.

Keyword Phosphate • Transporter • Symbiosis • Legume • Periarbuscular membrane • Root • Plasma membrane • Proton-coupled symport

Abbreviation

AM   Arbuscular mycorrhiza

1   Introduction

In the arbuscular mycorrhizal (AM) symbiosis the reciprocal exchange of nutrients results in a nutritional benefit for both symbionts. The fungus acquires carbon from plant and the plant obtains mineral nutrients from the fungus. While there is

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evidence for the transfer of phosphorus (P), nitrogen, zinc and copper, current data suggest that P is transferred in the highest quantities and that symbiotic P transfer occurs in the vast majority of AM symbioses (Smith et al. 2003, 2004; Smith and Read 2008).

Plants require large quantities of P, which they obtain as phosphate (Pi) from the solution phase of the soil. While many soils have a high P content, it occurs mostly as complex organic or inorganic forms that are not directly accessible to the plant, and Pi levels in the soil solution are below 10 μM (Richardson 2009). Additionally, Pi diffusion through the soil is slow and consequently, Pi depletion zones develop rapidly around the roots. In the AM symbiosis, the additional volume of soil which can be explored by the fungal extra-radical hyphae is significant, and the nutritional advantage conferred by symbiotic Pi transfer can be considerable (Schachtman et al. 1998; Smith et al. 2003).

In the early 1970s radiotracer experiments demonstrated that in an AM symbiosis, Pi acquired by the fungal extra-radical hyphae was transferred to the plant (Sanders and Tinker 1971). Pi transfer between symbionts was assumed to occur between the arbuscules and their host cortical cell, a prediction that was later supported by various lines of cellular, physiological and molecular data. Subsequently, radiotracers and compartmental growth systems were used to compare the relative amounts of Pi entering the plant via the AM fungus and directly through the plant root transport systems (Pearson and Jakobsen 1993; Smith et al. 2003, 2004). These studies were important because they revealed that Pi can be transferred from the fungus to the plant even in absence of a visible growth response, an original criterion used to estimate benefit for the plant (Smith et al. 2003, 2004).

Symbiotic Pi transfer, i.e., the acquisition of Pi from the soil by the extra-radical hyphae and subsequent transfer to the plant cell, requires transport proteins to move Pi across the membrane of the AM fungus and plant. In recent years, there has been tremendous progress in the identification of plant and fungal Pi transporter proteins, and coupled with the physiological data a greater understanding of symbiotic Pi transfer has emerged. Here we summarize the current data about Pi transporters and their expression patterns and roles in AM symbiosis.

2 Pi Transporters in AM Fungi

In the AM symbiosis Pi uptake from the soil by the extra-radical hyphae, and Pi efflux across the arbuscule membrane, are of major significance in symbiotic Pi transport (reviewed in Smith and Smith (1990); Karandashov and Bucher (2005); Bucher (2007). Currently, several transporters that likely mediate Pi uptake have been identified while those involved in Pi efflux from the arbuscule remain to be discovered.

Initial studies of Pi transport in AM fungi were carried out in germ-tubes of an AM fungus, *Gigaspora margarita.* In this system, Pi uptake measurements revealed uptake kinetics that were consistent with the presence of two Pi uptake systems:

a high affinity, active transport system (Km 1.8–3.1 μM) and a low affinity, passive transport system (Km 10.2–11.3mM) (Thomson et al. 1990). These findings parallel the data reported for *Neurospora crassa* and *Saccharomyces cerevisiae* where genes encoding high affinity Pi transporters had been identified. The sequence information from *S. cerevisiae PHO84* enabled the identification of a Pi transporter gene (*GvPT*) from an AM fungus, *Glomus versiforme,* and subsequently from two other AM fungi, *Glomus intraradices* (*GiPT*) and *Glomus mosseae* (*GmosPT*) (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001; Benedetto et al. 2005). The GvPT protein shares 47.9% identity with PHO84 and 45% identity with PHO-5 of *N. crassa.* The identity between the three AM fungal Pi transporters is greater than 70%.

Expression of *GvPT* in a yeast Pi transport mutant complemented the Pi transport defect, and Pi transport in the transformed yeast cells followed Michaelis-Menten kinetics with an apparent Km of 18 μM, which suggested that GvPT is a high affinity Pi transporter. Addition of carbonyl-cyanide-*m*-chlorophenyl-hydrazone (CCCP), which causes uncoupling of the proton gradient across the membrane, prevented further Pi uptake (Harrison and van Buuren 1995). This suggested Pi transport occurs via proton-coupled symport, the same mechanism as that of the yeast PHO84 transporter.

GvPT is expressed primarily in the extra-radical hyphae, which is consistent with its proposed role of Pi uptake from the soil. However, a small amount of expression in the intra-radical hyphae was also observed (Harrison and van Buuren 1995). In G. *mosseae, GmosPT* transcript levels were similar in the intra-radical and extra-radical hyphae, whereas there was no *GmosPT* expression in either dormant or germinated spores (Benedetto et al. 2005). Although the primary purpose of GvPT and GmPT may be to take up Pi from the soil, a secondary purpose may be to control the amount of Pi delivered to the plant, by competition between efflux and influx of Pi in the peri-arbuscular space. Additional research will be needed to address this possibility, and to identify how these Pi transporters are regulated in the intra-radical mycelium.

Regulation of Pi transporter gene expression in the extra-radical hyphae has been investigated in *G. intraradices* (Maldonado-Mendoza et al. 2001). This fungus is particularly useful for such studies because it can be maintained in *in vitro* root organ culture and the environment surrounding the extra-radical hyphae can be readily manipulated (St-Arnaud et al. 1996; Maldonado-Mendoza et al. 2001). Using this system, it was shown that *GiPT* expression is regulated in response to Pi levels in the external environment, and is induced by micromolar Pi concentrations that are typical of levels in the soil solution. These data suggest that *G. intraradices* has a mechanism of sensing Pi in the environment surrounding the extra-radical hyphae. Treatment with vanadate, a transition-state analog of Pi, also induces *GiPT* expression which supports the hypothesis that Pi itself is responsible for *GiPT* induction, rather than a downstream metabolite such as ATP (Maldonado-Mendoza et al. 2001).

The Pi status of the plant also impacts *GiPT* expression and expression was lower in roots that had previously received high Pi media and presumably had a

high Pi status. Perhaps the Pi status of the plant alters the rate of Pi efflux from the fungus, which then regulates Pi uptake in the extra-radical hyphae. Alternatively, Pi uptake by the extra-radical hyphae may be regulated by a signal from the plant.

3 Plant Pi Transporters of the PHT1 Family

Plants can acquire Pi directly from the soil via transport systems in their roots, or alternatively it is delivered to the root cortical cells by their AM fungal symbionts. Subsequently, Pi is translocated throughout the plant and transported into each cell and then to each organelle and subcellular compartment within each cell. Consequently plants have a wide variety of transporter proteins capable of transferring Pi across the different cell membranes. For reviews, the reader is referred to (Raghothama 1999; Rausch and Bucher 2002; Javot et al. 2007b; Miller et al. 2009). Here we focus only on the Pht1 family of Pi transporters. Members of this family are involved in Pi uptake from the soil while others are implicated in symbiotic Pi transport. Overall, there is significant differential regulation of the Pht1 transporters during AM symbiosis. For ease of reading, we have used the Pi transporter gene names as described in the original literature. An official nomenclature for Pi transporters has been established (Bucher et al. 2001; Karandashov and Bucher 2005) and a recent review lists the original and official names (Javot et al. 2007b).

Genes encoding Pi transporters of the Pht1 family were cloned initially from Arabidopsis (Muchhal et al. 1996) and then subsequently from a wide array of plant species (Table 1 and references within). Analyses in yeast showed that they encode functional Pi transporters but the individual proteins varied widely in their Pi transport kinetics ranging from high- to moderate- to relatively low-affinity transporters (Leggewie et al. 1997; Daram et al. 1998; Liu et al. 1998, 2008). Whether the Pi transport data obtained in yeast provide an accurate reflection of the kinetics *in planta* has remained a highly debated question. Nevertheless, the yeast data demonstrate that the proteins are capable of Pi transport, and all the data point to proton-coupled symport as the mode of operation. Currently, the full complement of Pht1 transporters is known only for Arabidopsis and rice, where complete genome sequences are available. These two species have nine and thirteen Pht1 family Pi transporter genes respectively (Mudge et al. 2002; Paszkowski et al. 2002). In Arabidopsis, eight out of nine members are expressed in roots (Mudge et al. 2002). Similarly in other plant species many of the Pi transporters are expressed in roots, and a significant proportion of these are induced in response to low-Pi conditions. However, while root expression predominates, some members of the Pht1 family are expressed in other tissues including leaves, flowers, cotyledons, pollinating germ tubes, hydathodes and siliques (Chiou et al. 2001; Nagy et al. 2005, 2006; Chen et al. 2007; Liu et al. 2008).

Because of the importance of Pi acquisition from the soil, there has been particular focus on the Pi transporter genes expressed in roots. Detailed expression analyses have been undertaken via a variety of approaches (Table 1 and references

Table 1 Expression patterns of Pht1 Pi transporter genes from a selection of dicotyledonous and monocotyledonous plant species

Plant species and Pi transporter	Transcript presence and location in non-mycorrhizal roots[a]	Expression in AM symbiosis (relative to non-mycorrhizal roots)[b]	Transcript location in AM symbiosis	Analysis methods[c]	References
Solanum lycopersicum					
LePT1	(+) E, RH, C	Down-regulated constitutive (Gi)	Cortical cell and cortical cell with arbuscule	Transcript, transcript (LM) in-situ hybrid	Liu et al. (1998), Nagy et al. (2005), Balestrini et al. (2007), Daram et al. (1998), Rosewarne et al. (1999), Xu et al. (2007)
LePT2	(+) C	Induced constitutive (Gi)	Cortical cell and cortical cell with arbuscule	Transcript, Transcript (LM)	Nagy et al. (2005), Balestrini et al. (2007), Xu et al. (2007)
LePT3	(+) (−)	Induced (Gi)	Cortical cell with arbuscule	Transcript, Transcript (LM)	Balestrini et al. (2007), Xu et al. (2007)
LePT4	(+) (−)	Induced (Gi)	Cortical cell with arbuscule	Transcript, Transcript (LM), Promoter-GUS	Nagy et al. (2005), Balestrini et al. (2007), Xu et al. (2007)
LePT5	(+)	Induced (Gi)	Cortical cell and cortical cell with arbuscule	Transcript, Transcript (LM)	Nagy et al. (2005), Balestrini et al. (2007)
Solanum tuberosum					
StPT1	(+)	Down-regulated (Gi)		Transcript	Leggewie et al. (1997), Nagy et al. (2005)
StPT2	(+) E, RH	Down-regulated (Gi)		Transcript	Nagy et al. (2005)

(continued)

Table 1 (continued)

Plant species and Pi transporter	Transcript presence and location in non-mycorrhizal roots[a]	Expression in AM symbiosis (relative to non-mycorrhizal roots)[b]	Transcript location in AM symbiosis	Analysis methods[c]	References
StPT3	(+) (−) C	Induced (Gi, Gc, Gim)	Cortical cell and cortical cell with arbuscule	Transcript In situ hybrid. Promoter-GUS	Gordon-Weeks et al. (2003) Nagy et al. (2005) Rausch et al. (2001) Karandashov et al. (2004)
StPT4	(−)	Induced (Gi)	Cortical cell with arbuscule	Transcript	Nagy et al. (2005)
StPT5	(−)	Induced (Gi)		Transcript	Nagy et al. (2005)
Petunia hybrid					
PhPT1	(+)	Constitutive (Gi)		Transcript	Wegmueller et al. (2008)
PhPT2	(+)	Constitutive (Gi)		Transcript	Wegmueller et al. (2008)
PhPT3	(+)	Induced (Gi)		Transcript	Wegmueller et al. (2008)
PhPT4	(−)	induced (Gi)		Transcript	Wegmueller et al. (2008)
PhPT5	(+)	Induced (Gi)		Transcript	Wegmueller et al. (2008)
Medicago truncatula					
MtPT1	(+) E, RH, C	Down-regulated (G.i. Gir, Gv) Constitutive (Gm)		Transcript In-situ hybrid Promoter-GUS Promoter-GFP	Liu et al. (1998) Chiou et al. (2001) Xiao et al. (2006) Grunwald et al. (2009)
MtPT2	(+) E, RH, C, V	Constitutive (Gi, Gr, Gm) Down-regulated (Gv)		Transcript Promoter-GUS Promoter-GFP	Xiao et al. (2006) Grunwald et al. (2009)
MtPT3	(+) V	Down-regulated (Gm, Gr, Gv, Gi)		Transcript Promoter-GUS	Liu et al. (2008) Grunwald et al. (2009)

Gene		Expression	Localization	Method	References
MtPT4	(−)	Induced (Gm, Gir, Gv, Gi)	Cortical cell with arbuscule	Transcript, Promoter-GUS	Harrison et al. (2002) Grunwald et al. (2009)
MtPT5	(+) E, C	Down-regulated (Gi,) Constitutive (Gm, Gr, Gv)	Epidermal, cortical cell and cortical cell with arbuscule	Transcript, Promoter-GUS	Liu et al. (2008) Grunwald et al. (2009)
MtPT6		Down-regulated (Gi, Gv, GiR, Gm)		Transcript	Grunwald et al. (2009)
Lotus japonicus					
LjPT1	(+)	Down-regulated (Gm)		Transcript	Maeda et al. (2006)
LjPT2	(+)	Down-regulated (Gm)		Transcript	Maeda et al. (2006)
LjPT3	(+)	Induced (Gm)	Cortical cell with arbuscule	Transcript, in situ hybrid.	Maeda et al. (2006)
LjPT4	nd	Induced (Gi)		Transcript	Takeda et al. (2009)
Oryza sativa					
OsPT1	(+)	Down-regulated (Gi)		Transcript	Paszkowski et al. (2002)
OsPT2	(+) V	Down-regulated (Gi)		Transcript	Paszkowski et al. (2002)
				Promoter-GUS	Ai et al. (2009)
OsPT3	(+)	Down-regulated (Gi)		Transcript	Paszkowski et al. (2002)
OsPT4	(+)	Constitutive (Gi)		Transcript	Paszkowski et al. (2002)
OsPT5	(+)	Constitutive (Gi)		Transcript	Paszkowski et al. (2002)
OsPT6	(+) E, C	Down-regulated (Gi)		Transcript	Paszkowski et al. (2002)
				Promoter-GUS	Ai et al. (2009)
OsPT7	(−)	Constitutive (Gi)		Transcript	Paszkowski et al. (2002)
OsPT8	(+)	Constitutive (Gi)		Transcript	Paszkowski et al. (2002)
OsPT9	(+)	Down-regulated (Gi)		Transcript	Paszkowski et al. (2002)

(continued)

Table 1 (continued)

Plant species and Pi transporter	Transcript presence and location in non-mycorrhizal roots[a]	Expression in AM symbiosis (relative to non-mycorrhizal roots)[b]	Transcript location in AM symbiosis	Analysis methods[c]	References
OsPT10	(+)	Down-regulated (Gi)		Transcript	Paszkowski et al. (2002)
OsPT11	(−)	Induced (Gi)	Cortical cell with arbuscule	Transcript, in situ hybrid.	Paszkowski et al. (2002); Glassop et al. (2007)
OsPT12	nd			Transcript	Paszkowski et al. (2002)
OsPT13	(−) (+)	Induced (Gi)(Sc)	Cortical cell with arbuscule	Transcript, in situ hybrid.	Paszkowski et al. (2002); Guimil et al. (2005); Glassop et al. (2007)
Hordeum vulgare					
HORvu;Pht1;1	(+) RH, E, V	Down-regulated (Gi, Gsp); Constitutive (Gi, Gg)	E	Transcript Promoter-GUS Promoter-GFP	Schunman et al. (2004); Rae et al. (2003); Glassop et al. (2005); Grace et al. (2009)
HORvu;Pht1;2	(+) RH, E, V	Down-regulated (Gi, Gsp); Constitutive (Gi, Gg)	E,V	Transcript Promoter-GUS Promoter-GFP	Schunman et al. (2004); Glassop et al. (2005); Grace et al. (2009)
HORvu;Pht1;3/1;4	(+)	Constitutive		Transcript	Glassop et al. (2005)
HORvu;Pht1;5				Transcript	Glassop et al. (2005)
HORvu;Pht1;6	(−)			Transcript	Rae et al. (2003); Glassop et al. (2005)
HORvu;Pht1;7				Transcript	Glassop et al. (2005)
HORvu;Pht1;8	(−) (+)	Induced (Gi, Gsp, Gg)	Cortical cell with arbuscule	Transcript In-situ hybrid.	Glassop et al. (2005); Grace et al. (2009)

Triticum aestivum					
TRIae;Pht1;myc	(−)	Induced (Gi, Gsp)	Cortical cell with arbuscule	Transcript / In-situ hybrid.	Glassop et al. (2005)
Zea mays					
ZEAmaPht1;1	(+)	Down-regulated (Gi)		Transcript	Wright et al. (2005)
ZEAmaPht1;2	(+)	Constitutive (Gi)		Transcript	Nagy et al. (2006)
ZEAmaPht1;3	(+)	Constitutive (Gi)		Transcript	Nagy et al. (2006)
ZEAmaPht1;4	(+)	Down-regulated (Gi)		Transcript	Wright et al. (2005)
ZEAmaPht1;5	nd			Transcript	Nagy et al. (2006)
ZEAmaPht1;6	(+)	Induced (Gi, Gsp)	Cortical cell with arbuscule	Transcript / In-situ hybrid.	Glassop et al. (2005) / Nagy et al. (2006)
ZmPT3	(+)	Down-regulated (Gi)		Transcript	Wright et al. (2005)

[a](+) transcript detected; (−) transcript absent; nd, not determined, E, epidermal cell; RH, root hair, C, cortical cell; V, vascular tissue

[b]The species of AM fungus is shown in brackets. *Glomus intraradices* (Gi), *Glomus versiforme* (Gv), *Glomus mosseae* (Gm), *Glomus geosporum* (Gg), *Glomus caledonium* (Gc), *Glomus species* (Gsp), *Gigaspora margarita* (Gim), *Gigaspora rosea* (Gir), *Scutellospora calospora* (Sc)

[c]Transcript indicates that transcripts were assessed by RT-PCR, Quantitative RT-PCR or northern blot analysis in RNA from whole roots; Transcript (LM) indicates transcript analysis on laser microdissected samples. In situ hybrid. indicates transcript location assessed by *in situ* hybridization; Promoter-GUS or Promoter-GFP indicates gene expression analysed by promoter-reporter gene fusion

within). Many of the genes are expressed in the epidermis and root hairs, while others are located in the cortical cells and vascular tissue. In general, there is some overlap in expression patterns between the different family members (Daram et al. 1998; Chiou et al. 2001; Glassop et al. 2005; Balestrini et al. 2007; Liu et al. 2008; Ai et al. 2009). In a few plants, the locations of the Pi transporter proteins have been examined by immunolocalization or analysis of GFP-fusion proteins. These studies indicated that the proteins were located in the plasma membrane (Chiou et al. 2001; Gordon-Weeks et al. 2003; Nagy et al. 2005; Liu et al. 2008) and in potato, the StPT2 protein showed an asymmetric distribution within the plasma membrane with most protein located on the side of the epidermal cell closest to the soil (Gordon-Weeks et al. 2003). Overall, the gene expression patterns and protein locations suggest roles in Pi uptake from the soil, and in translocation of Pi through the cortex to the vascular tissue. These predictions were confirmed in Arabidopsis where mutation of either *AtPht1;1* or *AtPht1;4*, two of the epidermal Pi transporters, lead to a small reduction in Pi uptake, and the double mutant showed a 75% reduction in Pi uptake (Misson et al. 2004; Shin et al. 2004) . Similarly rice plants in which *OsPT2* and *OsPT6* were down-regulated by RNAi showed reduced Pi uptake and Pi translocation to the shoots (Ai et al. 2009).

4 PHT1 Family Transporters in AM Symbiosis

Are the Pht1 family Pi transporters differentially regulated in mycorrhizal roots? This question has been addressed by many researchers in a wide variety of plant species. In general, the Pi-starvation inducible transporters that are expressed in the root hair and epidermal cells are down-regulated during AM symbiosis (Table 1 and references within). However, the extent to which down-regulation occurs varies with the environmental conditions and the fungal symbionts involved (Chiou et al. 2001; Rausch et al. 2001; Chen et al. 2007; Liu et al. 2008; Grace et al. 2009; Grunwald et al. 2009). The gene expression data correlate well with radiotracer studies (Smith et al. 2003, 2004) and together the data support the hypothesis that during the symbiosis, the plant switches its Pi acquisition strategy and favors Pi acquisition from the AM fungal symbiont over acquisition through its roots/root hairs. Although down-regulation of root Pht1 transporters occurs during AM symbiosis, this pattern is not true of all root Pht1 transporters. For example, in *M. truncatula* promoter-GUS fusion studies indicate that expression of at least one Pi transporter is maintained in the epidermis, even in mycorrhizal roots suggesting that the plant retains some level of Pi acquisition via its root hairs (Liu et al. 2008). Similarly, there are examples of Pi transporters whose transcript levels remain constant in mycorrhizal roots of rice, petunia, barley and maize (Table 1) (Paszkowski et al. 2002; Glassop et al. 2005; Nagy et al. 2005, 2006; Wright et al. 2005; Maeda et al. 2006; Balestrini et al. 2007; Liu et al. 2008; Grace et al. 2009; Grunwald et al. 2009). The roles of these Pi transporters in the AM symbiosis remain to be determined.

5 PHT1 Family Transporters Induced During AM Symbiosis

Several Pht1 transporter genes show expression either exclusively during AM symbiosis or alternatively, their expression is up-regulated substantially in mycorrhizal roots relative to non-colonized control roots (Table 1) (Rausch et al. 2001; Harrison et al. 2002; Paszkowski et al. 2002; Karandashov et al. 2004; Glassop et al. 2005; Nagy et al. 2005, 2006; Maeda et al. 2006; Wegmueller et al. 2008; Grace et al. 2009). Consequently, these transporters are prime candidates for a role in symbiotic Pi transport. Phylogenetic analyses indicate that AM-induced Pi transporters are dispersed throughout the Pht1 family, which suggests that they have arisen in different ways in the different plant families (Fig. 1). However, there is one exception. One clade within the Pht1 family, referred to as Pht1, subfamily 1 (Nagy et al. 2005), contains only AM-induced transporters. With the exception of Arabidopsis, a non-mycorrhizal species, most plants have at least one gene in subfamily 1 (Table 1 and Fig. 1). MtPT4, a well-characterized member of this subfamily, shows low-affinity Pi transport kinetics in yeast. In mycorrhizal roots, *MtPT4* is expressed only in cells with arbuscules and the protein was localized to the periarbuscular membrane, specifically to the regions around the fine branches of the arbuscules (Harrison et al. 2002; Pumplin and Harrison 2009). It is assumed that the other transporters of this subfamily will show periarbuscular membrane localization also.

Most plants have a single Pi transporter gene in subfamily 1 except for members of the Solanaceae in which a duplication event has given rise to two paralogs. In potato, and petunia, *StPT4* and *PhPT4* are expressed only in AM roots, while the paralogs, *StPT5* and *PhPT5* are expressed in non-mycorrhizal roots, but significantly upregulated in AM symbiosis. In tomato, both paralogs, *LePT4* and *LePT5* are expressed in non-mycorrhizal roots but transcripts are upregulated in mycorrhizal roots and are present in cortical cells containing arbuscules and in non-colonized cortical cells (Nagy et al. 2005; Balestrini et al. 2007; Chen et al. 2007; Xu et al. 2007; Wegmueller et al. 2008). In additional to the AM-inducible expression, *LePT4* is regulated in response to Pi level and expression is highest in mycorrhizal plants grown in low Pi conditions (Nagy et al. 2009).

In addition to the subfamily 1 Pi transporters, most plant species have at least one additional AM-induced Pht1 transporter. In the Solanaceae, potato *StPT3* and orthologs*, LePT3* of tomato and *PhPT3* of petunia are expressed in roots but upregulated significantly in AM symbiosis. In mycorrhizal roots, the *StPT3* promoter is active in cells with arbuscules and transcripts for *LePT3* were likewise detected only in colonized cortical cells (Nagy et al. 2005; Balestrini et al. 2007; Wegmueller et al. 2008). Analysis of *StPT3* during symbioses with different AM fungi indicated that in an *Arum*-type AM symbiosis, the promoter is active in cells containing arbuscules, while in the *Paris*-type symbiosis, the promoter is active in cells containing hyphal coils. These data support earlier suggestions that the coils observed in the *Paris*-type symbiosis are functionally analogous to the arbuscules of the *Arum*-type associations (Karandashov et al. 2004).
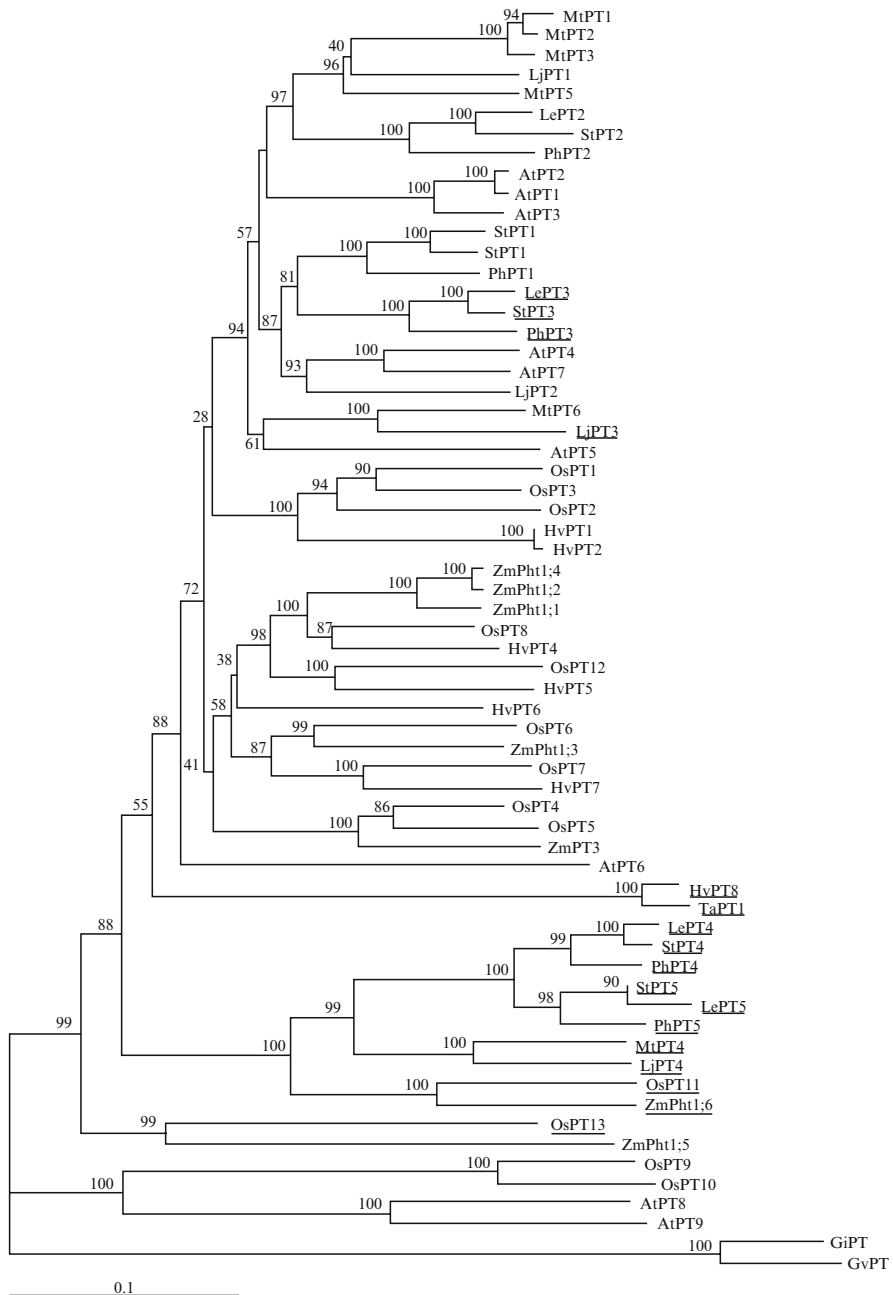
Fig. 1 Amino acid sequences of Pht1 family members from *Medicago truncatula* (Mt), *Lotus japonicus (Lj), Arabidopsis thaliana (At), Oryza sativa (Os), Zea mays (Zm), Hordeum vulgare (Hv), Triticum aestivum (Ta), Petunia hybrida (Ph), Solanum tuberosum (St) and Solanum lycopersicum (Le) were aligned with ClustalW* (Larkin et al. 2007). Distances were calculated using the neighbor joining method and bootstrap values were obtained with 100 trials. The tree was created

In *Lotus japonicus*, an AM-induced transporter *LjPT3* shows similar expression patterns to the *StTP3* group but clusters elsewhere in the Pht1 family with *MtPT6* of *M. truncatula*, a gene that is down-regulated during AM symbiosis (Maeda et al. 2006; Grunwald et al. 2009). Likewise, *OsPT13* of rice, *HORvu;Pht1;8* of barley and *TRIae;Pht1;myc* of wheat are all AM-inducible Pi transporters, that do not cluster in Pht1 subfamily 1. *OsPT13* and *TRIae;Pht1;myc* transcripts were detected only in mycorrhizal roots, while *HORvu;Pht1;8* transcripts were present in roots and significantly upregulated in AM symbiosis. In AM symbiosis, both *TRIae;Pht1;myc* and *HORvu;Pht1;8* transcripts were detected only in arbuscule containing cells. The expression of *TRIae;Pht1;myc* is correlated with colonization of the root system (Glassop et al. 2005), while *HORvu;Pht1;8* is highly expressed in colonized roots regardless of the levels of fungal colonization (Glassop et al. 2005; Grace et al. 2009).

6   Regulation of AM-Inducible PHT1 Transporter Genes

As indicated, several of the AM-inducible transporters show expression exclusively in cells containing arbuscules and appear to be regulated in a cell autonomous fashion (Table 1 and references within). Promoter switching experiments involving the promoters of *S. tuberosum StPT3*, *M. truncatula MtPT4* and *Oryza sativa OsPT11* showed that eudicot species promoters directed the correct AM-inducible, cell-type specific expression of the reporter GUS gene in five different eudicot host plants, while the monocot rice promoter did not work in eudicot plants (Karandashov et al. 2004). From these results, it is evident that the eudicot AM-inducible Pi transporter gene promoters and their regulation are highly conserved in phylogenetically distant plant species (i.e. *Solanales*, *Apiales*, and *Fabales*). Consequently, they should potentially have the same cis-acting elements in their promoter regions and similar regulatory pathways. To identify candidate regulatory cis-acting elements in these promoters, phylogenetic footprinting was performed with the 129 bp region (−639/−510) of *StPT3* promoter and approximately 1 Kb region of promoters from 3 other mycorrhiza-specific Pi transporter genes (*LePT4*, *MtPT4*, *OsPT11*), one mycorrhiza-induced glutathione-S-transferase (*MtGST1*), and two non-mycorrhiza associated Pi transporters from Arabidopsis (*Pht1;6* and *Pht1;8*). From this phylogenetic footprinting, six putative cis-regulatory elements (TGTT-motif, AAA-motif, TGCA-motif, TAAC-motif, CTTC-motif, and TAAT-motif) were found. The CTTC motif was prominent in the promoters of *LePT4*, *MtPT4* and *MtGst1*, but not in *OsPT11* or in the Arabidopsis *Pht1;6* and *Pht1;8* promoters. Only the promoters

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Fig. 1   (continued) in TREEVIEW (Page 1996). Two AM fungal transporters, GvPT and GmPT were used as outgroups. Scale bar represents 0.1 substitutions per site. Mycorrhizal-induced Pi transporters are underlined. LePT5, StPT5 and ZmPT3 sequences were not full length. To keep the names short within the tree, Barley Pi transporters, whose names in Table 1 are HORvu;Pht1;1 etc. are abbreviated HvPT1 etc. The single wheat Pi transporter, TRIae;Pht1;myc is abbreviated as TaPht1;myc

of the three mycorrhiza-specific Pi transporter genes from the eudicots contain the TAAT motif (Karandashov et al. 2004). The phylogenetic footprinting provides a useful guide for future analyses of the regulatory elements controlling expression of the mycorrhiza-inducible Pi transporters. While the elements controlling expression still remain to be determined, one of the signals capable of inducing expression of the transporters has been identified. In potato, lipid extracts prepared from mycorrhizal roots induced expression of *StPT3*. The active molecule was identified as lyso-phosphatidylcholine and application to tomato suspension cultures induced expression of *LePT4* (Drissner et al. 2007). This suggests that the tomato cell cultures contain all components of the signaling pathway for expression of the AM-inducible Pi transporters and infers that other AM-specific components are not required. Lyso-phosphatidylcholine is a common lipid component of both plant and fungal cells and currently the origin of the active signal molecule is unknown.

7   Function of AM-inducible Pi Transporters

The expression patterns and membrane location of the AM-induced Pi transporters suggests that these proteins play a role in symbiotic Pi transport. To determine the importance of the transporters, researchers analyzed plant mutants lacking the individual transporter proteins. So far, such experiments have been undertaken for only three AM-induced transporters but the results are exciting and reveal that Pi transporters are in fact central and crucial for the symbiosis.

The first evidence for a mycorrhizal phenotype caused by disrupting a Pi transporter came from work on *L. japonicus LjPT3* which is AM-induced and expressed strongly in cells with arbuscules, but is not a subfamily1 ortholog (Table 1 and Fig. 1). RNAi plants in which *LjPT3* transcripts were down-regulated to 15% of the native level were generated and while uninoculated RNAi plants grew similarly to a vector control, down-regulating *LjPT3* reduced the effectiveness of the symbiosis. In control *L. japonicus* plants, AM colonization provided a five-fold increase in plant biomass, while *LjPT3* RNAi plants increased biomass only about 2.5-fold after colonization. In addition, following inoculation, one of the two *LjPT3* RNAi lines showed reduced shoot Pi levels relative to the control, and radio-labeled P[33] accumulated around arbuscules, suggesting that Pi uptake at the plant-fungal interface was impaired. This phenotype was accompanied by a small but significant reduction in arbuscule number within roots and an increase in idioblasts, possibly a defense response by the plant, and an increase in fungal penetration attempts at the root surface. Finally, these RNAi plants were impaired in nodule formation, but only when also colonized by AM fungi, suggesting the impaired AM symbiosis had a broader effect on root symbioses (Maeda et al. 2006).

In tomato, a line with a transposon insertion in *LePT4*, a subfamily 1 member (Table 1 and Fig. 1), was identified but functional characterization of this gene was complicated because of the paralog, *LePT5*, and also an additional AM-induced transporter, *LePT3*. While it was originally reported that *lept4* loss-of-function

mutants did not display a phenotype caused by this insertion, further characterization of *lept4* in which other co-segregating transposon insertions had been removed revealed a mild phenotype (Nagy et al. 2005; Xu et al. 2007). After AM colonization, *lept4* mutants showed a moderate but statistically significant decrease in shoot mass and shoot Pi accumulation relative to controls but effects on fungal development were not reported. Interestingly, it was also reported that *lept4* mutants show impaired Pi uptake relative to control plants in the absence of AM colonization. This was attributed to a basal level of *LePT4* expression in wild-type non-mycorrhizal roots (Xu et al. 2007).

The most severe mycorrhizal phenotype caused by loss of Pi transporter function comes from disruption of *MtPT4*, which is the predominant Pi transporter expressed in *M. truncatula* cells with arbuscules (Harrison et al. 2002; Javot et al. 2007a). Plants in which MtPT4 was down-regulated by RNAi showed no increase in shoot mass or shoot Pi in response to fungal colonization, while control plants more than doubled in shoot mass and increased shoot Pi three to six fold in response to colonization. In addition, in the MtPT4 RNAi lines and in a loss-of-function *mtpt4* EMS mutant, the levels of colonization were much lower than the controls. Detailed analysis of the arbuscules indicated that their lifespan in the *mtpt4* mutants or MtPT4 RNAi lines was reduced relative to the controls. The data indicated that MtPT4 function was required to enable the arbuscules to maintain their normal lifespan and consequently for the fungus to become established within the root (Javot et al. 2007a).

Functional experiments on Pi transporters in the AM symbiosis are indicating that symbiotic Pi transport is essential in the AM symbiosis and that the consequences of losing these activities have impacts beyond nutrient exchange. While symbiotic Pi transporter mutants are impaired in mycorrhizal growth benefits, the data indicated that symbiotic Pi transport may be required to maintain AM fungal development within roots. One interpretation is that plants have evolved a mechanism to sense when a symbiont is not contributing sufficient nutrients and eliminate it. An alternate interpretation is that Pi delivery by the fungal symbiont is required to trigger carbon release by the plant. In the absence of symbiotic Pi transport, carbon would not be available to sustain the fungal symbiont, and arbuscule degeneration may simply be the natural fungal response to an environment that lacks available carbon. Additional analyses will be necessary to fully understand the phenotypes observed.

8   Conclusions

The AM symbiosis is formed by the vast majority of land plants and while initial studies have revealed common themes to symbiotic Pi transport, they also indicate that there is significant variation between plant families and species. Analyses of the roles of the individual Pi transporters in symbiotic Pi transport, and in regulation of the AM symbiosis are just beginning. Further research is required to

determine the mechanisms underlying their roles in regulating maintenance of the symbiosis, and the extent to which these aspects are conserved across all plant species. Currently, our understanding of regulation of symbiotic Pi transporter gene expression is limited, and the signaling pathways and transcription factors that control the AM-inducible, cell-type specific expression remain to be discovered.

We have learned a small amount about one class of Pi transporters that operate in the extra-radical hyphae of the AM fungal symbionts, but we know nothing about the transporters or channels that mediate of Pi from the arbuscule, or how these are controlled. The sequencing of the first AM fungal genome is in progress and this may assist in the identification of proteins involved in Pi efflux. There is still much to discover before we have a complete understanding of Pi transport in the AM symbiosis.

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Chapter 7
Nutrient Uptake: The Arbuscular Mycorrhiza Fungal Symbiosis as a Plant Nutrient Acquisition Strategy

Elke Neumann and Eckhard George

Abstract Symbiotic association of roots with arbuscular mycorrhizal (AM) fungi is a very widespread strategy by which plants facilitate their acquisition of mineral elements from the soil. Studies employing sophisticated methodology in the fields of in vitro culture of AM colonized roots, microscopy, isotope labeling and molecular biology have shed light into the physiology of AM fungal nutrient uptake, transport and delivery to the host plant. In addition to the direct contribution to element uptake via the symbiotic pathway, AM mycelia have also been shown to affect root morphology and functioning, as well as mycorrhizosphere soil properties. This may lead to indirect effects of the AM association on plant nutrient availability and uptake. With their thin diameter, AM hyphae might be able to access smaller soil pores, and better compete with soil microbes for nutrient resources, compared with plant roots. Alone or in collaboration with associated hyphosphere bacteria, AM mycelia might also promote chemical mobilization of nutritional elements from sparingly plant available resources. Similar with plant root systems, AM mycelia appear to differ considerably in their architecture and physiological activities depending on their genotype. Whether such inherent traits represent different strategies in nutrient acquisition in collaboration with functionally compatible host roots, still remains speculative. Not much is known about how the AM fungal symbiosis is integrated into particular plant nutrient acquisition strategies, but it can be assumed that individual symbiotic strategies are highly diverse. The AM mycelium might assist the roots in spatial and/or chemical soil nutrient resource exploitation in a complementary and/or synergistic way. Knowledge about what

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factors determine the extent of functional compatibility between individual plant nutrient acquisition strategies and certain AM fungal traits would contribute to our understanding of ecosystem functioning, and might assist further development of mycorrhiza technology for plant production.

Keywords Chemical nutrient mobilization • Ecological niche • Extraradical mycelium • Functional compatibility • Hyphal nutrient transport • Hyphosphere • Mycorrhizosphere • Nutrient acquisition strategy • Spatial nutrient resource exploitation

Abbreviations

AM   Arbuscular mycorrhiza
BAS   Branched absorbing structure
C   Carbohydrate

1   Introduction

Fossil founds revealed that some of the first land plants were already associated with fungi resembling the Glomeromycota (Remy et al. 1994; Redecker et al. 2000). It is tempting to speculate that fungal assistance in water or mineral element acquisition was a prerequisite for plant colonization of land masses. Since these times, approximately 450 Mio years ago, land plants have evolved from primitive clubmoss-like forms of life to organisms with highly diversified adaptations to almost all terrestrial habitats. While the first land plants most likely had no roots at all, root systems of modern higher plants appropriate a highly regulated set of strategies to exploit various mineral nutrient resources. Nowadays, for up to 80% of all land plants, nutrient acquisition strategies still involve the mutualistic association with AM fungi (Smith and Read 2008). Compared with their plant partners, the latter appear to have changed morphologically relatively little. However, studies comparing contributions of individual AM fungal strains to plant element uptake under different environmental conditions or from different nutrient resources, revealed a considerable diversity in AM fungal adaptations and services. Here we review some of the key features of AM fungal mycelia, and means by which these take up, transport and transfer nutritional elements to their host plant. We postulate that 450 Mio years of coevolution between plants and AM fungi have led to the evolution of a broad variety of integrative strategies by which plant roots and fungal symbionts jointly exploit soil nutrient resources. Possible means by which AM fungi may complement and/or assist plant roots in spatial or chemical mobilization of sparingly available nutrients in the rooting zone are proposed.

2 Physiology of Uptake, Transport and Delivery to the Host Plant of Nutritional Elements by Arbuscular Mycorrhiza Fungal Mycelia

2.1 Development and Morphology of Extraradical Arbuscular Mycorrhiza Hyphal Networks

Though a network of AM fungal hyphae is present in almost every terrestrial eco-system, knowledge about its morphology and development is still scarce. A major reason for this may lie in methodological difficulties to extract and observe AM mycelia from the soil. Blending, wet sieving, and decanting of soil samples is a common method to extract fungal hyphae (Hanssen et al. 1974; Li et al. 1991a; Jakobsen et al. 1992), but particularly on soils rich in organic or porous particles, may not yield the complete mycelium. Artificial growth media, such as sand or glassbead substrates with nutrient solution have thus frequently been used as growth substrates for mycorrhizal plants (Redecker et al. 1998; Jentschke et al. 1999; Chen et al. 2001). These allow for the extraction and analysis of AM myce-lium samples, but it cannot be excluded that morphology and activities of AM hyphae in nutrient solution differ considerably from those in soil. Several studies of extraradical AM development employed axenic cultures of AM fungi with Ri T-DNA transformed roots (Bago 2000; de Souza and Declerck 2003). Similar with other artificial systems, conclusions from observations on agar plates need to be drawn with certain reserves.

A substrate consisting of 40 μm wet sieved soil and glassbeads, which allows for the complete extraction of intact AM mycelium, has been proposed as growth sub-strate for AM mycelia by Neumann and George (2005a). This substrate may simu-late soil conditions better than previously used artificial growth substrates. However, by this technique, biomass of AM hyphae can only be completely separated from that of roots when grown in a distinct compartment. In greenhouse microcosms, fungal compartments are usually separated from the root compartment by a 30 μm mesh membrane (Fig. 1). The narrow mesh width allows only fungal hyphae, but not roots to penetrate and to enter the compartment. However, since there is evidence that AM mycelium development in proximity and distance of roots may differ con-siderably (St-Arnaud et al. 1996; Neumann et al. 2009), it may be inappropriate to draw general conclusions on activities or morphology of the extraradical part of the AM symbiosis from data obtained for mycelium from fungal compartments.

AM mycelia constitute of morphologically different types of hyphae. Relatively coarse and thick-walled hyphae with a diameter between 5 and 20 μm appear to func-tion mainly in nutrient transport and extension of the fungal colony. At rates of 1–2 mm per day (Harinikumar and Bagyaraj 1995; Olsson and Wilhelmsson 2000), so called 'runner-hyphae' elongate and form hyphopodia in parallel with the root axis (Friese and Allen 1991). Coarse hyphae extending radially around the root can reach distances of up to 24 cm (Drew et al. 2006) and may rapidly spread AM infection over
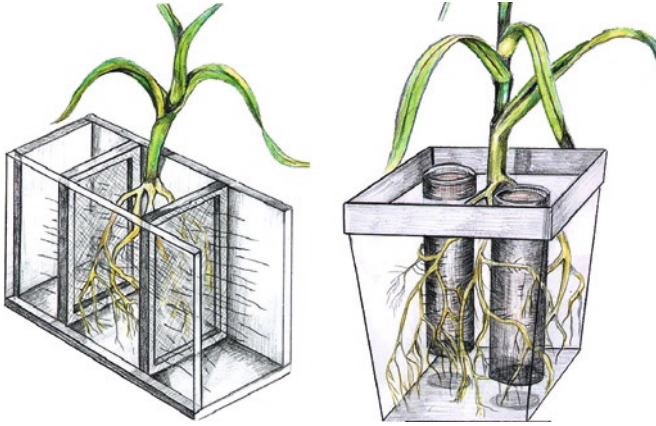
Fig. 1 Compartmented planting pots used in the study of AM fungal extraradical development and contribution to plant element uptake. *Left*: Two outer fungal compartments are separated from the middle compartment where the mycorrhizal plant is growing by a membrane with a mesh width of 30 µm. The small mesh width allows only AM hyphae, but not roots to penetrate and to grow into the fungal compartments. This type of compartmented planting pot is frequently used in nutrient uptake studies where isotopically labeled elements are supplied exclusively to the fungal compartments. *Right*: Two fungal compartments constructed from perforated tubes surrounded by a 30 µm mesh membrane are inserted into the soil where a mycorrhizal plant is growing. These types of fungal compartments are often used to compare AM fungal extraradical proliferation in, and nutrient uptake from different substrates or nutrient resources in the rooting zone

noncolonized neighboring roots of the same or of a different host plant (Drew et al. 2006; Voets et al. 2009). In most undisturbed plant communities, plants may thus connect with their roots to a pre-existing AM hyphal network in the soil shortly after germination, and neighboring mycorrrhizal plants of the same or of different species may be interconnected by common AM hyphal networks (Selosse et al. 2006). Arbuscular mycorrhiza hyphal bridges between neighboring roots may also be formed by anastomosis of hyphae of the same AM species (Croll et al. 2009).

In nutrient uptake experiments where AM mycelia need to establish from inoculum consisting of colonized root fragments and/or AM spores, AM fungal strains may differ in the time they require for the establishment of a fully functional AM extraradical hyphal network (Dodd et al. 2000). Differences between AM species in their net contributions to plant nutrient uptake assessed during vegetative growth of annual plants in such experiments may thus reflect differences in speed of re-establishment after soil disturbance rather than differences in nutrient uptake capacity between fully established AM symbioses.

The coarse extraradical AM hyphae appear to function as backbones for the formation of clusters of finely branched, thin-walled hyphae with a diameter below 4 µm (Bago et al. 1998; Bago 2000). When observed on petriplates, the latter often resemble in their morphology and life-span of 5–7 days intraradical arbuscules (Karandashov et al. 2000; Bago and Cano 2005; Fig. 2), and show high metabolic
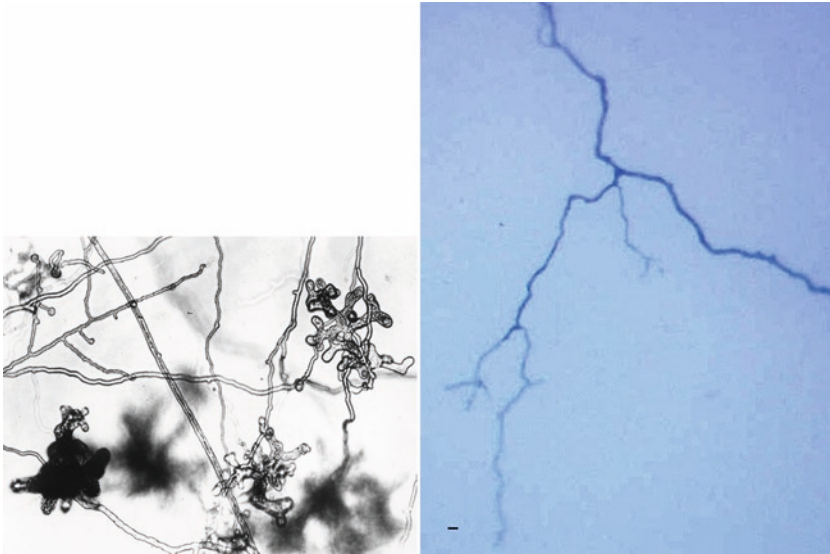
Fig. 2 Finely branched extraradical AM hyphal structures, presumably sites of fungal nutrient uptake from the soil. *Left*: Arbuscule-like branched structures of the external mycelium of *Glomus caledonium* growing under axenic conditions with Ri T-DNA transformed carrot roots (Karandashov et al. 1999, unpublished; for growing conditions see Karandashov et al. 2000) *Right*: Typical morphology of a branch of fine hyphae observed in extraradical mycelium of *Glomus intraradices* extracted from fungal compartments filled with soil and glassbeads. Hyphae were stained with trypan blue in lactic acid (Bieler and Tong 2009, unpublished; for methodology see Neumann and George 2005a)

activity (Bago 2000; Bago et al. 2002). Since these structures are presumably the major site of AM nutrient uptake from the soil, they have been termed ‚Branched absorbing structures, BAS’. Structures similar to BAS are also observed in extraradical mycelium of *Glomus intraradices* obtained from fungal compartments filled with wet sieved soil and glassbeads (Fig. 2). These typically appear less branched compared with BAS described by Bago et al. (1998), and the length of individual fine hyphae appears longer.

Whether the number of BAS or the length of fine AM hyphae in the soil is correlated with the number of arbuscules in associated host roots, or the contribution of the AM symbiosis to plant element uptake, deserves further investigation. Arbuscular mycorrhiza mycelium associated with tomato plants and extracted from fungal compartments filled with wet sieved soil and glassbeads showed a decreasing proportion of fine hyphae in response to an increasing soil P fertilization level (Fig. 3). This suggests that the soil nutrient availability and/or the plant nutritional status may affect the morphology and possibly activities of the extraradical AM mycelium. More knowledge about the physiological mechanisms behind such effects might lead to improved means of predicting or even manipulating the outcomes of the AM association in terms of plant nutrient uptake and plant growth.
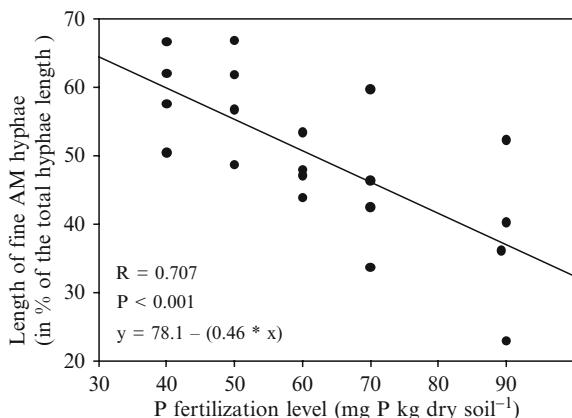
Fig. 3 Length of fine AM hyphae in percent of the total AM hyphal length. Extraradical mycelium of *Glomus mosseae* in symbiosis with *Lycopersicon lycopersicum* Cv. 'Micro-Tom' was obtained from fungal compartments filled with wet sieved soil and glassbeads. Plants were grown in greenhouse microcosms for 3 months, and soil in the plant and fungal compartment was fertilized either with 40, 50, 60, 70 or 90 mg P kg dry soil^{-1} at the beginning of the experiment. Hyphae length was obtained by a modified gridline intersection method according to Neumann et al. (2009). Hyphae with a diameter <5 μm were considered as fine (Hahn and Neumann 2009, unpublished)

2.2 Nutrient Uptake and Transport by Arbuscular Mycorrhizal Hyphae

Improved total P uptake and growth of mycorrhizal plants compared with corresponding nonmycorrhizal controls is a common observation in greenhouse studies, particularly when plants are grown on a soil with a low nutrient availability. Investigations on the uptake, transport and transfer to the host plant of mineral elements by AM mycelia often employed in vivo or in vitro microcosms where AM colonized plant roots and extraradical mycelium were grown in distinct compartments. Significant depletion of the fungal compartment of a certain nutritional element along with its increased uptake by the associated host plant compared with a corresponding nonmycorrhizal control, indicate symbiotic transfer of this element via the AM mycelium. Several studies have employed radioactive or stable isotopes of nutritional elements in combination with compartmented microcosms. When isotopically labeled nutritional elements supplied exclusively to the AM fungal mycelium can be detected in the associated host plant tissues some time after fertilization, evidence of a direct contribution of the AM mycelium to plant uptake of the respective element is furnished.

By means of such experimental approaches, uptake, transport and delivery to the host plant of P and N has been demonstrated for various plant/AM fungal combinations, and under different environmental conditions (e.g. Hattingh et al. 1973; Ames et al. 1983; George et al. 1992; Smith et al. 2000; Wang et al. 2002).

Other studies have also confirmed the uptake of Zn (Bürkert and Robson 1994; Jansa et al. 2003), S (Rhodes and Gerdemann 1978; Allen and Shachar-Hill 2009) and Fe (Caris et al. 1998) via the AM pathway. An increase in plant uptake of Cu in response to AM root colonization has frequently been observed (Li et al. 1991b; Lee and George 2005) but the transport and transfer to the host plant of nutritional elements other than P, N, Zn, Fe and S via the AM mycelium has not yet been unequivocally verified.

Recently, molecular biological methods have complemented classical nutrient uptake studies by characterizing AM fungal transport proteins involved in the uptake of P (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001; Benedetto et al. 2005) or N (López-Pedrosa et al. 2006) by the extraradical mycelium. Molecular mechanisms behind AM fungal contribution to plant P uptake are described in detail in Chapter 6.

Similar with ectomycorrhizal fungi (Allaway and Ashford 2001), AM extraradical hyphal cytoplasm is characterized by an extensive vacuolar system (Uetake et al. 2002). It has been suggested that long, tubular vacuoles may be compartments for transport of P in form of dispersed poly-P through AM hyphae, while spherical vacuoles might function in storage of Poly-P and other elements, particularly in coarse hyphae (Kuga et al. 2007).

Nitrogen can be taken up by the AM extraradical mycelium as NH_4^+ or NO_3^- (Johansen et al. 1992; Subramanian and Charest 1999), but also in form of amino acids (Hawkins et al. 2000). The uptake of NH_4^+ by AM hyphae can lead to substrate acidification in the hyphosphere with a positive impact on P acquisition from Ca-P (Li et al. 1991a; Bago et al. 2004). Recent results indicate that NH_4^+ is the preferred form of N taken up by AM fungal hyphae when NO_3^- as well as NH_4^+ is present in the soil (Toussaint et al. 2004; Tanaka and Yano 2005). It is thus possible that in AM fungi, physiological hyphosphere acidification in response to NH_4^+ uptake may contribute to P mobilization even under conditions where NH_4^+ is not the dominant form of N in the soil.

Arginine seems to constitute the major form in which N is transported through the AM hyphae, and the extraradical AM mycelium shows considerable built up of this amino acid when supplied with mineral N (Govindarajulu et al. 2005; Jin et al. 2005; Cruz et al. 2007). In the intraradical mycelium, arginase and urease break down arginine, and NH_4^+ is transferred to the plant without carbon (Govindarajulu et al. 2005; Chalot et al. 2006). It has been suggested that NO_3^- is reduced in the extraradical AM mycelium immediately after uptake, and used for the synthesis of arginine (Govindarajulu et al. 2005). However, results of Hildebrand et al. (2002) indicated that N might also be transferred to the plant in form of NO_3^-. Further investigations are required to clarify, whether direct transport and transfer of NO_3^- is possible in AM mycelia, or not.

Recently Allen and Shachar-Hill (2009) presented the first detailed study on transport forms of S through the AM mycelium. Cysteine and methionine, as well as SO_4^{2-} can apparently be taken up and transported through AM hyphae. Their study provided evidence for the transfer of S to the host plant in reduced form, or as SO_4^{2-}.

Mechanisms of translocation of nutritional elements other than P, N and S through the AM mycelium are not known to date. It is possible that metal cations are transported for charge equalization along with poly-P in AM fungal vacuoles (Christie et al. 2004; Cavagnaro 2008), but clear evidence for a coupled transport of P and cations through AM hyphae is lacking up to now (Liu et al. 2000; Lee and George 2005).

2.3 Arbuscular Mycorrhiza Fungal Nutrient Transfer and Plant Uptake of Elements via the Symbiotic and Asymbiotic Pathway

In nonmycorrhizal plants, nutritional elements can be taken up from the soil solution into the cytoplasm by epidermal root cells. The surface for epidermal element uptake may be increased by the formation of root hairs. After their uptake, elements are transported radially through the root cortical symplasm towards the central cylinder. Alternatively, elements may move through the root apoplast before being taken up into the cytoplasm at the endodermis (Van Iren and Boers-van der Sluijs 1980). In mycorrhizal plants, nutritional elements can also be transported into the root cortex via the intraradical AM mycelium. Upon release by the AM fungal hyphae, elements can be transferred into the plant cytoplasm through membranes adjacent to AM hyphal structures. Plant nutrient transporters expressed at the periarbuscular membrane, and most likely involved in plant symbiotic element uptake, have been identified (Rausch et al. 2001; Harrison et al. 2002; Paszkowski et al. 2002).

There is evidence that in mycorrhizal plants, the functioning of asymbiotic and symbiotic nutrient uptake pathways is interdependent. The transfer of certain nutrients into the plant root cytoplasm via the asymbiotic route may affect nutrient uptake of the same or of other elements via the symbiotic pathway, and vice versa. This implies that for a given nutritional element, plant capacities of asymbiotic and symbiotic nutrient uptake are not necessarily additive upon mycorrhization. It has indeed been observed that in some plant species, AM fungi can contribute almost 100% to total plant uptake of P without increasing total plant P content over that of a nonmycorrhizal control (Smith et al. 2003, 2004). The precise physiological mechanisms behind such effects are not known to date. It has been suggested that plants downregulate functional components of asymbiotic P uptake, e.g. expression of high affinity P transporters in epidermal cells, upon AM fungal root colonization (Smith et al. 2009). However, results obtained so far cannot rule out the possibility that the presence of AM mycelia in root cortical tissues physiologically affects asymbiotic uptake of elements in some plant species, e.g. by decreasing rates of radial symplasmic or apoplasmic element flux. High rates of element uptake into arbusculated cortical cells might affect ion concentration gradients in the symplasmic continuum, and thus symplasmic ion flux. Modifications in the element binding capacity of the apoplast have also been

observed upon AM root colonization (Zhang et al. 2009). Decreasing rates of symplasmic and/or apoplasmic radial transport of mineral elements through the root cortex would lead to a decreased transfer of elements from root to shoot. A poorer root-to-shoot translocation of mineral elements in mycorrhizal compared with nonmycorrhizal plants is indeed a frequent observation (Li et al. 1991a; Joner and Leyval 2001; Chen et al. 2003). In some cases this could be attributed to retention of these elements in the intraradical and extraradical AM mycelium within and attached to the analyzed roots (Johansen et al. 1992; Ravnskov and Jakobsen 1995). However, when concentrations of macroelements were analyzed in AM mycelium (Neumann and George 2005a; Neumann et al. 2009; Neumann et al. 2009, unpublished), these were usually in the same range or even lower compared with root tissues.

Decreased root-to-shoot translocation was observed for various nutritional elements in tomato plants of the cultivar 'Golden Queen' upon AM root colonization (Fig. 4). In the same experimental approach, element concentrations in roots of the mycorrhiza defective mutant 'rmc' (Barker et al. 1998) were unaffected by extensive surface colonization with AM extraradical hyphae and hyphopodia (Neumann and George 2005b). This indicates that element retention in extraradical AM
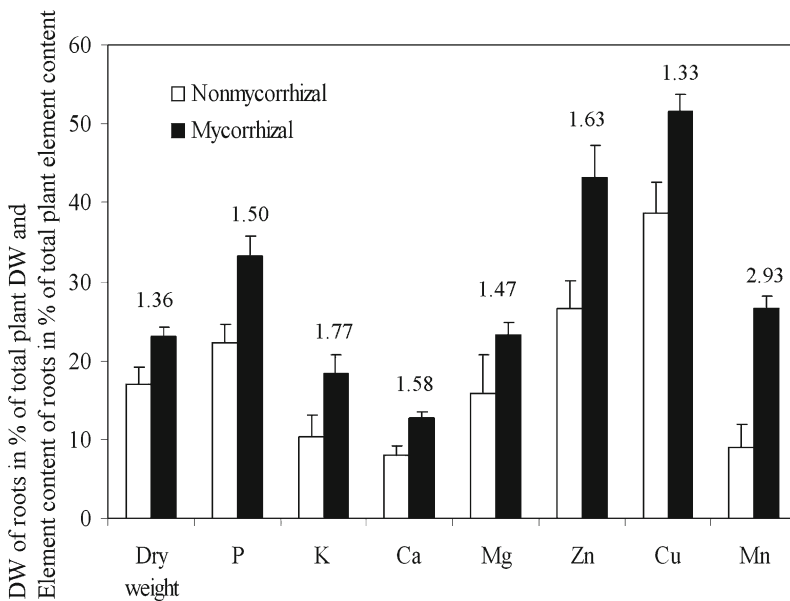
Fig. 4 Dry weight of roots in percent of the total plant dry weight, and mineral element contents of roots in percent of total plant mineral element content. Wildtype tomato plants were grown in presence (mycorrhizal) or absence (nonmycorrhizal) of a mixture of *Glomus mosseae* and *G. intraradices* for 7 weeks in the greenhouse (for growing conditions see Neumann and George 2005b). Shown are the mean values ± standard deviation. Numbers above the columns show the results of the divisions of the mean values for mycorrhizal plants by the corresponding values for nonmycorrhizal controls (After Neumann and George 2005b)

mycelium attached to the roots was not the major reason for differences in element translocation observed between mycorrhizal and nonmycorrhizal wildtype plants in this experiment. In *Arum*-type mycorrhizas, arbuscules develop preferentially close to the central cylinder. Blee and Anderson (1998) suggested that this might facilitate carbon supply to the AM symbiosis. It might, however, also be the evolutionary consequence of a decreased ability to radially transport certain elements through the root cortex in some plants upon mycorrhization.

The ability of the plant to take up elements in absence of the mycorrhizal symbiosis is usually negatively correlated with the contribution of the symbiosis to the uptake of these elements in the symbiotic state. Intra- and extraradical AM development is often negatively affected by an increasing plant P and N nutritional status (Thomson et al. 1991; Bruce et al. 1994), or increasing soil solution concentrations of these elements (De Miranda and Harris 1994; Nagahashi et al. 1996). It has been suggested that under conditions where AM contribution to nutrient uptake is not required, plants decrease photoassimilate supply to their fungal symbiont (Graham and Eissenstat 1994; Olsson et al. 2002; Schaarschmidt et al. 2007).

Though a low degree of AM root colonization is usually observed even under very high soil P fertilization levels, contribution of the AM symbiosis to plant uptake of mineral elements has rarely been reported under these conditions.

As long as the soil P and N availability is low, concentrations of other mineral elements in the soil solution do usually not have big effects on the development of the AM symbiosis (except when in a toxic range). However, availability of a certain element for plant uptake via the asymbiotic pathway may affect its uptake via the AM pathway by other means than symbiosis development. Increased availability of Fe (Caris et al. 1998), P (Maldonado-Mendoza et al. 2001), Zn (Chen et al. 2003), N (Azcón et al. 2008) and S (Allen and Shachar-Hill 2009) to plant roots has been shown to decrease symbiotic transfer of these elements, irrespective of intra- or extraradical AM fungal development. To which extent and by which physiological mechanisms host plants and AM fungi regulate symbiotic nutrient transfer, is not yet completely understood.

The effect of the plant element supply status on AM element transport and transfer activities becomes even more evident when certain elements are available in the soil in excessive amounts. While the AM symbiosis facilitates plant uptake of Zn, Cu and Mn under conditions of low availability of these elements, it may decrease their uptake by plants when supply is excessive (Liu et al. 2000; Chen et al. 2003).

Though symbiotic plant uptake of elements other than P, N, Zn and Cu has rarely been studied in the past, there is some evidence that the contribution of the AM symbiosis to their uptake may also strongly depend on their availability to asymbiotic roots. Increased uptake of basic cations such as K^+, Mg^{2+} or Ca^{2+} of mycorrhizal compared with nonmycorrhizal plants has almost exclusively been observed on acidic soils naturally poor in these elements (Clark et al. 1999a, b; Alloush et al. 2000). In contrast, plant uptake of Ca and Mg may be decreased in response to AM root colonization on alkaline soils (Khalil et al. 1994; Bermudez and Azcón 1996).

An improved iron nutrition of mycorrhizal compared with nonmycorrhizal plants has been observed on soils with a neutral pH and a poor plant availability of Fe (Liu et al. 2000; Wang et al. 2008). At pH > 8 and low substrate Fe content, AM development may be negatively affected by host plant Fe deficiency (Treeby 1992; Wang et al. 2008).

2.4 Aspects of Functional Compatibility Between Symbiotic Partners in Arbuscular Mycorrhizal Nutrient Acquisition

Under experimental conditions where plants are grown in presence of one particular AM fungal isolate, the net outcome of the mycorrhizal symbiosis in terms of plant nutrient uptake not only depends on environmental conditions such as soil properties, but also on the genotypes of the plant and AM fungal strain involved (Hamel et al. 1997; Jakobsen et al. 2001). The ability of two symbiotic partners to establish an association that leads to improved plant performance compared with a nonmycorrhizal control under given environmental conditions has been termed 'functional compatibility' (Gianinazzi-Pearson 1984; Pearson and Jakobsen 1993a, b; Ravnskov and Jakobsen 1995). To date, the view on functional compatibility has been largely plant-centered, leaving out of consideration that a symbiotic association where the AM fungus does not or even negatively affect plant performance, might be at least temporarily beneficial ('compatible') for the fungal partner in terms of, e.g. spore production or foraging for new host roots. Whether, in associations particularly beneficial from the plant perspective, plants might temporarily 'cheat' on their AM symbiont by depriving the AM mycelium of mineral elements or transferring insufficient amounts of C for adequate AM fungal reproduction, has not been investigated so far.

The precise physiological reasons behind a high or low extent of functional compatibility between plants and AM fungi are not yet understood. It is possible that in some plant/AM fungal combinations, establishment of a symbiotic interface allowing for nutrient exchange at high rates is not possible due to physiological incompatibility between the two organisms at the cellular level. However, in most previous experiments, differences in functional compatibility between a given host plant and various AM fungal strains were associated with differences in AM life-cycle strategies or mycelia development (Bürkert and Robson 1994; Graham and Abbott 2000; Smith et al. 2000, 2004). Similar with plant species, AM fungal strains may exhibit different strategies to spatially and/or chemically exploit soil nutrient resources. We hypothesize that functional compatibility in AM symbioses increases with increasing synergism between plant root and AM mycelium nutrient mobilization and acquisition strategies. Whether roots and associated AM mycelia are able to coordinate their activities in soil exploitation for mineral elements by exchange of particular signal molecules deserves further investigation.

3 Exploitation of Soil Nutrient Resources by Arbuscular Mycorrhizal Root Systems

3.1 Coordination of Nutrient Absorbing Surface Formation Between Plant Roots and Arbuscular Mycorrhiza Mycelia

The size of the nutrient absorbing root surface is an important determinant of plant nutrient acquisition capacity, particularly with respect to elements with a low mobility in the soil solution (Marschner 1995). An increase in root branching, fine root elongation or root hair formation is thus often observed when plants are growing in soils with a low nutrient availability. Such root growth responses are sometimes accompanied by a decrease in the shoot/root ratio compared with corresponding individuals growing in well fertilized soil (Gutschick 1993; Ciereszko et al. 1996). The extent of such adaptive growth responses may vary considerably depending on the plant species and the identity of the mineral elements limitative to plant growth. Plants have been shown to respond to a low availability of N, P and S with a decrease in the shoot/root-ratio, whereas a low supply with K, Mg or microelements usually has no or even negative effects on root biomass in relation to that of the shoot (Marschner et al. 1996; McDonald et al. 1996). The establishment of the AM symbiosis constitutes another strategy by which plants extend their belowground surface area for nutrient acquisition. However, to date not much is known about how extraradical AM development is coordinated in response to the plant nutritional status, the soil nutrient availability, or root traits.

Root system growth and architecture are under shared control of the root and the shoot (Desnos 2008). Phytohormones, products of photosynthesis, as well as the nutritional elements themselves function as signals in the coordination of root growth in response to soil nutrient availability or the plant nutritional status (Zhang and Forde 2000; López-Bucio et al. 2003; Ma et al. 2003). In some studies, AM root colonization and additional P fertilization had very similar effects on root morphology and the shoot/root-ratio (Fusconi et al. 2000; Vigo et al. 2000). This suggests that in some plant species, effects of the AM symbiosis on root growth and architecture are mainly the consequence of an improved plant nutritional status in response to AM contribution to element uptake. However, direct effects of the AM symbiosis on the plant phytohormonal status, or source/sink relations are also possible. Differences in the phytohormonal balance between mycorrhizal and nonmycorrhizal plants have frequently been observed. Some of these appear to be mainly associated with the coordination of plant accommodation of the AM fungus (Herrera-Medina et al. 2007; Gutjahr and Paszkowski 2009), but it cannot be excluded that AM induced changes in cytokinine, auxine or abscisic acid levels also influence host root system morphology.

Fitter (2006) suggested a proportional exchange of delivered nutrients for carbohydrates between plant roots and AM fungi. This would lead to a decreasing availability of photoassimilates for root growth when plants acquire nutrients via the AM symbiosis. One could indeed assume that investment into root growth would be less

feasible under conditions where plants acquire a major proportion of nutritional elements via the symbiotic pathway. However, an increase in the shoot/root ratio upon AM colonization is sometimes (Trotta et al. 1991; Atkinson et al. 1994), but by far not always observed (Al-Karaki et al. 1998; Fig. 4). Strategies of integration of the AM symbiosis into individual plant nutrient acquisition strategies may vary depending on the plant genotype. For example, root hairs and AM mycelia have been shown to exploit different nutrient resources in the mycorrhizosphere of barley (Jakobsen et al. 2005), and thus complementary use of symbiotic and asymbiotic pathways in parallel might be a successful strategy in this plant species. A decreased growth of root apices and an increase in the formation of lateral and adventitious roots is frequently observed in mycorrhizal compared with corresponding nonmycorrhizal plants (Berta et al. 1990, 1993; Yano et al. 1996; Torrisi et al. 1999). Upon AM colonization, root length densities have been shown to decrease in some plant species (Hetrick et al. 1988; Fusconi et al. 1999), and to remain unaffected or increase in others (Yano et al. 1996). It needs to be considered that fine roots not only provide surface area for asymbiotic uptake of mineral elements, but also cortical tissues able to symbiotically interface with AM mycelia. It has thus been suggested that an increase in overall length of lateral roots observed in mycorrhizal compared with nonmycorrhizal rice root systems might serve the provision of additional cortical tissues to host the symbiotic AM fungal colony (Gutjahr et al. 2009).

While effects of AM formation and/or the plant nutrient availability on root system architecture have been addressed by several studies in the past, only little is known about how the plant genotype and its nutritional status affect AM extraradical mycelium architecture.

There are indications that the soil nutrient availability and/or the plant nutritional status might affect the development of the nutrient absorbing surface area in AM mycelia in a similar way as in roots. In a pot experiment employing fungal compartments filled with wet sieved soil and glassbeads, we grew tomato plants in presence of the AM fungus *G. mosseae* under a low P supply (60 mg P kg dry soil^{-1}), and either a low (80 mg N kg dry soil^{-1}) or a high (250 mg N kg dry soil^{-1}) N fertilization level. While the extent of AM fungal root colonization was unaffected by the N fertilization level (data not shown), extraradical AM mycelium obtained from the fungal compartments showed a considerable increase in the calculated hyphal surface area in response to low N fertilization (Fig. 5). The sites of control over such a response in AM mycelia remain a matter of speculation, but an increase in the number of spores per unit AM hyphal surface in response to a low N supply level suggest that amounts of C supplied to AM fungi might be higher when the plant N availability is low (Fig. 5). The direct involvement of root exudates such as strigolactones or phytoestrogenes, which have been shown to promote presymbiotic AM hyphal branching (Nair et al. 1991; Akiyama et al. 2005), can be largely excluded with respect to our results, since the AM mycelium grew in absence of roots in fungal compartments.

According to our estimations, fine hyphae contributed only a relatively small proportion of approximately 25% to the total AM hyphal surface area. In this
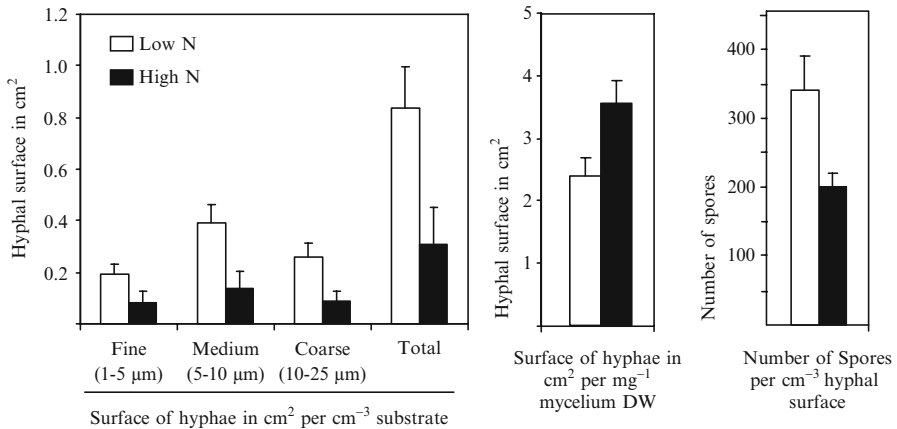
Fig. 5 Distribution of calculated AM hyphal surface area over different AM hyphal size classes, and in relation with the AM mycelium dry weight and the number of AM spores in response to two different soil N fertilization levels. Extraradical mycelium of *Glomus mosseae* in symbiosis with *Lycopersicon lycopersicum* Cv. 'Micro-Tom' was obtained from fungal compartments filled with wet sieved soil and glassbeads. Plants were grown in greenhouse microcosms for 3 months, and soil in the plant and fungal compartment was fertilized either with 80 or 250 mg N kg dry soil^{-1} at the beginning of the experiment. Hyphal length was estimated by a modified gridline intersection method for three diameter classes separately, and the hyphal surface was calculated under the assumptions that the hyphal shape was cylindrical, and that the distribution of hyphal length over different diameters within each size class was equal. Shown are the mean values ± standard error (Hahn and Neumann 2009, unpublished)

context, the rapid turnover and thus much shorter life span of fine compared with coarse hyphae needs to be considered (Bago et al. 1998). Similar with fine AM hyphae, fine plant roots tend to have a higher turnover compared with coarse roots (Hodge 2006; Joslin et al. 2006). In plants, however, the life-span of fine roots may also differ between parts of the same root system, depending on the nutrient availability in the soil around the root, and the plant nutritional status (Pregitzer et al. 1995; Hodge et al. 1999). Should fine hyphae indeed be the major site of nutrient uptake in AM extradical mycelia, and BAS turnover rates also vary depending on the soil conditions, measurements of hyphal length densities might be an inaccurate tool to predicting contributions of the AM mycelium to plant element uptake.

3.2 Collaboration Between Roots and Arbuscular Mycorrhiza Fungal Mycelia in Spatial Exploitation of Soil Nutrient Resources: Costs and Benefits

With respect to the high surface area/dry weight ratio of AM mycelia, it has been suggested that from the plant perspective, the nutrient absorbing surface area provided by the AM mycelium might be more cost-efficient compared with that of

roots in terms of photoassimilate and mineral element expenditure (Hodge 2006). However, under the assumption that the trophic position occupied by symbionts such as AM fungi is analogous to that of herbivores (Clay 2001), it needs to be considered that in trophic cascades only approximately 10% of energy provided by one trophic level can be processed by the next. In addition, the AM symbiont may partition C acquired from the host between symbiotic contribution to nutrient uptake, and own reproduction. Comparative measurement of C allocations to mycorrhizal and nonmycorrhizal roots indicate that AM plants allocate indeed up to 70% more photoassimilates to their roots (Hawkins et al. 1999; Douds et al. 2000). Nevertheless, it can be assumed that AM mycelia require almost no investment of nutritional elements by the plant. A decreased net expenditure of mineral elements per unit nutrient absorptive surface might be of particular advantage for plants under conditions where growth is limited by nutrient supply.

The net contribution of the AM symbiosis to plant element uptake may be particularly high when a large proportion of nutrients in the growth substrate is available exclusively to the AM mycelium (Janos 2007). Such a situation is often created in experiments employing compartmented planting pots where only AM hyphae have access to a compartment supplied with nutrients. When mycorrhizal plants were grown in presence of a hyphae compartment supplied with easily available mineral N, up to 80 % of total N in the plant were taken up via the AM mycelium (George et al. 1992; Frey and Schüepp 1993; Johansen et al. 1994). However, when roots and AM hyphae share the same soil volume, total plant N uptake usually appears to be unaffected by AM root colonization. An increased net uptake of P, Zn and Cu by mycorrhizal compared with nonmycorrhizal plants has, nevertheless, often been observed in greenhouse experiments using non-compartmented pots (e.g. McArthur and Knowles 1993; Liu et al. 2000). Most likely, root uptake rates of mineral elements easily mobile in the soil solution, such as NO_3^-, far exceed that of AM mycelia when roots and AM hyphae share the same soil volume. In contrast, narrow depletion zones of more immobile elements such as P, Zn, Cu or NH_4^+ are usually observed around plant roots (Jungk 1987), indicating that a considerable soil volume remains unexploited for uptake of these elements by nonmycorrhizal roots, even when root densities are high. In this respect it should be considered that artificial, sand/perlite-based growth substrates containing only little or no binding sites for P and other elements, may allow for almost free diffusion of most nutritional elements. Under such conditions, additional nutrient absorptive surface area provided by the AM mycelium may be of no advantage for plant element uptake, even when element concentrations in the nutrient solutions supplied to the pots are very low.

Plant species which are non-hosts to AM, are often characterized by the formation of an extensive system of fine roots, or the formation of long, dense root hairs when grown in a soil with a low nutrient availability. In contrast, some plant species with coarse, less branched root systems appear to be unable to acquire sufficient amounts of nutrients in absence of an AM symbiosis, even under conditions of a reasonable soil nutrient availability (Blal et al. 1990; Habte and Manjunath 1991). Beside such 'obligate' mycotrophic plant species, there are some species termed

'facultative' mycotrophs, which may benefit from the AM symbiosis under some, but not under all environmental conditions (Graham and Eissenstat 1994). However, since the establishment of the AM symbiosis may have a considerable feedback on other plant nutrient acquisition strategies, it would most likely be an oversimplification to describe AM contribution to spatial plant nutrient availability as a function of overall root system morphology (Smith et al. 2009). Similar with plant root systems, AM extraradical mycelia display distinct morphological patterns depending on the AM genotype. This may concern initial colonization establishment (Graham and Abbott 2000; Hart and Reader 2001), extension of the mycelium from the root surface (Jakobsen et al. 1992; Smith et al. 2000), pattern of anastomosis formation (de la Providencia et al. 2004), or growth into and P uptake from nutrient rich patches in the soil (Cavagnaro et al. 2005).

Nutrient resources are usually not homogeneously distributed in the soil, and nutrient availability may vary considerably within the rooting zone of a plant. Some plants respond to a heterogeneous distribution of elements in the soil by preferential allocation of resources to root formation in nutrient rich patches, whereas other plants appear to respond only little (Farley and Fitter 1999; Hodge 2009). Symbiotic AM fungi might assist their host plant in the exploitation of heterogeneously distributed nutrient resources either by exploiting the nutrient rich patches, or by compensating for a decreased root proliferation and thus nutrient uptake capacity outside the patch. The coordination of individual exploitation strategies might not only depend on the inherent traits of the symbiotic partners involved, but also on the size of the patch and its chemical composition. Most research conducted so far suggests that AM fungi do not actively forage for soil microsites with a high availability of mineral P (Olsson and Wilhelmsson 2000; Olsson et al. 2003). Plants exposed to a patchy distribution of mineral elements in their rooting zone benefited from the AM symbiosis in terms of growth and nutrient uptake, but this was not due to AM hyphal activities in exploiting nutrient resources within the nutrient rich patches (Cui and Caldwell 1996). It has been suggested that AM fungal mycelia might be particularly important for the exploitation of nutrient patches too small to be perceived or exploited by a plant root (Tibbett 2000). In most previous experiments, however, nutrient rich patches were in a much larger range (usually > 1 cm in diameter), and thus experimental evidence for this assumption is still lacking. In the field, nutrient rich microsites are often associated with decomposing organic material. A stimulation of AM hyphal growth by the presence of decomposing plant material in the soil has often been observed (St. John et al. 1983; Gryndler et al. 2005). Increased uptake of mineral elements from patches of dead plant material by mycorrhizal compared with nonmycorrhizal root systems does, however, apparently only occur under certain environmental conditions, such as, e.g., interspecific plant competition (Hodge et al. 2000; Hodge 2003a, b).

Arbuscular mycorrhiza fungal strains may not only vary in their extraradical mycelium morphology or developmental plasticity, but also in their tolerance towards environmental conditions that negatively affect root proliferation or activities. When the relative extent of negative impact of an environmental stress factor on belowground development and nutrient uptake activity is different for plants compared with

AM fungi, relative contributions of the AM symbiosis to plant performance might differ accordingly. For example, under conditions where root growth is restricted due to a high plant availability of heavy metals, Al or salinity, AM fungal strains tolerant to particular harmful elements in the soil might compensate for a low nutrient absorptive root surface of their host plant (Ojala et al. 1983; Leyval and Binet 1998; Meharg and Cairney 2000). Under such conditions, plants might benefit from the AM symbiosis even on soils where availabilities of nutritional elements would be sufficient for optimal plant growth in absence of environmental stress. Arbuscular mycorrhizal fungi isolated from polluted soils are usually more tolerant towards harmful elements compared with AM fungi from non-polluted sites (Coperman et al. 1996; Hildebrandt et al. 1999; Del Val et al. 1999), but the precise physiological mechanisms behind individual adaptations are not known to date.

Neumann and George (2004) observed that the net contribution of the AM symbiosis to plant growth and P uptake from a well fertilized soil increased three to five fold upon exposure of roots to partial rootzone drying. Similar results were obtained by Neumann et al. (2009; Fig 6). Even on well fertilized soils, a low soil moisture regime may severely decrease plant availability of mineral elements (Gahoonia et al. 1994). With their small diameter, AM hyphae have access to small soil pores, which remain filled with soil solution even under low levels of soil moisture (Faber et al. 1991). This may render the AM fungal mycelium less sensitive towards drought compared with a plant root. However, AM fungi are also dependent on their host plant for C supply, and environmental factors negatively affecting plant
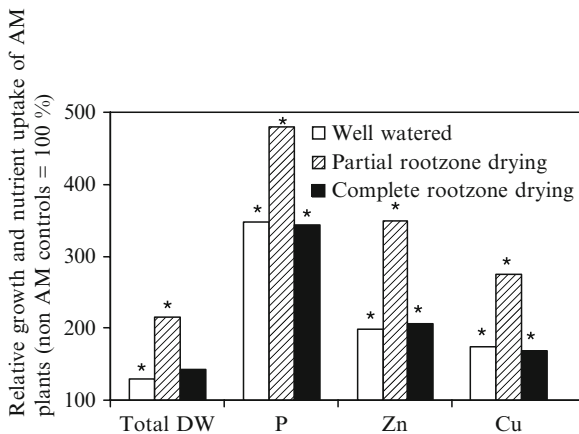
Fig. 6 Growth and nutrient content of AM colonized plants in percent of corresponding nonmycorrhizal controls. Sweet potato plants were grown in two compartment split root pots, constructed from conventional planting pots fastened together, side-by-side. After plants had established, the soil water content in one (partial rootzone drying), or in both (complete rootzone drying) root compartments of each split-root pot was decreased to impose drought stress. Controls remained well watered. Plants were harvested 4 weeks after beginning of the drought stress period. A star indicates a significant difference between the mean, absolute values obtained for mycorrhizal and corresponding nonmycorrhizal plants (t-test, $P < 0.05$; After Neumann et al. 2009)

photosynthesis may have an indirectly negative impact on AM development and activities. Limitation of photosynthesis by water deficiency might thus have been a reason why net AM contributions to nutrient uptake were smaller under complete compared with partial rootzone drying (Fig. 6).

Whether in natural ecosystems, individual plants are preferentially colonized by AM fungal strains that complement their individual root traits best in terms of nutrient acquisition and stress tolerance, remains speculative. However, preferential colonization of roots of certain plant species by particular AM fungal species seems to be a common phenomenon (Eom et al. 2000; Helgason et al. 2002). In this respect it also needs to be considered that plants in the field are usually colonized by more than one AM fungal strain, and that these may occupy different ecological niches within the same root system. Whether different types of roots (e.g. fine roots/coarse roots), or roots of different age are colonized by different AM fungal strains, has not been investigated so far. Nevertheless, there is evidence that the composition of the AM community colonizing the root system of a given plant may vary depending on the soil depth (Oehl et al. 2005) or the growing season (Pringle and Bever 2002). Koide (2000) hypothesized that AM fungal strains able to exploit nutrient resources in different parts of the mycorrhizosphere might spatially complement each other. Simultaneous root colonization by both AM fungal strains would in such a case be superior in terms of nutrient acquisition compared with colonization by either strain alone.

3.3 Effects of AM Fungal Mycelia on Chemical and Physical Mycorrhizosphere Properties, and Implications for Mineral Element Availability

The soil in the vicinity of a plant root is altered in its chemical, physical or biological properties by the activities of the root. Plant nutrient uptake may lead to decreased concentrations of some elements, while others may accumulate when their massflow towards the root surface exceeds plant uptake (Marschner 1995). Substances released by plant roots into the soil might feed certain soil bacteria, or mobilize mineral elements from sparingly soluble pools (Walker et al. 2003). The soil under the direct influence of plant root activities usually extends 1–6 mm from the root surface. However, when plants are mycorrhizal, sites of nutrient uptake, as well as flux of plant C into the soil may extend far beyond this so-called 'rhizosphere'. Taking the activities of the extraradical AM mycelium into account, the term 'mycorrhizosphere' was established to describe the soil part under the influence of roots and extraradical AM mycelia. The mycorrhizospheres of neighboring roots may be connected via common AM hyphal networks, making it impossible to spatially assign mycorrhizospheres to individual roots or plants. It has been suggested that the presence of an AM hyphal network, allowing for long-distance transfer of mineral elements may attenuate soil heterogeneity in terms of mineral nutrient availability (Hodge 2006).

Experiments employing isotopically labeled N and P have revealed that these nutrients can even be transferred between plants interconnected by a common AM hyphal network (Frey and Schüepp 1992; Martins and Read 1996; Martins and Cruz 1998; Leake et al. 2004). So far it is not clear by which mechanism nutrients are released by the donor plants before they are taken up for transfer to the receiver via AM hyphae. It cannot be excluded that AM fungal mycelia mainly recapture nutrients released by passive efflux, or upon death or damage of host root cells. Dying roots or root parts may be an important source of mineral elements in soils of ecosystems where nutrient availability limits plant growth. Once an AM colonized root dies, it may thus immediately become part of the mycorrhizosphere of its neighbors (Ritz and Newman 1985). Whether intraradical AM mycelia surrounded by dead, disintegrating cortical cells may even switch direction and become active in nutrient uptake after having been active in nutrient release while the root was alive, deserves further investigation.

Decay of dead plant or animal material, breakdown of its organic components and incorporation of mineral nutrients by living organisms is a crucial step in nutrient cycles of terrestrial ecosystems. So far it is not completely clear, how and to which extent AM hyphal networks contribute to this process. It has been shown that AM hyphae can acquire mineral nutrients from decomposing organic material, and transfer these to their host plant (Hodge et al. 2001; Leigh et al. 2009). Mineralization of organic material has been shown to accelerate in presence of AM roots or AM mycelia, compared with the bulk soil (Hodge, et al. 2001; Hodge 2001; Phillips and Fahey 2006).

Different from some ecto- and most ericoid mycorrhizal fungi, release of lignolytic or proteolytic exoenzymes has not been observed in AM mycelia investigated so far. Some experiments provided evidence for the ability of AM extraradical hyphae to excrete phosphatases, enabling the fungus to release and acquire P from organic P resources (Koide and Kabir 2000), including phytate. Other experiments, however, could not confirm extraradical phosphatase release by AM fungi (Dodd et al. 1987; Joner and Jakobsen 1995).

Glucanases may as well be released by AM hyphae, but their function has so far mainly been related with the ability of AM hyphae to penetrate the host apopolast (Garcia-Garrido et al. 1992). It might, however, similarly enable AM hyphae to penetrate and spatially access dead non-host roots or litter. Colonization of dead leaf tissues by extraradical AM hyphae has indeed been observed (Aristizábal et al. 2004). Even if AM fungi should be largely unable to cleave higher molecular weight organic compounds such as polypeptides by their own exoenzymes, their small diameter and large surface area might enable them to rapidly access dead plant tissues and exploit them for nutritional elements in mineral form, or in form of low molecular weight organic compounds. The diameter of the nutrient absorbing AM hyphae is in a similar range as that of other soil microorganisms, and thus the AM hyphae might be better able than roots to spatially compete with bacteria or saprophytic fungi for nutritional elements within dead organic material. Hyphae of AM fungi grown in non-sterile soil are often coated with bacteria (Bianciotto et al. 2001). Thus, not only competitive, but also collaborative interactions between other soil microorganisms and AM mycelia in the exploitation of sparingly available

nutrient resources in the soil have been proposed (Bianciotto et al. 2002). These are addressed in more detail in Chapter 10.

The excretion of organic acids or phenolic compounds is a widespread strategy of plant roots to mobilize P, Ca or micronutrients from their soil matrix binding sites (Masaoka et al. 1993; Dakora and Phillips 2002). It has been shown that relatively high concentrations of organic acids are necessary to mobilize P from Al-/ Fe- oxides (Johnson and Loeppert 2006). Thus, plants usually excrete these organic compounds in a high concentration in a discrete root section shortly behind the root tip, where decomposing bacteria are not yet abundant, and root hairs ensure rapid uptake of mobilized elements (Roemheld et al. 1984; Marschner 1995). It has been shown that the mycelia of some AM fungi proliferate mainly in proximate vicinity of the root (Smith et al. 2000). Whether these fungi might assist the plant in retrieving mineral elements mobilized from sparingly available pools by the release of organic acids or enzymes deserves further investigation. There are indeed indications that root release of phosphatases is promoted in response to AM root colonization in some plant species (Ezawa et al. 2005). In a study of Shibata and Yano (2003), AM fungal hyphae only contributed to uptake of P from sparingly available resources when root exudates were present.

Most studies undertaken so far do not point to the ability of AM fungal mycelia themselves to excrete organic acids in order to mobilize mineral elements (Jayachandran et al. 1989; Antunes et al. 2007). Hyphosphere acidification has frequently been observed in AM fungi, but this was usually associated with hyphal uptake of NH_4^+ and subsequent release of H^+ (Li et al. 1991a; Bago et al. 2004). Substantial energy loss is associated with transfer of carbohydrates from one trophic position to the next within the trophic cascade. Thus, nutrient mobilization by release of organic acids or enzymes may be up to ten times more expensive via AM mycelia compared with plant roots in terms of photoassimilate expenditure. It is further likely that soil in the vicinity of root cells and AM hyphae does not differ much in the amount of nutritional elements mobilized per unit organic acid or enzyme added. Thus, it could be speculated that the release of organic compounds into the AM hyphosphere would not increase plant nutrient availability enough to justify its presumably high carbohydrate costs. Plant growth (Passioura 2002) and activities of soil microorganisms (Andrade et al. 1998) depend to a large extent on the structure and stability of the soil matrix. Soils with a crumbly structure usually show better water infiltration, water holding capacity and resistance towards wind and water erosion compared with weakly structured soils. Such conditions are favorable for root growth and physiological activity of many plant species, and usually allow for aerobic bacterial decomposition of organic material. The formation of soil aggregates is a complex process, in which both, abiotic and biotic factors are involved (Oades 1984). Arbuscular mycorrhiza fungal hyphae and fine roots have often been shown to contribute to soil aggregate stability, e.g. by physically entangling soil microaggregates into larger units, or by increasing the abundance and activity of microbes involved in soil aggregation processes (Miller and Jastrow 1990; Rillig 2004; Rillig et al. 2005). There is experimental evidence that facilitation of soil aggregation may be an important mechanism by which AM

improve water and nutrient availability to their host plants, and possibly even nonmycorrhizal neighbors, under field conditions (Hamel et al. 1997; Cavagnaro et al. 2006; Van der Heijden et al. 2006).

The AM hyphae may also have the ability to improve the physical contact between plant roots and the soil. This might be of particular advantage under conditions where plant water uptake temporarily exceeds water flux towards the root surface, and the rhizosphere falls dry (Davies et al. 1992). Under such conditions, AM hyphal networks may assist the plant in maintaining hydraulic conductivity between the root surface and the soil solution beyond the rhizosphere. Whether water films around living or even dead AM hyphae contribute significantly to water or mineral element flux towards the root surface is still speculative. The small diameter of AM hyphae might not allow for the flux of substantial amounts of soil solution (George et al. 1992). However, polyphosphates have been observed in the AM hyphal apoplast (Kuga et al. 2007), suggesting that this compartment might potentially play a role in AM transport or storage of mineral elements. The hyphae of some AM species have also been shown to produce glomalin, a hydrophobic glycoprotein (Wright et al. 1996). Glomalin is very stable towards decomposition (Rillig et al. 2006), and it has been suggested that this glue-like protein also promotes the formation of water-stable soil aggregates (Wright and Upadhyaya 1998). Recent findings indicate that glomalin constitutes a cell wall component of AM hyphae rather than a substance excreted into the hyphosphere (Driver et al. 2005). The precise physiological functions of the protein are not known to date, but it may be involved in bacterial attachment to AM hyphae, or protection of the mycelium from soil animal grazing or desiccation in dry soil parts (Purin and Rillig 2007). Through its effect on chemical and physical mycorrhizosphere soil properties, the AM mycelium may influence plant availability of water and mineral elements, irrespectively of the hyphal activities in their uptake, transport and transfer to their host via the symbiotic pathway.

4 Conclusions

The AM symbioses are a key component of nutrient cycles in almost all terrestrial ecosystems. The extent by which plants successfully compete with neighboring plants or soil microbes for prevailing soil nutrient resources, is an important determinant of plant occupation of particular ecological niches. Plant competitiveness may depend on the symbiosis with AM fungal strains functionally compatible with the plant life-cycle and its other nutrient acquisition strategies. This may be a major reason for the close interdependence of plant and AM fungal community composition observed in ecosystems (Hart et al. 2003; Wardle et al. 2004).

The establishment of functionally compatible symbioses may be achieved by host selectivity and preferential colonization of a particular plant species by compatible AM fungal genotypes. However, exchange of signals between plant roots and AM fungi might also be involved in the coordination of joint nutrient resource

exploitation strategies. Despite considerable progress in the study of AM symbiotic nutrient uptake in plants, knowledge on how AM symbioses are integrated into individual plant nutrient resource exploitation strategies in an ecosystem context is still incomplete. Such data would not only broaden our understanding of the functioning of natural ecosystems, but also facilitate the development of strategies to manage AM in agroecosystems.

In the past, the investigation of AM functioning at the ecosystem level has been hampered by methodological difficulties such as, e.g., the establishment of nonmycorrhizal controls in the field, or the simulation of field conditions in climate chambers. In the future, however, the considerable methodological progress achieved in the ecophysiological, biochemical and molecular methods to the study of AM symbioses should be applied in integrated approaches to extend our understanding of the functioning of the AM symbiosis in plant nutrient uptake from model systems towards (agro-)ecosystems.

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Chapter 8
Hormonal Responses in Host Plants Triggered by Arbuscular Mycorrhizal Fungi

Jutta Ludwig-Müller

Abstract Plant hormones are essential factors for the development of plants. They also constitute signals for the interaction of plants with microbes, including both pathogens and symbionts. The role of different classes of hormones in symbiosis is just beginning to unravel. The major advances in our knowledge on hormones in arbuscular mycorrhiza (AM) development have come from the analysis of increasing number of mutants and transgenic plants, which are available also for mycorrhizal plants now, as well as more sensitive analytical techniques. Auxins might be necessary for root growth, while cytokinins could be involved in recognition as well as in establishment of functional mycorrhiza. Jasmonic and abscisic acids are necessary for proper arbuscule formation, but for most hormones functional analysis is missing. Here, the knowledge on possible functions of different phytohormones is summarized, some data on function as far as available, are discussed and finally some thoughts about belowground signals to aboveground tissues and their effects in relation to the possible role of hormones in the upper part of the plant are given.

Keywords Abscisic acid • Arbuscular mycorrhiza development • Auxin • Cytokinin • Ethylene • Gibberellin • Hormone metabolism • Jasmonic acid • Plant root • Salicylic acid

Abbreviations

ABA   Abscisic acid
ACC   1-Aminocyclopropane-1-carboxylic acid
AM   Arbuscular mycorrhiza
AMF   Arbuscular mycorrhizal fungus (fungi)

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AOC   Allene oxide cyclase
CK   Cytokinin
COI1   Coronatine insensitive 1
ET   Ethylene
GA   Gibberellin
GUS   β-Glucuronidase
IAA   Indole-3-acetic acid
IBA   Indole-3-butyric acid
iPA   Isopentenyladenine
JA   Jasmonic acid
LR   Lateral root
MeJA   Methyl-jasmonate
NCED   9-*cis*-epoxycarotenoid dioxygenase
P   Phosphorus
PAA   Phenylacetic acid
PR   Pathogenesis related
SA   Salicylic acid
TFIBA   *trifluoro*-indole-3-butyric acid
TIBA   *tri*-iodobenzoic acid
ZR   Zeatin riboside

1   Introduction

Within the recent years a tremendous effort has been made using combined methods of hormone analysis, mutants, transgenic plants and transcriptome data to unravel the role of plant hormones during the interaction of plants with arbuscular mycorrhizal fungi (AMF). However, there are still huge gaps in our knowledge on how both partners in the symbiosis can contribute to the regulation of hormone production. While plant hormones regulate a plethora of events during the development of a plant, they constitute also ideal signalling molecules to control the establishment of a symbiosis (Fig. 1, Table 1). Auxins are known to be involved in regulating plant architecture in shoots (Reinhardt and Kuhlemeier 2002) and roots (Doerner 2000). For example, auxins might be responsible for lateral root formation on the host plant and therefore trigger early events (Kaldorf and Ludwig-Müller 2000), whereas jasmonates and abscisic acid are necessary for arbuscule development (Herrera-Medina et al. 2007; Isayenkov et al. 2005). For vesicle and spore formation no distinct hormone has been described so far to be specifically involved. These later changes in the development of the fungus might therefore be triggered by autonomous signals of the fungus itself. Also, plant hormones can be involved in transient defense responses necessary to establish a homeostasis between host plant and AMF (Ginzberg et al. 1998; Shaul et al. 1999; Garcia-Garrido and Ocampo 2002), or they could induce systemic resistance to protect the host from other pathogens (Pozo et al. 2002).
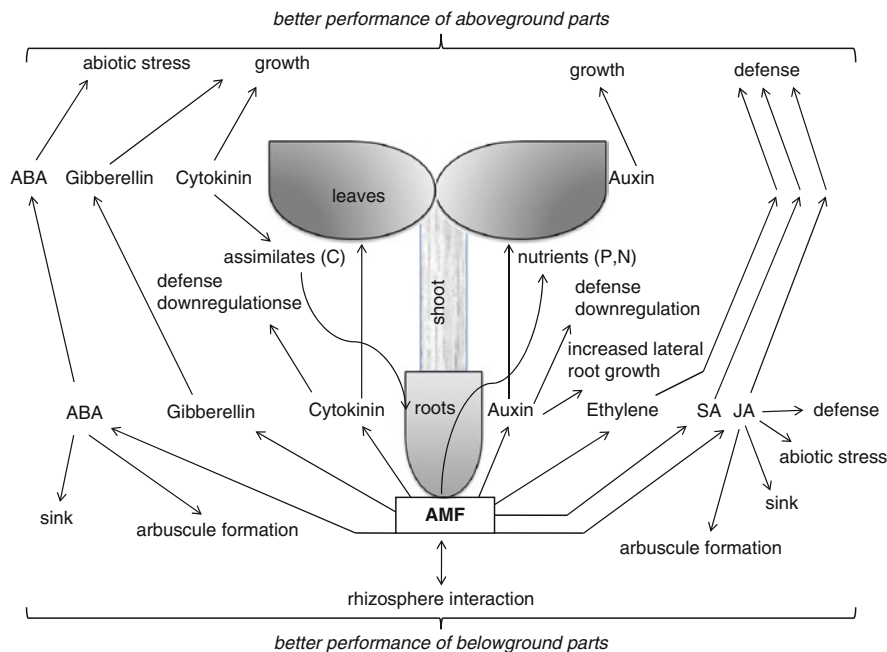
Fig. 1 Model on the interaction of plant hormones in belowground and aboveground development of the host plant

Table 1 Differential regulation of hormone levels in various plant species where the particular hormone was analyzed

Plant Hormone	Plant species	Organ	PH in Fungus
IAA	Maize, tobacco	Roots	Glomus spores
	Tropaeolum (↔)	Roots	
	Soybean, leek, litchi (↑)		
IBA	Maize, Tropaeolum	Roots	
	Medicago (↑)	Leaves	
	Maize (↑)		
PAA	Tropaeolum (↑)	Roots	
Auxin	Maize (↑)	Roots, leaves	
Conjugates	Tropaeolum (↑)	Roots	
Cytokinins (general)	Flax, sour orange	Roots	Glomus
	Bouteloua, Plantago (↑)	Root exudate	Mycelium
	Citrus (↑)		
ZR	Leek (↑)	Roots	
iPA	Tobacco (↑)	Roots	
	Litchi (↑)	Roots, leaves	
Gibberellins	Bouteloua, flax (↑)	Leaves	Glomus culture filtrate

(continued)

Table 1 (continued)

Plant Hormone	Plant species	Organ	PH in Fungus
	Bouteloua (↓)	Roots	
	Leucaena (↑)	Root exudate	
	Tobacco, Agropyron (↑)	Roots	
	Tobacco, Agropyron (↔)	Leaves	
Abscisic acid	Maize, soybean	Roots	Glomus
	Medicago, Tropaeolum (↑)		Hyphae, spores
Jasmonic acid	Barley, soybean	Roots	
	Medicago (↑)		
JA-Isoleucine	Barley (↑)	Roots	
Ethylene	Flax, papaya (↑)	Roots	
	Tomato (↔)	Roots	
	Snapdragon (↓)	Flowers	
Salicylic acid	Tobacco (↓)	Roots	
	Rice (↑)	Roots	

(↑) Mostly upregulation (↓) mostly downregulation (↔) analyzed but no difference found. IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; PAA, phenylacetic acid; ZR, zeatin riboside; iPA, isopentenyladenine; PH, plant hormone

Finally, plant hormones also regulate sink-source relationships (Balibrea Lara et al. 2004) where they could be important for nutrient exchange between plant and fungus (Schaarschmidt et al. 2007). This would then result in return in better growth or stress tolerance of host plants. Since many hormones are involved in a variety of processes, overlapping functions need to be taken into account.

2 Auxins: Triggers of Organ Formation?

2.1 Auxin Production by Mycorrhizal Fungi

Auxins can be produced by several fungi, which form ectomycorrhizal associations with host plants (Gay and Debaud 1987; Ho 1987) and thus influence the host plant to increase the root system (Splivallo et al. 2009). The growth promoting fungus *Piriformospora indica* (Sebacinaceae), whose interaction with host roots resembles in some aspects an AM, also produces indole-3-acetic acid (IAA) (Sirrenberg et al. 2007; Vadassery et al. 2008), and IAA was discussed to be one factor for the increased growth of the host plant because the root system resembled that after auxin treatment (Sirrenberg et al. 2007). Spores of the AMF *Glomus intraradices* produce small amounts of IAA but not indole-3-butyric acid (IBA), another naturally occurring auxin (Ludwig-Müller et al. 1997). Neither auxin was detected in hyphae grown in the vicinity of a host plant (Jentschel et al. 2007).

2.2   The Occurrence of Auxins in the Arbuscular Mycorrhiza Symbiosis

Increased levels of IAA or IBA were observed in different plant-AM interactions. Even though several AM host plants have been investigated by now, there is no clear pattern on the modulation of auxin (Table 1). In maize (*Zea mays*) roots, IAA was not increased (Danneberg et al. 1992), whereas IBA increased especially in young maize roots inoculated with AMF (Ludwig-Müller et al. 1997; Kaldorf and Ludwig-Müller 2000). In addition, Fitze et al. (2005) reported an increase in IAA amino acid conjugates in AM inoculated roots but not ester conjugates. Also, a systemic increase of IBA and IBA conjugates was noted.

Since IBA could be identified in other plant species besides maize (Ludwig-Müller and Cohen 2002; Campanella et al. 2008), the levels of different auxins were determined in two additional plant species, *Tropaeolum majus* (Jentschel et al. 2007) and the model legume *Medicago truncatula* (Campanella et al. 2008). In addition to IAA and IBA, phenylacetic acid (PAA), a compound with auxin activity (Ludwig-Müller and Cohen 2002), which is presumably derived from high levels of benzylg-lucosinolate in *T. majus*, is present albeit in much smaller amounts. At very early stage, free IAA and IBA were lower in infected than in control roots, whereas PAA concentration was higher in infected roots than in controls. At later stages, especially IBA was increased in colonized roots (Jentschel et al. 2007). These patterns indicate that maybe different auxins are responsible for different stages of colonization. In *M. truncatula* the measurements of IAA and IBA during development of AM showed a significant increase of IBA over the time of AM development, whereas IAA was only at one later time point significantly increased (Ludwig-Müller and Güther 2007; Campanella et al. 2008). In another legume, soybean (*Glycine max*), free IAA was increased in AM-colonized roots of a split-root system, whereas in the non-mycorrhized part of the system no increase in IAA was found (Meixner et al. 2005, 2007). This shows that IAA is produced and retained at the site of colonization. An increase in IAA was also reported in mycorrhizal roots of leek (*Allium porrum*) compared to controls (Torelli et al. 2000) and in litchi plant roots inoculated with either *Glomus intraradices* or *Gigaspora margarita* (Yao et al. 2005).

Hormone profiling in tobacco (*Nicotiana tabacum*) AM roots revealed no changes in IAA levels (Shaul-Keinan et al. 2002). However, using a tobacco line with an auxin-responsive promoter-reporter construct (*GH3::GUS*) showed increased GUS activity in AM inoculated roots compared to controls (Jentschel et al. 2007). Since IBA can also induce these reporters and such constructs do not really measure auxin levels, rather auxin responsiveness, this is not in disagreement with the findings of Shaul-Keinan et al. (2002). In transgenic tobacco plants over-expressing an apoplastic invertase, free and conjugated IAA correlated in one experiment with an increased mycorrhization in the transgenic plants compared to controls, but this was not reproducible in a second experiment (Schaarschmidt et al. 2007). This might indicate that other factors, e.g. environmental or developmental, could be responsible for differences measured in IAA levels.

2.3   Control of Auxin Homeostasis in AM Symbiosis

The control of auxin levels in plant tissues is essential because otherwise proper development of the plant is disturbed. The maintenance of the levels of free IAA and IBA is a combination of biosynthesis, transport, degradation, and conjugation/hydrolysis to/from inactive conjugates (Ljung et al. 2002). At present, it seems that AMF do not produce enough auxin themselves to disturb auxin homeostasis, therefore, the plant seems to be in charge of controlling auxin levels.

   The synthesis of IBA was higher in AM colonized maize roots than in controls (Ludwig-Müller et al. 1997), which co-incided with the endogenous IBA levels. In *T. majus* the synthesis of IAA, IBA and PAA was investigated in control and AM-inoculated roots using in vivo labelling techniques (Jentschel et al. 2007). Not much difference in the IAA and PAA labelling patterns between controls and AM-inoculated roots were found, whereas IBA synthesis was slightly higher in AM-inoculated roots (Jentschel et al. 2007). In *M. truncatula* roots the increase in auxin was accompanied with differential upregulation of several transcripts belonging to a family of auxin amino acid conjugate hydrolases (Campanella et al. 2008). This could be an indication for the involvement of auxin conjugate hydrolysis to contribute to high auxin levels. Contrary, in experiments with maize the enzymatic hydrolysis of auxin conjugates did not play a role in the increase of auxins (Fitze et al. 2005).

2.4   Pharmacological Approaches to Understand the AM Symbiosis

Interestingly, the lateral root (LR) system of maize increased concomitantly with IBA and it resembled the phenotype of a root system treated with low IBA concentrations (Kaldorf and Ludwig-Müller 2000). For rice (*Oryza sativa*) it was shown that large LRs were preferentially colonized, whereas fine LRs were immune to arbuscular mycorrhizal colonization (Gutjahr et al. 2009). Fungal preference for large LRs also occurred in *sym* mutants that block colonization of the root beyond rhizodermal penetration. Interestingly, in rice the LR formation was functionally connected to IBA and not IAA (Chhun et al. 2004). Even though changes in root morphology after inoculation with AMF have been reported by several groups for other than monocots (Tisserant et al. 1996, Yano et al. 1996), other plants did not show this change in the root system after AM inoculation, e.g. *M. truncatula* (Ludwig-Müller and Güther 2007; Campanella et al. 2008).

   It seems, however, that not all plants can respond to AM inoculation by the production of sufficient hormones. The addition of IAA was able to increase the infection rate in treated roots of *Allium sativum* compared to controls (Bareen et al. 1988). Interestingly, IAA also enhanced the germination rate and hyphal growth of axenically grown spores of *Glomus mosseae* (Barea 1986). IBA application to mycorrhizal seedlings of *Citrus aurantium* significantly increased the percentage of AMF colonized roots compared to control plants (Dutra et al. 1996). On the contrary, the

growth of intraradical hyphae of *Glomus fistulosum* and *Glomus mosseae* decreased by IAA at physiological concentrations (Gryndler et al. 1998). Maybe auxins could suppress high proliferation rates of the symbiont during early establishment, whereas higher colonization rates might be connected to LR formation. It could be speculated that maybe different auxins such as IAA and IBA could have different roles in these processes. Since in several plant species IBA and not IAA was increased in AM colonized roots (see above), future analyses should also take IBA into account.

Work on a potential role of auxin transport in AM formation is still scarce. Xie et al. (1997) showed that colonization of *Lablab purpureus* roots with *Glomus mosseae* was increased after treatment with the auxin transport inhibitor *tri*-iodobenzoic acid (TIBA). Shortly afterwards, these findings were confirmed using wild type and mutant peas (Müller 1999). Whether the application of TIBA resulted in the accumulation of IAA in treated roots was, however, not determined. Other evidence for transport comes from the systemic increase of IBA in maize leaves, although direct transport of the hormone was not determined (Fitze et al. 2005). Similarly, Yao et al. (2005) reported the increase in IAA in leaves of AM inoculated litchi (*Litchi chinensis*) plants. Mathesius (2003) hypothesized that the perturbance of the root auxin balance could be mediated by plant flavonoids, the only plant derived auxin transport inhibitors known, which also stimulate mycorrhizae formation (Xie et al. 1997).

A pharmacological approach has yielded indications for IBA not only in lateral root formation of the host, but also in the number of intraradical structures of the symbiont. A fluorinated IBA analog which acts as antagonist in maize root growth provided evidence that after TFIBA treatment the intraradical hyphae and arbuscule number of *Glomus intraradices* was reduced (Kaldorf and Ludwig-Müller 2000). Whether this is due to the reduction of entry sites or whether auxin is necessary for initial steps of colonization could not be elucidated with this approach.

Indications of the down-regulation of a chitinase gene by AM and auxin in *M. truncatula* gave an indication that auxins might be involved in the down-regulation of defense reactions at the beginning of the symbiosis (Ludwig-Müller and Güther 2007). However, the attempt to co-localize the auxin response using an auxin-inducible promoter-reporter line of tobacco with distinct mycorrhizal structures failed (Jentschel et al. 2007). Therefore, in contrast to the co-localization of jasmonic acid inducible genes with arbuscules (Hause et al. 2002), the increase in auxin response did not co-localize with fungal structures.

3   Cytokinins: Initial Recognition, Repressors of Defense Responses or Regulators of Growth?

The first contact between a fungal hyphae and the host root is essential for recognition. The involvement of strigolactones in these initial responses has recently been discovered and will be described in the chapter by Becard (this book). However, for different beneficial microorganisms it has been shown that cytokinin (CK) receptors are essential for the establishment of the symbiosis. For example, in the interaction of *Arabidopsis thaliana* with *P. indica*, a specific CK receptor combination was

necessary for successful interaction as shown by the growth promoting effect of the fungus (Vadassery et al. 2008). *P. indica* can synthesize CKs in high amounts, thereby probably triggering the plant response. Whether CK perception plays a role during initial phases of AM colonization in host roots has yet to be established, but can be assumed likely.

It is obvious that plant tissues inoculated with AMF need to balance the invasion of the fungus and establish the symbiosis. CKs are thought to play a role in the repression of defense responses during establishment of the symbiosis (Ginzberg et al. 1998; Shaul et al. 1999). Several pathogenesis-related (PR) proteins were downregulated during AM and also downregulated after CK treatment in previously induced plants. In addition, it is known that CKs can redirect assimilates (Walters and McRoberts 2006) and induce invertase (Ehness and Roitsch 1997), thereby directly contributing to C-distribution and by this also to nutrition of the fungus (arbuscules). Invertase seems to play a role in AM formation (Schaarschmidt et al. 2007). Also, carbohydrates could be involved in the adjustment of the osmotic potential during drought, thereby protecting AM colonized plants better than noncolonized ones (Augé 2001). Details on carbon metabolism in AM symbiosis will be covered in another chapter of this book in detail.

Cytokinin-like substances were produced by AMF in axenic mycelial cultures (Barea and Azćon-Aguilar 1982). Gryndler et al. (1998) reported that, in contrast to auxin, only high CK concentrations had an inhibitory effect on hyphal growth, indicating that this effect has no physiological role in the plant. It was assumed that the increase in CK levels could be due to increased phosphate nutrition in AM roots (Barker and Tagu 2000). In leek treatment with phosphorous (P) increased the levels of zeatin riboside (ZR) more than mycorrhizal colonization, whereas AM and P increased IAA at substantially similar values (Torelli et al. 2000). These results on CKs are in agreement with several studied of CK content in AM plants (Allen et al. 1980; Edriss et al. 1984, Dixon et al. 1988; Baas and Kuiper 1989, Danneberg et al. 1992; Drüge and Schönbeck 1992). ZR is considered a transport form for CKs (Dixon et al. 1988) and therefore may be involved in the root-to-shoot communication (Kuiper et al. 1988, 1989). Shaul-Keinan et al. (2002) determined a more complex CK pattern in tobacco roots, indicating an additional role for isopentenyl adenine (iPA) like substances in AM. This was confirmed in litchi plants (Yao et al. 2005), where also iPAs were increased after inoculation with two different AMF (*Glomus* and *Gigaspora*). Interestingly, the increase in IAA was much higher than the increase in CKs in this study. However, an increase not only in roots but also leaves was reported and the authors attributed at least part of the growth stimulation of AM colonized plants to the increase in plant hormones (Yao et al. 2005).

Alternatively, AM was discussed to be important for the maintenance of CK levels under drought stress (Drüge and Schönbeck 1992; Goicoechea et al. 1995). This was partially attributed to the assumption that CKs are synthesized mainly in the root system and that changes in the roots would correspond to changes in the capacity to synthesize CKs. The ratio of ABA to CKs was thereby identified as critical in roots of AM inoculated *Medicago sativa* where it was lowered in comparison to control plants (Goicoechea et al. 1995).

4 Gibberellins: Hormones Without Obvious Function in AM Formation?

Despite the long history of hormones and AM formation, still not much is known about the function of gibberellins (GA) in this symbiosis. While for nodulation (Ferguson et al. 2005) and the interaction with the growth promoting fungus *P. indica* a crucial role for GAs for example in modulating basal defense (Schäfer et al. 2009) have been recently established using mutants, this evidence is still lacking for the arbusular mycorrhiza. Early reports indicated the production of GA-like substances from *Glomus* species and one was tentatively identified as gibberellic acid (Barea and Azćon-Aguilar 1982). Also the increase in a GA-like substance in leaves was reported in *Bouteloua gracilis* (Allen et al. 1982) and *Linum usitatissimum* (Dugassa et al. 1996), whereas in AM roots of *Bouteloua gracilis* the GA levels rather decreased (Allen et al. 1982). Mada and Bagyaraj (1993) reported the exudation of GAs from inoculated roots of *Leucaena sp.* plants, indicating that the lack of accumulation of GAs could be explained partially by secretion of these compounds. Later, Shaul-Keinan et al. (2002) reported an increase in various GAs in roots but not shoots of AM colonized tobacco plants using sensitive GC-MS methods, confirming in more detail the findings of Clapperton et al. (1985) in *Agropyron thrachycaulum* infected with a *Glomus* species. Mycorrhizal colonization caused most of the GAs of the early-13-hydroxylation biosynthetic pathway to be increased in root samples of 5 week old plants. The authors discussed their findings in light of the possiblity that GAs could alter the sink for carbohydrate in the roots for the fungi (Blee and Anderson 1998) instead of promoting the growth of the upper parts of the plant.

Addition of GAs inhibited strongly the AM development in pea (*Pisum sativum*) (El Ghachtouli et al. 1996). Treatment of citrus rootstocks with the GA biosynthesis inhibitor paclobutrazol increased the AM colonization (Michelini et al. 1989), again indicative of a negative GA effect. In contrast, *P. indica* colonization was dependent on functional GA synthesis in barley (*Hordeum vulgare*), as shown by the reduction of colonization in GA biosynthesis mutants (Schäfer et al. 2009).

5 Abscisic Acid: Involved in Stress Protection and Arbuscule Formation

Fungal hyphae of *Glomus intraradices* were shown to produce abscisic acid (ABA) (Esch et al. 1994), which was confirmed by GC-MS (Ludwig-Müller 2005). This might constitute an early signal to increase the synthesis of IBA in young roots to enhance the number of lateral roots and thereby the possible entry sites for the fungus (Ludwig-Müller et al. 1997; Kaldorf and Ludwig-Müller 2000), because the synthesis of the auxin IBA was inducible by ABA (Ludwig-Müller et al. 1995). This could be an example where a hormonal signal produced by the symbiont could influence the plant hormone synthesis.

On the other hand, ABA is a well known stress hormone, which increased also during later time points of symbiosis in roots of maize (Danneberg et al. 1992), soybean (Meixner et al. 2005) as well as *M. truncatula* and *T. majus* (Ludwig-Müller 2010), where its increase might contribute to stress tolerance in the roots, for example against drought. Mycorrhizal and non-mycorrhizal *Lactuca sativa* plants exhibited contrasting responses to exogenous ABA during drought stress and recovery (Aroca et al. 2008a). The general beneficial effect of ABA during drought stress is the induction of stomatal closure as well as synthesis of drought protecting proteins (Bray 1997, 2002, Zhang et al. 2006). The beneficial effect of AM symbiosis under drought stress has been attributed to changes in transpiration rate and increasing root water absorption (Augé 2001, 2004). It has also been noted that AM and non-AM plants regulate the expression of drought stress related genes differently (Porcel et al. 2005; Ruiz-Lozano et al. 2006). Other water related genes differentially regulated during drought under the influence of AM are aquaporins (Porcel et al. 2006; Aroca et al. 2007). The fact that a key gene for ABA synthesis is also differentially expressed in AM roots (Jahromi et al. 2008) corroborates the importance of ABA during AM symbiosis. The changes in stress and ABA related genes have been attributed to the different ABA levels in AM and control plants (Ruiz-Lozano et al. 2006). Also, it has been reported that AM symbiosis regulated ABA contents of the host plant under drought conditions (Estrada-Luna and Davies 2003).

To gain further insight into gene expression patterns induced by ABA and AM fungi, Grunwald et al. (2009) carried out transcriptome analysis using *M. truncatula*. When *Gigaspora rosea* was used as fungal partner, the overlapping expression pattern between colonized and hormone treated roots was small, but the overlap between transcripts induced by *Gigaspora* and two *Glomus* species was also quite low (Hohnjec et al. 2005; Grunwald et al. 2009). These results indicate that hormonal changes could be highly dependent on the symbiotic fungus that was used for inoculation. Alternatively, the increase of this phytohormone during mycorrhization is too low for a regulation of a significant number of genes, or the response is restricted to a few cells. Among the commonly upregulated genes by *Gigaspora* and ABA were two leghemoglobin genes (Grunwald et al. 2009), which could play a role in detoxification in mycorrhizal and nodulated roots as well as also under non-symbiotic conditions by binding nitric oxide (Vieweg et al. 2005). Since nitric oxide is involved in ABA signalling in stress responses (Ruan et al. 2004), leghemoglobin expression could be involved in fine-tuning of the system during ABA response (Grunwald et al. 2009).

Recent research has indicated that ABA is much more for the establishment of the AM symbiosis than a stress signal. Tomato (*Solanum lycopersicon*) mutants (*sitiens*) deficient in ABA synthesis were reduced in the amount of vital AM, especially at the level of arbuscules (Herrera-Medina et al. 2007). This effect was restored by the application of ABA, correlating with increased mycorrhization by treatment of wildtype roots with ABA. This points to an involvement of plant-derived ABA in the establishment of a functional symbiosis. Root ABA might be necessary for a sustained colonization by AMF, particularly under conditions which

are not favourable for the plant, i.e. drought stress. This might ensure that roots become colonized and the plants benefit in the stress situation from the symbiosis (Fester and Hause 2007). Also, the *sitiens* mutant showed differential gene expression, e.g. the gene encoding NCED, the rate limiting enzyme in ABA synthesis, in roots after AM colonization suggesting that the gene expression is dependent on the ABA phenotype (Aroca et al. 2008b). The gene expression study together with levels of stress metabolites such as proline suggested that only the wild type tomato line responded positively to AM colonization (Aroca et al. 2008b). On the contrary, the low abscisic acid level tomato mutant *notabilis* did not show any effect after AM colonization (Zsögön et al. 2008), leaving the question as to which extent the genetic background and not the hormone deficiency, or the fungal partner, is responsible for the effects seen.

Recent work points to the involvement of ABA also in the establishment of a sink for carbon (Schaarschmidt et al. 2007). Transgenic plants with slightly increased leaf invertase activity showed a stimulation of mycorrhization, compared with wildtype, which was accompanied by a higher density of all fungal structures and a higher level of *Glomus intraradices*-specific rRNA. The roots had an increased ABA content compared with wildtype (Schaarschmidt et al. 2007) suggesting a connection to carbon partitioning.

6   Jasmonic Acid Contributes to the Life Cycle of the AM Fungus

The role of jasmonic acid (JA) in AM formation has been elucidated within the recent years (Hause et al. 2007). Initial results relied on the addition of JA to plants and produced somewhat contradictory results by either promoting or inhibiting AM formation in roots depending on the concentrations of JA used. While low concentrations promoted AM formation (Regvar et al. 1996), high concentrations were inhibitory (Ludwig-Müller et al. 2002). Colonization of roots with AMF resulted in an increase in JA as shown for several plant species, i.e. barley (Hause et al. 2002), soybean (Meixner et al. 2005) and *M. truncatula* (Stumpe et al. 2005), implying a role for JA during the symbiosis.

Colonization of barley roots with *Glomus intraradices* led also to the accumulation of the JA conjugate with isoleucine (Hause et al. 2002), which was shown to be active in the interaction with the JA receptor COI1 (Katsir et al. 2008; Browse 2009). It should be noted that this is in contrast to auxin conjugates, which are considered as inactive (Ljung et al. 2002). Therefore, JA and auxin conjugation might have completely different functions during AM formation. The increase in JA was accompanied by an increase in transcripts encoding for genes in JA biosynthesis, which were localized specifically to cells harbouring arbuscules (Hause et al. 2002). Because JA is also induced during osmotic stress, the following scenario was hypothesized. Sugars produced in source tissues have to be transported to sink tissues, in the case of AM colonization to the roots. Thereby, possibly

osmotic stress is transiently induced, leading to the induction of JA biosynthesis genes (Hause et al. 2002). An additional feature of sink tissues is the expression of defense-related genes (Roitsch 1999), which may contribute to the increase of the overall defense status of the plant. Jasmonates may modulate such a defense status by induction of stress and defense related genes (Wasternack and Hause 2002).

Since for JA it has been demonstrated that the up-regulation of biosynthesis takes place in arbuscule-containing plant cells of *M. truncatula* (Isayenkov et al. 2005) a transgenic approach was taken to investigate the role of JA in AM formation. Silencing the gene encoding allene oxide cyclase (AOC) in hairy roots of *M. truncatula* reduced JA levels and concomitantly suppressed AM colonization mainly at the level of arbuscule formation, but these did not exhibit an altered structure (Isayenkov et al. 2005). In *MtAOC* antisense plants also the upper part did not respond positively with growth to AM colonization (Isayenkov et al. 2005). Therefore, Hause and coworkers suggested several possible mechanisms for the involvement of JA in AM formation, i.e. induction of defense related genes, reorganization of cytoskeleton, alteration of sink status of the root, and increase in plant fitness, maybe due to increased CK levels (Hause et al. 2007), but also an effect on host cell wall expansion has been suggested (Gutjahr and Paszkowski 2009), which is important for the development of arbuscule-containing cells. An additional function for JA in defense comes from transcriptome analysis where JA related transcripts involved in defense reactions and secondary metabolism were upregulated in AM colonized roots (Hohnjec et al. 2005). These results are corroborated by the findings of Grunwald et al. (2009) who showed that a small overlapping number of genes between JA treatment and colonization of *M. truncatula* roots by *Gigaspora* in a transcriptome analysis mainly resulted in the upregulation of genes involved in secondary metabolism and stress response.

Tomato mutant plants defective in JA synthesis (*spr2*) also showed reduced AM colonization (Tejeda-Sartorius et al. 2008). The degree of mycorrhization correlated with changes in the transcriptional regulation of a number of genes involved in sucrose hydrolysis and transport, as well as cell wall invertase activity in roots. The results obtained suggest that one of the mechanisms by which JA might operate to modulate the mycorrhization process could be through its influence on the regulation of C partitioning in the plant (Tejeda-Sartorius et al. 2008). The significant colonization increase observed in mycorrhizal *spr2* plants supplied with exogenous methyl-JA (MeJA) supported its role as a positive regulator of the symbiosis (Tejeda-Sartorius et al. 2008). On the contrary, tomato plants overexpressing prosystemin, which should result in higher JA levels did not show alteration in AM colonization, with the exception of a slight increase in arbuscule number (Tejeda-Sartorius et al. 2008). The JA-insensitive tomato mutant *jai-1* was more susceptible to fungal infection, showing increased frequency and intensity of fungal colonization (Herrera-Medina et al. 2008). MeJA was effective in reducing mycorrhization and mainly affected fungal phosphate metabolism and arbuscule formation, showing that AM colonization in tomato is reversely controlled in leaves and roots by the JA signalling pathway (Herrera-Medina et al. 2008).

7   Defense Related Hormones

Since the colonization of plant roots with AMF can enhance resistance to other plant pathogens (Pozo et al. 2002), the colonization should lead to the production of defense-related signals/hormones. Induced systemic defense responses after AM inoculation correlated with the production of PR proteins such as 1,3-β-glucanases and chitinases (Pozo et al. 1998, 1999, 2002). PR proteins are also inducible by salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Pieterse et al. 2002; Pieterse and van Loon 2004). Since auxins and CKs were able to suppress transcripts of PR proteins (Ginzberg et al. 1998; Shaul et al. 1999; Ludwig-Müller and Güther 2007), these two signals might antagonize the "classical" defense hormones. On the other hand, if JA would contribute to the increase in CKs, since it was shown to induce CKs in potato plants (Dermastia et al. 1994), then it could downregulate defense by itself. It is obvious that probably the defense related signals might have dual functions in AM, as discussed for JA, triggering defense but also as signals for the maintenance of AM.

7.1   *Ethylene*

Few studies are available describing changes in ET itself during AM formation. There are more reports on the treatment of plants with ET and the outcome of AM is monitored thereafter. A decrease in colonization after ethrel (an ET generator) treatment was observed in *M. sativa* after inoculation with *Glomus mosseae* (Azcon-Aguilar et al. 1981). Similarly, ET inhibited colonization of *Poncirus trifolata* (Ishii et al. 1996) and of pea and leek by AMF (Geil et al. 2001; Geil and Guinel 2002). Since ET application caused changes in root morphology, one could speculate that decreased colonization could be due to reduction in the root system, as it was vice versa reported for auxin (Kaldorf and Ludwig-Müller 2000). This hypothesis is corroborated by the observation that the appressoria appeared abnormal (Geil et al. 2001). Morales Vela et al. (2007) analyzed pea symbiotic mutants and found a correlation between the mutant with highest ET content, which showed lowest colonization rate.

An alternative approach to reduce ET levels is the co-inoculation with rhizobacteria expressing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, thus reducing ET precursors and subsequently ET levels in the plant (Gamalero et al. 2008). Co-inoculation increased mycorrhizal colonization and especially arbuscule abundance after *Gigaspora* inoculation in cucumber roots, again supporting the inhibitory role of ET during AM colonization. In agreement, a mutated bacterial strain without ACC deaminase did not show this effect (Gamalero et al. 2008).

In an ET sensitivity tomato mutants (*Never ripe*) and one with high ET production (*epinastic*) root colonization by *Glomus clarum* was inhibited under low P conditions,

when compared to controls, and the effect of ET was associated to the control of defense-related gene expression (Zsögön et al. 2008). Under low P conditions, the steady state levels of transcripts encoding a class I basic chitinase were higher in mycorrhizal *epinastic* and *Never ripe* mutant roots as compared to controls. In contrast the steady state levels of a class III acidic β-1,3-glucanase transcripts in mycorrhizal *epinastic* mutant roots were significantly lower than in mycorrhizal control roots. Root colonization in *epinastic* mutants was accompanied by several alterations in fungal morphology (Zsögön et al. 2008). The inhibition under "natural" high ET levels is in agreement with the results from treatment experiments.

In *L. usitatissimum* roots inoculated with *Glomus intraradices* ET formation was increased (Dugassa et al. 1996), which was not detected in tomato inoculated with *Glomus mosseae* (Vierheilig et al. 1994). Levels of ACC and ET were determined in roots of papaya (*Carica papaya*) after AM colonization (Cruz et al. 2000). Under well-irrigated conditions, the ET concentration in the roots was increased by the presence of AM, although there was no significant difference between AM and non-AM roots in ACC levels. ACC increased in both AM and non-AM roots under water-stress conditions, adding another layer of control to ACC and ET induction. The effect of mycorrhizal colonization on ET production by flowers of snapdragons (*Antirrhinum majus*) was determined and revealed that AM colonization reduced ET production of the flower as shown by longer vase life (Besmer and Koide 1999), which indicates also systemic effects on ET production.

Sebacina vermifera, a growth promoting endophytic fungus promotes the growth and fitness of *Nicotiana attenuata* by inhibiting ET signalling (Barazani et al. 2007). Also, plants without ET production were less promoted in growth by the fungus, which led to the hypothesis that the fungus increases plant growth by impairing ethylene responses. This recent report is in accordance with earlier work showing low ET levels in AM tomato roots (Vierheilig et al. 1994).

The interaction between *N. attenuata* and *Glomus intraradices* is different from others in the sense that the host does not perceive the benefits of AM colonization (Riedel et al. 2008). Colonization of this species resulted in decreased growth, which was attenuated when ET signalling or synthesis was impaired in mutant plants. Release of ET was slightly decreased in colonized plants compared to controls, whereas levels of JA, JA-Isoleucine and SA were not changed (Riedel et al. 2008). Transgenic plants silenced either in JA biosynthesis or signalling did not show any changes concerning AM colonization. The authors therefore concluded that ET signalling plays a role on the growth phenotype seen during the interaction.

7.2   Salicylic Acid

During AM formation, modulation of plant defense responses occurs, potentially through cross-talk between SA and JA dependent signalling pathways (Pozo and Azcon-Aguilar 2007). This modulation may also impact plant responses to potential enemies by priming the tissues for a more efficient activation of defense mechanisms

in addition to conferring a balance between the AMF and the host. Although there is not much known in AM about cross talk of JA and SA, investigation on the reactions of plants to different pathogens indicate antagonism of JA and SA in defense responses to biotrophic pathogens (SA pathway) and necrotrophic pathogens (JA pathway) and often the activation of one signalling pathways suppress the other (see e.g. review by Robert-Seilaniantz et al. 2007). The authors also hypothesized that auxins and CKs could positively influence the JA pathway which would in turn have a negative influence on SA signalling, resulting in the promotion of biotrophic interactions by auxins and CKs, whereas GAs act vice versa. These antagonistic signalling pathways could possibly also explain some of the contradictory results for these hormones observed in AM symbiosis.

The mycorrhization of tobacco with *Glomus mosseae* resulted in reduced SA levels in tobacco plants and colonization was suppressed by constitutive SA synthesis, whereas *NahG* plant constitutively expressing SA hydroxylase showed increased colonization which was attributed to the low SA levels (Medina et al. 2003). However, this was only a delay in colonization because in old roots wild type and transgenic high and low SA plants did not differ. This could be interpreted as a repression of early infection events that is, at later stages, compensated by extensive spread of fungal intraradical hyphae. In this scenario, SA would be important for the first cell-to-cell contact between the symbionts to reduce colonization (Gutjahr and Paszkowski 2009). It was found that in *M. truncatula* hairy roots a pathogenesis-related protein of class 10 was upregulated in root cells close to the hyphopodium of AMF and subsequently repressed during formation and fungal passage of the prepenetration apparatus, further indicating that early defense responses are important (Siciliano et al. 2007).

On the other hand, SA levels increased after infection of rice with *Glomus mosseae* during early stages of the interaction (Blilou et al. 2000), coinciding with an increase in proteins discussed to be involved in defense. Also, exogenous SA reduced inoculation with AM (Özgönen et al. 2001; Ludwig-Müller et al. 2002). In mycorrhiza defective *myc–* mutants, SA levels were enhanced in response to AMF, whereas the accumulation was low and transient in mycotrophic plants (Garcia-Garrido and Ocampo 2002). This indicates that the hormonal balance is highly important for the coordinated defense response, leading eventually to AM formation. The sequence of events leading to the development of the symbiosis involves the regulation of defense-related genes, which are transiently upregulated during the early establishment of the AM symbiosis (Garcia-Garrido and Ocampo 2002; Liu et al. 2003; Balestrini and Lanfranco 2006), but later on downregulated in expression maybe through hormonal signals such as auxin (Güther and Ludwig-Müller) and CK (Ginzberg et al. 2008) or suppressors of the symbiotic partner (Gianinazzi-Pearson 1996). The defense reaction might be important first in the roots to restrict AM colonization before establishment of the symbiosis, later on when the symbiosis is established, the defense reactions in the roots are downregulated. Contrary, systemic defense would still be activated to increase the performance of the leaves producing assimilates, which are also consumed by the fungus. AM also induces resistance more in roots than shoots against other pathogens. For the former, the increase in SA in roots might be also important, whereas accumulation in PR proteins, SA or marker proteins associated with systemic acquired resistance have not been reported from systemic tissues (Pozo and Azcon-Aguilar 2007).

8 The Interaction of Different Hormones Is Necessary for the Beneficial Effects of the Symbiosis on the Host Plant

The focus of the investigations on the role of plant hormones in AM symbiosis has naturally been on the belowground root system. However, it is known that the AM symbiosis alters the physiology and metabolism of the aerial parts of the host plant as well (Toussaint 2007). Nevertheless, there are few indications for systemic signalling available, which contribute to the overall improvement of growth, health, as well as stress and pathogen tolerance also aboveground.

It is not easy to draw conclusions from all the different plant species involved so far in the work on the role of plant hormones in the establishment of an AM symbiosis, but an attempt is made to integrate the data from the different species. The same holds true for the AMF involved, but for simplicity, this factor is not taken into account.

A model is summarizing some functions of the different plant hormones as known up to now (Fig. 1). Auxins and CKs could regulate early responses by changing the plant architecture. Also, they may be involved in repression of defense responses. Both would contribute to the growth of the AMF and thereby to the beneficial effect for the belowground and aboveground parts of the host. In addition, CKs play a role in the establishment of a sink in the roots. The role of GAs in this respect is less clear but they may also be involved in growth regulation of the upper plant parts. ABA and JA have now been shown to be necessary for arbuscule formation in addition to their role in abiotic and biotic stress responses, respectively. Again, protection of the plant from stresses will ultimately lead to better growth performance of the aboveground part.

Also, ET and SA could be involved in the regulation of AM colonization by transiently increasing defense responses of the plant in the root, but they also contribute to the overall defense system of the plant with respect to other leaf and root microbes/pathogens which could colonize the plant, thereby inducing local and systemic defense responses. JA could also contribute to the latter. This highly coordinated regulatory network has to be active in all parts so that the host plant gets full benefit from the colonization (Fig. 1). Finally, plant hormones interact with each other and can influence their endogenous contents, which leads to another level of complexity.

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Part III
Influence of the Symbiosis on the Host and its Surrounding: Host Response to Biotic Stress

Chapter 9
Impact of Arbuscular Mycorrhizal Symbiosis on Plant Response to Biotic Stress: The Role of Plant Defence Mechanisms

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Abstract Arbuscular mycorrhizal associations imply a remarkable reprogramming of functions in both plant and fungal symbionts. The consequent alterations on plant physiology have a clear impact on the plant response to biotic stresses. In this chapter we discuss the effects of the mycorrhizal symbiosis on plant susceptibility/resistance to potential deleterious organisms, including root and shoot pathogens, root parasitic plants and phytophagous insects, highlighting the mechanisms that may be operating in each particular case. Special attention is given to the modulation of plant defence responses in mycorrhizal systems, as it may affect all interactions. Finally we focus on the priming of jasmonate regulated plant defence mechanisms that seem to mediate the induction of resistance by arbuscular mycorrhizas.

Keywords Biotic stress • Bioprotection • Induced resistance • Priming • Plant defence • Biocontrol • Defence signalling • Jasmonates • Pathogens • Insects

Abbreviations

AM   Arbuscular mycorrhiza
AMF   Arbuscular mycorrhizal fungi
MIR   mycorrhiza-induced resistance
JA   jasmonate
SA   Salicylic acid
Nm   Non-mycorrhizal plants
Gm   *Glomus mosseae* colonized plants
Hpi   hours post inoculation

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1   Introduction

The establishment of the arbuscular mycorrhizal (AM) symbiosis implies remarkable changes in the physiology of the host plant. The changes span from alterations in the hormonal balance and transcriptional profile to altered primary and secondary metabolism (Hause et al. 2007; Liu et al. 2007; Schliemann et al. 2008; López-Ráez et al. 2010). This global reprogramming of plant functions has an impact on the plant interaction with the environment, modifying its responses to biotic and abiotic stresses. As a result, mycorrhizal plants are generally more tolerant to environmental stresses. The consequences go beyond the individual level as they may influence plant diversity and productivity in terrestrial ecosystems (van der Heijden et al. 2008).

It should be noted that the impact of the symbiosis in terms of resistance/tolerance to biotic stresses differs among AM fungal isolates for a given plant-pathogen interaction. Moreover, such impact can be modulated by environmental conditions. Despite of this variability, general trends emerge from the multiple studies dealing with mycorrhiza in diverse pathosystems. Generally, enhanced resistance to soil-borne pathogens has been reported in AM plants. Furthermore, the symbiosis can also impact plant interactions with above-ground attackers. In this case, the outcome ranges from enhanced resistance to increased susceptibility, largely depending on the attacker life-style (Pozo and Azcón-Aguilar 2007).

Early works on mycorrhizas and biotic stresses were mostly descriptive (for reviews see Schonbeck and Dehne 1989; Sharma et al. 1992; Linderman 2000). Generally, reports have focussed on beneficial effects of the symbiosis, aiming at using AM as potential biocontrol agents in integrated management programmes for disease control (Sharma and Adholeya 2000; Harrier and Watson 2004; Whipps 2004; Mukerji and Ciancio 2007).

A key factor determining the effect of the symbiosis on interactions with other organisms seems to be the extension of root colonization by the AM fungi (AMF). With some exceptions (Caron et al. 1986; García-Garrido and Ocampo 1988; St-Arnaud et al. 1997; Kapoor 2008), reports on mycorrhizal protection against pathogens show the requirement of a well established symbiosis prior to the challenge with the attacker (Rosendahl 1985; Cordier et al. 1998; Slezack et al. 2000; Khaosaad et al. 2007). The first mechanism proposed to be involved in mycorrhiza-induced protection was the improvement of plant nutrition and the consequent compensation of the damages caused by the pathogen. However, studies including nutrient supplemented controls showed that AM effects cannot be regarded as a mere consequence of improved phosphorus nutrition (Trotta et al. 1996; Fritz 2006; Liu et al. 2007). As we advance in our knowledge on the physiology and regulation of the AM symbiosis, we start to understand the diversity of mechanisms underlying the impact of the symbiosis on plant interactions with other organisms. In addition to the nutritional aspects, changes in the plant architecture, root exudation and in the microbial populations in the rhizosphere, and the activation of plant defence mechanisms may all be relevant. Their individual contribution to the final outcome will depend on the organisms involved and the timing of the interactions

(Azcón-Aguilar and Barea 1996; Whipps 2004). In this chapter we will discuss the possible mechanisms affecting the different types of plant-attacker interactions, with special emphasis in those involving plant defence responses.

2 Impact of the AM Symbioses on Soil-Borne Pathogens

It is widely accepted that AM symbioses reduce the damage caused by soil-borne pathogens. Many studies revealed a reduction of the incidence and/or severity of diseases as root rot or wilting caused by diverse fungi such as *Fusarium, Rhizoctonia, Macrophomina* or *Verticillium,* bacteria as *Erwinia carotovora*, and oomycetes as *Phytophthora, Pythium* and *Aphanomyces*. A comprehensive review of those studies was compiled by Whipps (2004). Similarly, a reduction of the deleterious effects by parasitic nematodes such as *Pratylenchus* and *Meloidogyne* has been reported in mycorrhizal plants (Pinochet et al. 1996; de la Peña et al. 2006; Li et al. 2006). Additional reports showed protection to other soil pathogens as *Armillaria melea* in grapevine (Nogales et al. 2009), broadening the range of pathosystems in which AM symbioses may have a protective effect. The effectiveness against such diverse range of attackers confirms the broad spectrum character of the induced resistance associated to the AM symbiosis.

Studies comparing different fungal species or isolates highlighted that the degree of protection is highly dependent on the AMF involved (Kobra et al. 2009). Interestingly, many studies point to a higher protector effect of *Glomus mosseae* in comparison to other AMF (Pozo et al. 2002; Utkhede 2006; Ozgonen and Erkilic 2007).

Several mechanisms may operate simultaneously in the enhanced resistance of mycorrhizal plants to soil pathogens. In addition to a possible competition for photosynthates between the AMF and the pathogen, competition for colonization sites has been demonstrated. For example, in tomato roots, full exclusion of *Phytophthora* from arbusculated cells was evidenced (Cordier et al. 1998). Mycorrhizal colonization is also known to induce changes in the root system architecture and morphology (Schellenbaum et al. 1991; Norman et al. 1996). These changes may alter the dynamics of infection by the pathogen, although direct evidences of such correlation are lacking. An altered pattern of root exudation may also impact the development of the pathogen. Mycorrhizal colonization leaded to modifications in root exudates composition that significantly reduced the sporulation of *Phytophthora fragariae* (Norman and Hooker 2000) and altered the chemotactic response of the zoospores of *Phytophthora nicotianae* (Lioussanne et al. 2008). Since root exudates are key factors in shaping soil microbial communities (Badri and Vivanco 2009), the changes in exudation into the mycorrhizosphere may result in alteration of the microbial communities including possible antagonistic organisms. This may be the reason underlying the biocontrol of pathogens in non-AM species by co-culture with mycorrhizal plants (St-Arnaud et al. 1997).

Because of the root localization of both attacker and AMF, it is difficult to discern the local or systemic character of the protection observed. However, the use

of split-root experimental systems allowing physical separation between AMF and pathogens has confirmed a reduction of disease symptoms in the non-mycorrhizal parts of the mycorrhizal root systems. Systemic protection at the root level has been demonstrated against *Phytophthora* and *Ralstonia* in tomato (Cordier et al. 1998; Pozo et al. 2002; Zhu and Yao 2004), against *Gaeumannomyces* in wheat (Khaosaad et al. 2007), and against parasitic nematodes in banana plants (Elsen et al. 2008). The systemic character of the induced resistance pointed to the involvement of plant defences. Because of its relevance in all kinds of interactions, the implication of plant defence mechanisms will be discussed in Section 7.

3 Effects of AM Symbioses on Root Parasitic Plants

Plants of the genera *Striga* and *Orobanche* parasitize different hosts around the world, constituting one of the most damaging agricultural pests. These obligate parasites attach to the roots of many plant species and acquire nutrients and water from their host (Bouwmeester et al. 2003). Studies in African fields infested with the hemiparasite *Striga hermonthica* evidenced that inoculation with AMF significantly reduced the amount of parasitic plants in maize and sorghum cultures. Accordingly, the use of mycorrhizas for integrated management of parasitic weeds was proposed (Lendzemo et al. 2005; López-Ráez et al. 2009a).

Strigolactones are germinating stimulants for the seeds of root parasitic plants (Bouwmeester et al. 2007). With the discovery of strigolactones as host detection signals for AMF in the rhizosphere (Akiyama et al. 2005; see chapter by Bécard et al.) a causal connection between AM and its impact on parasitic plants could be established. Indeed, follow up experiments under controlled conditions confirmed that AMF inoculation leads to a reduction of the impact of *Striga*, apparently related to a reduction in strigolactones production (Lendzemo et al. 2007). Similarly, we have observed that extracts from tomatoes colonized by *G. mosseae* induce less germination of *Orobanche ramosa* seeds than those from non-mycorrhizal plants (López-Ráez et al. 2009b). Moreover, a reduced production of strigolactones in a tomato mutant correlated with less susceptibility to *Orobanche* (López-Ráez et al. 2008). All in all, it seems likely that a reduction in strigolactone production underlies the decrease in the incidence of root parasitic plants on mycorrhizal plants.

4 Impact of AM Symbioses on Above-Ground Interactions

Studies dealing with AM effects on above-ground diseases are less abundant, and apparently less conclusive. Early reports associated AM symbioses with enhanced susceptibility to viruses (Whipps 2004), and it was generally accepted that AM plants are more susceptible to shoot pathogens. However, recent studies dealing with pathogens of different life styles have evidenced a more complex reality.

Biotrophic pathogens, such as powdery mildew and rust fungi (*Blumeria*, *Oidium*, *Uromyces*) seem to perform better in mycorrhizal plants, although increased tolerance was often observed in terms of plant mass and yield (Gernns et al. 2001; Whipps 2004). Concerning hemibiotrophs, the impact of the symbiosis varies from no effect to reduction of the disease, for example, against *Colletotrichum orbiculare* in cucumber (Lee et al. 2005; Chandanie et al. 2006). Finally, several studies evidenced a positive effect on plant resistance to other shoot pathogens, including the bacteria *Xanthomonas campestris* in *Medicago* (Liu et al. 2007), and the necrotrophic fungus *Alternaria solani* in tomato (Fritz et al. 2006; De La Noval et al. 2007). We have also confirmed that symbiosis with *G. mosseae* in tomato induces systemic resistance to the necrotrophic fungus *Botrytis cinerea* (Jung et al., 2009) (see Section 7). Recently, a positive effect of *G. mosseae* against *Botrytis cinerea* has also been shown in roses, although dependent on environmental factors (Moller et al. 2009).

Phytoplasma are specialized obligate parasites of phloem tissue transmitted by insect vectors. AM establishment in tomato lead to a reduction of the disease symptoms caused by a phytoplasma of the Stolbur group (Lingua et al. 2002). Because they inoculated through grafting with infected scions, potential effects on the insect vector were ruled out. Thus, the protection is related to physiological changes in the mycorrhizal plant. Tolerance to phytoplasma disease was also reported in pear (García-Chapa et al. 2004). Recently, a reduction in the titre of the Chrysanthemum yellows phytoplasma has been shown in mycorrhizal chrysanthemum (D'Amelio et al. 2007), confirming that mycorrhization can confer resistance to this type of pathogens.

In above-ground interactions of mycorrhizal plants, two main mechanisms may be operative. One would be the potential changes in nutrient levels of the host plant and alterations of the source-sink relation within it, that may affect the suitability of the plant for shoot attackers. The other would be the modulation of plant defence mechanisms, as discussed in Section 7.

5 Effects of AM Symbioses on Phytophagous Insects

The mycorrhizal status of the host plant can also influence insect herbivore performance, but the magnitude and direction of the effect depend upon the feeding mode and life style of the insect (Hartley and Gange 2009; Koricheva et al. 2009).

Many different studies cover an ample range of mycorrhizal plant-insect interactions under controlled or field conditions. Upon a comprehensive review of the published data, Hartley and Gange (2009) concluded that, generally, mycorrhizas have strong negative effects on rhizophagous insects, but effects on shoot-feeding insects are weaker and more variable. Despite of this variability, some general patterns emerge: generalist insects are usually adversely affected by mycorrhizas, whereas specialist insects may often benefit. Furthermore, aphids usually perform better on AM plants while leaf-chewing insects are usually negatively affected by the symbiosis.

Such patterns may arise from the differential impact of nutritional and defence aspects in the insect. While generalist insects are sensitive to plant defence mechanisms, specialist herbivores are likely to be able to circumvent the defences of their host and remain undetected. As a result, generalists may be affected by the enhanced defence capacity of AM plants, while specialists will circumvent the defences and may benefit from the improved nutritional status of the plant. The negative effect on leaf-chewers is likely related to their susceptibility to jasmonate-dependent defences (Peña-Cortés et al. 2004) potentiated in mycorrhizal plants (see Section 8). In addition, AM can also have an impact on herbivores by affecting the performance of their predators and parasitoids: in tomato, the volatile blends released by AM plants can be more attractive to aphid parasitoids than those from non-mycorrhizal ones (Guerrieri et al. 2004).

6   AM Symbiosis Modulate Host Defence Responses

As discussed earlier in this book and reviewed elsewhere (Gianinazzi-Pearson 1996; Harrison 2005; Parniske 2008) the establishment of a successful mutualistic interaction requires a high degree of coordination between both partners. Plant and fungus actively engage in the process of colonization, and a tight control of plant defence mechanisms is necessary. Interestingly, the plant is able to restrict AMF colonization once plants are already mycorrhizal, a phenomenon known as *auto-regulation* (Vierheilig et al. 2008). The mechanisms operating in such autoregulation may also impact plant interactions with pathogens.

The levels of several phytohormones (mainly salicylic acid (SA), jasmonates (JAs), ethylene (ET) and abscisic acid (ABA)) fine-tune the defence responses in plants through an intricate regulatory network (Pieterse et al. 2009). Remarkably, the levels of these hormones seem to be altered in mycorrhizal plants (Hause et al. 2007; López-Ráez et al. 2010), probably affecting plant defence mechanisms. There is evidence for the accumulation of defensive plant compounds in mycorrhizal roots, although to a much lower extent than in plant-pathogen interactions. Activation of phenylpropanoid and oxylipin metabolism, accumulation of reactive oxygen species and of specific isoforms of defence-related enzymes has been reported in mycorrhizal roots (García-Garrido and Ocampo 2002; De Deyn et al. 2009; López-Ráez et al. 2010). These reactions, generally localized, may control the development of the fungus inside the roots (Pozo et al. 2002; Dumas-Gaudot et al. 2000; García-Garrido and Ocampo 2002). Indeed, as obligate biotrophs, AMF share similarities with biotrophic pathogens (Paszkowski 2006) and transcriptional profiling of plant responses to AMF revealed some overlap with responses to biotrophic pathogens (Güimil et al. 2005). Coherently, SA, a key regulator of plant defences against biotrophs (Glazebrook 2005), seems to have a negative effect on AM colonization (García-Garrido and Ocampo 2002; López-Ráez et al. 2010). Thus, it is plausible that AMF repress SA-dependent responses in the host in order to achieve a compatible interaction. Indeed, a delay in the accumulation of PR-1 proteins, common markers of SA-dependent responses, has been observed in mycorrhizal

roots (Dumas-Gaudot et al. 2000). Even repression of defence responses triggered upon pathogen attack has been reported when *G. intraradices* was co-inoculated with *Rhizoctonia solani* (Guenoune et al. 2001). Although AMF are able to trigger plant defence responses as evidenced in *myc* mutants, only weak and transient defence responses are activated during compatible AM interactions (Liu et al. 2003). Thus, AM establishment seems to require inhibition of certain SA-regulated defence responses. Remarkably, inhibition of SA responses is also necessary for the *Rhizobium*–legume symbiosis (Stacey et al. 2006).

Modulation of plant defences during AM formation does not only occur in the roots, but also in the shoots. Accumulation of insect anti-feedant compounds (Gange 2006; Pozo et al. 2009) and transcriptional up-regulation of defence-related genes (Liu et al. 2007; Pozo et al. 2009) have been described in leaves of mycorrhizal plants. Also a repression of certain defences may take place: a delay in the systemic accumulation of PR1 upon treatment with SA or analogs has been reported in mycorrhizal tobacco shoots (Shaul et al. 1999) and suppression of certain chemical defences has also been reported (Bennett et al. 2009). This modulation may affect the interaction with shoot attackers. To this regard, the reciprocal influence of below-ground and above-ground interactions through their impact on plant defences is receiving increasing interest (Bezemer and van Dam 2005; Erb et al. 2009). An additional level of complexity is related to the altered volatile profile released by AM plants (Guerrieri et al. 2004; Rapparini et al. 2008). Volatiles may play key roles in defence, for example, by attracting natural enemies of potentially harmful insects, or by priming distal parts of the plant for a more efficient activation of defences (Heil and Ton 2008).

We have recently shown that mycorrhizal colonization in tomato leads to increases in the expression of defence related genes known to be regulated by JA (Pozo et al. 2009). JA is a key regulator of plant defences against insects and necrotrophic pathogens (Peña-Cortés et al. 2004; Pozo et al. 2005). Because of SA and JA signalling pathways are interconnected, mostly in an antagonistic way (Pieterse et al. 2009) their interplay may explain the pattern of enhanced resistance/susceptibility of AM plants. If AM inhibits SA-regulated responses, the plant would be more susceptible to pathogens resisted through these responses, i.e. biotrophic pathogens. On the contrary, an induction of the JA signalling pathway would make mycorrhizal plants more resistant to necrotrophic pathogens and JA-sensitive insects (Pozo and Azcón-Aguilar 2007). Such pattern is more obvious in shoot interactions, where modulation of plant defences seems to be the main mechanism. In roots, the relevance of this altered balance will be lower since other mechanisms are operating simultaneously (see Section 3), and a reduction of the disease is the most general outcome.

7 AM Symbiosis Primes JA-Dependent Responses

Upon detection of a potential attacker, a rapid and strong activation of the defence mechanisms is crucial for resistance. Accordingly, pre-conditioning of plant tissues for a quick and more effective activation of defences upon attack has important

ecological fitness benefits, and seems to be a common feature of the plant's immune system. This boost of basal defences is known as *priming* (Conrath et al. 2006; Goellner and Conrath 2008). Priming seems to be the strategy followed by several beneficial micro-organisms to enhance resistance in plants, avoiding a direct activation of defences which would be too expensive for the host in the absence of challenging attackers (Pozo et al. 2005; Van Wees et al. 2008).

Evidences for primed defence responses in mycorrhizas were first reported in root tissues. Mycorrhizal transformed carrot roots displayed stronger defence reactions at challenge sites by *Fusarium* (Benhamou et al. 1994). Similarly, mycorrhizal potatoes showed amplified accumulation of phytoalexins upon *Rhizoctonia* infection (Yao et al. 2003). Priming for callose deposition seems to be responsible for the protection achieved by *G. intraradices* against *Colletotrichum* in cucumber (Lee et al. 2005). Recently, primed accumulation of phenolic compounds in AM date palm trees has also been related to protection against *F. oxysporum* (Jaiti et al. 2008). Remarkably, priming is not restricted to AMF colonized areas of the roots, but to the whole root system. This was first illustrated in tomato plants during *P. parasitica* infection (Cordier et al. 1998; Pozo et al. 2002). Only AM plants, even in non-mycorrhizal parts of the root system, formed papilla-like structures around the sites of pathogen infection, preventing further spreading of the pathogen. They also accumulated more PR-proteins than non-mycorrhizal plants upon challenge (Cordier et al. 1998; Pozo et al. 1999). Mycorrhizal protection of grapevine against *Meloidogyne incognita* has also been associated with primed systemic expression of a chitinase gene in response to the nematode (Li et al. 2006). But the primed response is not restricted to the root system. Recently, we have shown priming of defences also in shoots of mycorrhizal plants (Pozo et al. 2009).

Evidence is accumulating that priming associated to systemic resistance induced by beneficial organisms is regulated by similar jasmonate signalling pathways (Van Wees et al. 2008). Indeed, studies on rhizobacteria induced systemic resistance (ISR) in *Arabidopsis* revealed the requirement of a functional JA signalling pathway for the efficient induction of resistance (Pieterse et al. 1998; Pozo et al. 2008). The JA signalling pathway is also required for rhizobacteria ISR in tomato (Yan et al. 2002) and for the induction of resistance by the beneficial fungi *Trichoderma* and *Piriformospora* (Shoresh et al. 2005; Stein et al. 2008). Interestingly, JA accumulation have been proposed to mediate plant "memory" of previous challenges (Galis et al. 2009), a possible basis for the primed state.

Jasmonates are key regulators in the AM symbiosis, and elevated endogenous levels of JA have been confirmed in mycorrhizal roots (reviewed in Hause et al. 2007 and Hause and Schaarschmidt, 2009). We have found a significant increase in JAs in mycorrhizal tomato roots (López-Ráez et al. 2010), but the levels were not altered in the shoots (López-Ráez and Pozo, unpublished). However, we found small, yet significant, increases in the expression of marker genes for JA responses, a result that may indicate an enhanced sensitivity to the hormone.

To confirm whether AM leads to priming of JA-dependent responses in the shoots, we compared the response of non-mycorrhizal and AM plants to foliar application of different defence-related stimuli. Transcript profiling of leaves 24 h
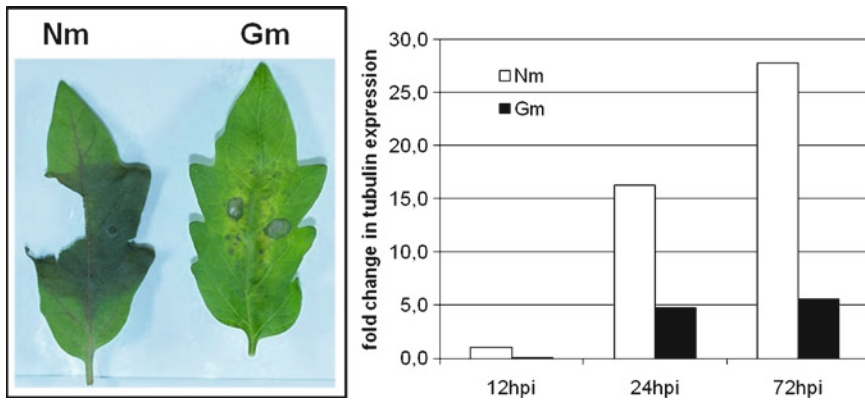
Fig. 1 Mycorrhizal tomato plants are more resistant to the necrotrophic foliar pathogen *Botrytis cinerea*. *Left* – Photograph of necrotic lesions developed 6 days after drop inoculation of tomato leaves with *Botrytis cinerea*. *Right* – Quantification of *Botrytis cinerea* in infected leaves by real time PCR analysis of expression of its tubuline gene. Nm: Non mycorrhizal plants, Gm: *Glomus mosseae*-colonized plants

after treatment with JA revealed a stronger induction of JA-regulated genes in mycorrhizal plants, particularly in *G. mosseae*-colonized plants, confirming a primed response in those plants (Pozo et al. 2009). To address the biological relevance of such primed response, we tested whether priming occurs during interactions with pathogens. AM and non-mycorrhizal plants were challenged with *B. cinera*, and the expansion of necrotic lesions was markedly lower in the leaves of AM plants (Fig. 1a). The levels of pathogen in the tissues were also lower in mycorrhizal plants at all time points tested (Fig. 1b), confirming induced systemic resistance. Remarkably, this pattern inversely correlated with the expression of the defence-related, JA-marker gene *Pin II*. *Pin II* expression was induced in response to *B. cinerea* in all plants, but this induction was markedly higher in AM plants (Jung et al. 2009). The results support that Mycorrhiza Induced Resistance (MIR) to *Botrytis* is associated to priming of JA-dependent defences.

8   Conclusions and Outlook

AM symbiosis affect the outcome of the host plant interaction with other organisms. Although the effect is influenced by plant, AMF and environmental factors, generally there is a clear protector effect of the symbiosis over soil-borne deleterious organisms. However, the impact on shoot interactions is more variable and relies largely on the attacker lifestyle. Besides nutritional effects and local changes in the plant (affecting mainly soil-borne pathogens), modulation of plant defences associated to AM seems to play a crucial role. Instead of constitutive activation of defences, MIR is mediated by priming for an efficient activation of defences.

Experimental evidences and the general spectrum of protection by mycorrhiza point to a central role of jasmonates in MIR. Despite of the relevance of this process, its precise regulation awaits elucidation. Increasing our knowledge on the modifications of plant physiology in AM, as well as in the biology of the potential attackers is essential in order to define markers of induced resistance and to generate predictive models for the outcome of particular mycorrhiza-pathogen interactions. Another challenge ahead is to decipher the connections in plant responses to biotic and abiotic stresses. Experimental evidences point to common regulatory nodes in the signalling pathways governing responses to both types of stresses, and those nodes may be the target of biotechnological strategies for optimization of plant protection by arbuscular mycorrhizas. Finally, it is important to consider mycorrhiza in a multitrophic context, as the impact of the symbiosis on plant interactions can be modified by other organisms in the system.

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Chapter 10
Biotic Environment of the Arbuscular Mycorrhizal Fungi in Soil

Jan Jansa and Milan Gryndler

Abstract Arbuscular mycorrhizal fungi (AMF) inhabit two different, highly contrasting kinds of environment: root cortex and soil. Roots inner volume offers rather stable conditions in terms of physics, chemistry and biology, with rather low diversity of microbes. The other environment, the soil, is one of the most diverse ecosystems on Earth, inhabited by various kinds of organisms. The soil is extremely variable in many different aspects and across a wide range of scales. In contrast to the interactions of AMF with roots, their interactions with soil and in particular with other soil microbes is less studied and understood. However, recent methodical advances allow slowly gaining insights into complex interactions between different inhabitants of the soil, where AMF appear to play an important role as carbon and nutrient highway between the plants and the soil. Moreover, the complex community of soil biota is involved in transformation of soil organic matter, resulting in production of biologically active substances affecting both the AMF and the root physiology. Understanding all these processes may prove critical for sustainable use of soil as a finite resource to cover various needs of human societies.

Keywords Soil • Microorganisms • Interactions • Diversity • Carbon • Rhizosphere • Ecology • Dynamics • Methods

Abbreviations

AMF   Arbuscular mycorrhizal fungi
P   Phosphorus

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N   Nitrogen
C   Carbon
CO_2 Carbon dioxide
O_2 Molecular oxygen

1   Introduction

Arbuscular mycorrhizal fungi (AMF) inhabit and interconnect two very different kinds of environments, namely the root's inner volume with the soil surrounding the roots (Helgason and Fitter 2009). Inner volume of the roots can be considered rather uniform environment, with respect to both abiotic and biotic conditions. Water, nutrients, and pollutant levels are maintained within the roots in rather narrow ranges, and the biomass concentration of plant cells is usually much higher than any other biological subject (e.g. endophytes, pathogens, symbionts) within the roots (Sturz et al. 2000; Wittenmyer and Merbach 2005; Amtmann and Blatt 2009). Thus the AMF within the roots are significantly interacting with only a limited range of organisms such as the plant itself, other AMF species, endophytes and parasites, and/or animals feeding on plant tissues.

On the other hand, the soil surrounding the roots and being under their influence (the rhizosphere) can greatly vary in its physical, chemical, and biological properties (Hinsinger et al. 2006). It has long been recognized that microbial activity is higher and the composition of microbial communities is different in the proximity of roots than in the soil further away from the roots (Gryndler 2000; Hinsinger et al. 2005). Therefore, these soil zones close to the roots can be considered hotspots of microbial activity, reaching some of the highest recorded diversities on Earth (Beare et al. 1995; Brussaard et al. 1997; Stockdale and Brookes 2006; Trinh et al. 2007). Due to high competitive pressure between the different microbial community members, soil surrounding the roots can be seen as a battlefield of a wide range of organisms like prokaryotes, fungi, and animals. The primary reason is the ample presence of reduced carbon (C) compounds having its origin in the photosynthesis carried out by the plants (Jones et al. 2009). This carbon may be present in different forms and available at different times and at different rates depending on the plant species, biological soil activity, as well as on the soil and climatic conditions (Marschner et al. 2004; Vale et al. 2005; Watt et al. 2006).

Arbuscular mycorrhizal fungi must colonize roots of the host plant to complete their life cycle, whereas their propagules are usually dispersed through soil (Smith and Read 2008). This means that interaction of AMF with roots is far the most important biotic interaction from the myco-centric point of view. However, this is handled in details in other chapters of this book and, therefore, is not included here. Outsides of the roots, AMF are particularly exposed to interactions with other soil microbes (and other organisms) in two main periods of their life cycle, namely (1) during spore germination and subsequent primary mycelium growth or hyphal extension from other soil-borne propagules such as colonized root or hyphal fragments

before reaching the root surface, and then (2) during formation of secondary mycelium network and formation of spores, sporocarps, and/or auxiliary cells in the soil. In this short review, we provide an up-to-date synthesis on the identity, mechanisms, and relevance of interactions between AMF and other organisms in soil for plant and ecosystem functioning, with some perspectives for the future research and outlook for exploitation of these results for human welfare. This review does not aim at exhaustive listing of all available knowledge, but should rather provide some examples, in order to show the breadth of the interactions and stimulate further research as well as thoughts on potential exploitation of these biological relationships. On purpose, we completely leave out interactions of AMF with bacteria involved in the symbiotic nitrogen (N) fixation (e.g. *Rhizobium*, *Bradyrhizobium*, *Frankia* etc.) since this topic inherently includes the roots and has been frequently addressed elsewhere (Ahmad 1995; Zahran 1999; Lum and Hirsch 2002; Vessey et al. 2005; Chalk et al. 2006).

2 The Players

Most organisms inhabiting the soil in the proximity of the AMF, which could be called rhizosphere in the broad sense of the word, or the mycorrhizosphere or hyphosphere more specifically (Johansson et al. 2004), do interact with the AMF to some extent. This is because phenomena like CO_2 production through root and microbial respiration, exudation of organic acids and other biologically induced changes in physico-chemical soil conditions, consumption of root and hyphal exudates etc. cannot be strictly confined within clear spatial borders. Moreover, the above phenomena are contributed to by very many different organisms, some of them being attracted or repelled, and some being only opportunists in these niches (Hartmann et al. 2009).

Therefore, here we will focus only on two types of organisms associated with the AMF in an important way. First, these will be microbes previously shown to be physically associated with the AMF (inhabiting surfaces of hyphae, spores, or even their cytoplasma), which would predispose them to undergo functional interactions with the AMF (Toljander et al. 2006). Second, we will pay attention to soil-borne microbes or other organisms, which have been shown to affect strongly the development and growth of the AMF. Even when applying these rather restrictive criteria, rather long list of organisms interacting with AMF in the soil emerges (Table 1).

This list highlights well-studied interactions between AMF and bacteria, some studies on interactions between AMF and other soil fungi, nearly missing knowledge on the AMF interactions with archaea, and complete absence of knowledge on interactions with viruses and soil eukaryotic algae (e.g. not including cyanobacteria; Buée et al. 2009a). Surprisingly little is also known on interactions between different mycorrhizal fungal species in soil, in contrast to studies on competition between different AMF (or AMF and ectomycorrhizal) species for root colonization (Vierheilig et al. 2000; Adams et al. 2006; Alkan et al. 2006; Jansa et al. 2008).

Table 1 Organisms interacting with the arbuscular mycorrhizal fungi (AMF) in soil

Group of interacting organisms	Identity of the interacting organism	Identity of the AMF (if known)	Niche	Strength of association	Reference	Note
Viruses	Unknown	None	Unknown	Unknown	None	No viruses described for AMF so far
Archaea	Oligonucleotide probe-reactive group	Field community	Unknown	Loose to none	Burke et al. 2002	In-situ quantification, no change due to AMF presence
Bacteria	*Burkholderia* sp., possibly others	*Gigaspora* and *Scutellospora*, *Glomus caledonium*	Spores and hyphae	Tight	MacDonald and Chandler 1981; Bianciotto and Bonfante 2002; Levy et al. 2003	Molecular methods and microscopy, endocellular bacteria
	Corynebacterium, *Streptomyces* and other actinomycetes	*Glomus* and *Gigaspora*	Spores, sporocarps	Tight to loose	Mayo et al. 1986; Lee and Koske 1994; Carpenter-Boggs et al. 1995; Filippi et al. 1998	Physical contact or not (volatiles involved), within cell walls (tunnels), cultivation and microscopy
	Pseudomonas and *Rhizobium*	*Gigaspora margarita*	Spore germination (primary mycelium)	Possibly loose, not specific	Bianciotto et al. 1996; Bianciotto and Bonfante 2002	In-vitro study, adhesion depends on polysaccharide production by bacteria
	Enterobacter, *Pseudomonas* sp., *Stenotrophomonas*, *Arthrobacter*	Various *Glomus* spp.	Spores, hyphae	Possibly tight	Mayo et al. 1986; Toro et al. 1997; Barea et al. 1998; Villegas and Fortin 2001, 2002; Bharadwaj et al. 2008	Promote spore germination, root colonization, hyphal growth in bioassays

		Hyphosphere (hyphae, spores, hyphal exudates)		References	Methods
Bacillus, Arthrobacter, Paenibacillus, Enterobacteriaceae, various β-proteobacteria etc.	*Glomus etunicatum, G. mosseae, G. intraradices*	Hyphae	Unknown, tight to loose	Andrade et al. 1997; Artursson et al. 2005; Toljander et al. 2007	Cultivation and molecular methods, lacking proof of tight association with hyphae
Gram–negative bacteria, no *Pseudomonas*	*Glomus fasciculatum*	Hyphae	Tight	MacDonald and Chandler 1981; Vančura et al. 1990	Cultivation and microscopy
Paenibacillus brasilensis, Pseudomonas fluorescens	*Glomus* spp.	Hyphae	Tight?	Toljander et al. 2006	Prefer vital hyphae. Molecular methods, microscopy
Bacillus cereus, Paenibacillus poeriae	*Glomus* spp.	Hyphae	Loose	Artursson and Jansson 2003; Toljander et al. 2006	Prefer non–vital hyphae, Molecular methods, microscopy
AMF *Glomus intraradices, G. proliferum, Gigaspora*	*Glomus intraradices, G. proliferum, Gigaspora*	Hyphae	No obvious antagonism	Cano and Bago 2005	*In-vitro* polyxenic system, artificial conditions (nutrient availabilities and lack of entire plants (nutrient sink)
Glomus mosseae, G. caledonium, G. intraradices	*Glomus mosseae, G. caledonium, G. intraradices*	Hyphae	Fusions, self-compatibility,	Mikkelsen et al. 2008; Croll et al. 2009	Both *in-vitro* and soil-based, microscopy, ^{33}P transfer as a proof of network continuity

(continued)

Table 1 (continued)

Group of interacting organisms	Identity of the interacting organism	Identity of the AMF (if known)	Niche	Strength of association	Reference	Note
Ectomycorrhizal (ECM) fungi	*Pisolithus tinctorius, Laccaria lateritia, Laccaria bicolor, Thelephora terrestris*	*Glomus etunicatum, G. caledonium, Acaulospora, Scutellospora*	Hyphae	Loose	Chilvers et al. 1987; Jones et al. 1998; Chen et al. 2000; Lodge 2000; dos Santos et al. 2001	Little evidence for direct hyphal interactions, competition for root colonization, AMF followed by ECM, sometimes synergistic effects of AMF + ECM on plants
Chytridiomycota	*Phlyctochytrium, Spizellomyces*	Various *Glomus* spp.	Spores	Tight	Ross and Ruttencutter 1977; Daniels and Menge 1980; Paulitz and Menge 1984	Possible parasitism of spores and/or saprophytes on dead spores
Saprophytic fungi	*Wardomyces, Paecilomyces, Gliocladium, Trichoderma*	*Glomus mosseae*	Sporocarps, spore germination	Loose to tight	Fracchia et al. 1998	Cultivation, bioassay, *Wardomyces* antagonistic, *Paecilomyces* synergistic
	Trichoderma harzianum, Exophiala, Aspergillus	*Glomus mosseae, G. intraradices*	Hyphae	Loose	McAllister et al. 1994; McAllister et al. 1995; Green et al. 1999; Tiunov and Scheu 2005	Saprophytes reduced upon AMF establishment, particularly upon collembolan grazing
	Trichoderma harzianum, Verticillum, Acremonium	*Glomus intraradices, Gigaspora gigantea*	Spores, hyphae	Tight	Lee and Koske 1994; Rousseau et al. 1996	Parasitism of spores and hyphae

Eukaryotic algae	Unknown	None	Unknown	Unknown	None	No interactions of AMF with soil algae (not including blue-green algae, which belong to bacteria) described yet
Animals	Collembola (*Folsomia* and field communities)	*Glomus* spp.., *Scutelospora*, *Acaulospora*	Hyphae	Tight	Warnock et al. 1982; Larsen and Jakobsen 1996; Klironomos and Moutoglis 1999; Seres et al. 2007; Bonkowski et al. 2009	Grazing, dispersal of propagules, indirect effects through multitrophic interactions
	Earthworms	*Glomus intraradices* and field AMF community	Spores, hyphae	Loose?	Gange 1993; Harinikumar and Bagyaraj 1994; McLean et al. 2006; Eisenhauer et al. 2009; Milleret et al. 2009	Disruption of mycelium network by burrowing, dispersal?
	Amoebas	*Glomus dimorphicum*	Spores	Tight	Boyetchko and Tewari 1991	Parasitism of walls of living spore, inference from microscopy and *in-vitro* biotest

Another feature apparent from the examples given in Table 1 is lack of coherent results among some of the studies. For example, *Bacillus* and *Pseudomonas* were sometimes postulated as tightly associated with the AMF hyphae and having significant effects on spore germination and hyphal growth, whereas other studies reported absence of tight physical interactions of these bacteria with the hyphae. Some *Paenibacillus* sp. preferred living hyphae, but some other preferentially associated with dead hyphae (Artursson et al. 2005; Toljander et al. 2006). Whereas some of these contradictions could be attributed to differences in experimental approaches (cultivation versus molecular methods, *in-vitro* versus soil systems) and model organisms in different studies (Andrade et al. 1997; Artursson et al. 2005; Jaderlund et al. 2008), it appears that great functional redundancy within rhizosphere (Beare et al. 1995; Misko and Germida 2002) might result into different trajectories of (myco-) rhizosphere development in time. Additionally, the complex network of interactions and feedback regulations within the rhizosphere, further modulated by multiple players (Hartmann et al. 2009; Lambers et al. 2009), might give rise to very different pictures so that the cause-effects relationships might be very difficult to decipher.

3   Mechanisms of Interactions

Great variety of interactions occurs between the AMF and the biota surrounding them (Table 2). These interactions can be classified according to different criteria, they may be direct or indirect, antagonistic or synergistic, specific or not etc. (Table 2). Identity and/or strength of these interactions, even between the same players, can change in space and time. This means that the interactions are shaped by physico-chemical properties of the soil and by patchiness of the mycorrhizosphere with respect to distribution of root and AMF hyphal exudates, gases, water, soil organic matter, occurrence of disturbance events (e.g. earthworm burrowing), as well as by the dynamic changes in the mycorrhizosphere (Smith and Smith 1996; Hinsinger et al. 2005; Watt et al. 2006; Hartmann et al. 2009). Whereas some of the interactions have already been elucidated down to the molecular levels, very many others are described only superficially, with their mechanisms remaining unknown. Surely, great majority of the interactions between AMF and other biotic components of the mycorrhizosphere still awaits its discovery.

Since most of the organisms inhabiting the mycorrhizosphere, including the AMF, are heterotrophs depending on external supply of reduced C compounds, the struggle for C is strong and one of the most important driver of the processes in the mycorrhizosphere (Hartmann et al. 2009; Narula et al. 2009). Different organisms are transferring C between themselves in form of exudates/leachates, they get eaten and parasitized, and they are poisoning and killing each other, and then utilizing the carcasses. Apart from competition for carbon, the different members of mycorrhizosphere biota are competing for inorganic nutrients, suitable conditions and space. Some of them may modify their immediate environment in terms of pH, water

Table 2 Categorization of biotic interactions involving the arbuscular mycorrhizal fungi (AMF), according to different criteria

Category	Class	Example	Reference
Effect	Competition (bearing costs for both)	Plant versus AMF for carbon, AMF versus soil microbes for soil available P, AMF and ECM for root competition of *Eucalyptus*, competition for carbon between mycorrhizosphere microbes	Green et al. 1999; Chen et al. 2000; Vierheilig et al. 2000; Medina et al. 2003; de Boer et al. 2005; Raiesi and Ghollarata 2006
	Grazing, parasitism (costs for one, benefits for the other)	Collembolans grazing on AMF hyphae, chytridiomycetes and other specialized microbes inhabiting and feeding on AMF spores and hyphae	Ross and Ruttencutter 1977; Larsen and Jakobsen 1996; Levy et al. 2003; Gormsen et al. 2004; Bennett et al. 2006; Purin and Rillig 2008
	Amensalism (harm/costs for one, no effect on the other)	Production of antifungal compounds by *Pseudomonas* is stimulated by AMF, though not affecting AMF colonization, whereas reducing root infection by *Gaeumannomyces graminis*, several AMF species suppress development of *Trichoderma harzianum* in soil	Fracchia et al. 1998; Green et al. 1999; Martínez-Medina et al. 2009; Siasou et al. 2009
	Comensalism (benefits for one, no effect on the other)	Dispersal of AMF propagules by collembolans, stimulation of AMF germination/hyphal growth by volatiles from actinomycetes	Carpenter-Boggs et al. 1995; Seres et al. 2007
	Mutualism (benefits for both sides)	Commonly seen type of association, AMF-plant interactions often mutually beneficial, AMF-endocellular bacteria might potentially confer N-fixation benefits to the fungus, N mineralization and P-solubilization by AMF-hyphae associated bacteria, which receive in return hyphal exudates and/or preferential access to dead hyphal stretches	Smith and Smith 1996; Johnson et al. 1997; Bianciotto and Bonfante 2002; Denison et al. 2003; Hamel 2004; Lumini et al. 2007; Richardson et al. 2009

Table 1 (continued)

Category	Class	Example	Reference
Strength	Tight	Parasitism of AMF spores by Chytridiomycota, possibly AMF–species or –group specific	Ross and Ruttencutter 1977; Daniels and Menge 1980; Paulitz and Menge 1984
	Loose	Bacteria and saprophytic fungi on dead mycelium, some collembolans grazing on AMF (not preferred food source)	Klironomos and Moutoglis 1999; Artursson and Jansson 2003; Toljander et al. 2006
	Casual	Competition for plant-derived carbon between AMF and archaea upon O_2 deficiency	Buée et al. 2009a
Complexity	Dual	Parasitism of AMF spores/hyphae by specialized microbes, competition between AMF and ECM for root colonization	Ross and Ruttencutter 1977; Chilvers et al. 1987; dos Santos et al. 2001; Purin and Rillig 2008
	Triple	Plant-AMF-hyphal associated microbes responsible for N mineralization/P solubilization, may include indirect interactions with AMF	Buée et al. 2009a; Richardson et al. 2009
	Multiple	Plant-AMF-associated bacteria-protozoan/bacteria-feeding nematodes, may include indirect interactions with AMF	Bonkowski 2004; Dong and Zhang 2006; Bonkowski et al. 2009
Extent	Local	Bacteria feeding on spore wall polymers of the AMF, nematodes feeding on bacteria in the mycorrhizosphere	Lee and Koske 1994; Filippi et al. 1998; Dong and Zhang 2006
	Remote	Diffusible signal/low molecular-weight molecules, chemical gradients	Carpenter-Boggs et al. 1995; Akiyama et al. 2005; Bouwmeester et al. 2007
Specificity	Specific signals	Specific range of recipients (breadth may differ, own species to interactions across phyla), specific molecules for signaling purposes, usually compound families with modification determining specificity range, antibiotics, flavonoids, strigolactones, quorum-sensing signals	Chabot et al. 1992; Buée et al. 2000; Brodhagen et al. 2004; Scervino et al. 2005; Shaw et al. 2006; Bouwmeester et al. 2007; Pierson and Pierson 2007; Standing et al. 2008; Faure et al. 2009
	Non-specific	Nutrient-induced proliferation, effects of catabolites (not produced primarily as signals), CO_2 and O_2 concentrations on soil organisms	Saif 1981; Bécard et al. 1992; Crawford 1992; Balaji et al. 1995; Gryndler et al. 1996; Jansa et al. 2003; Gryndler et al. 2009

holding capacity, antimicrobial activities etc. They may attract their kin or their associates/symbionts to settle a niche or to facilitate competition with antagonists.

Grazing/parasitism on many different levels has been observed or postulated, and for the specific case of AMF it has been reviewed thoroughly elsewhere (Purin and Rillig 2008). Some examples are provided in Table 2. However, not only the bodies of (micro-)organisms are parasitized by other biota, but also processes such as enzymatic release of nutrients or siderophores can be (at least potentially) utilized/benefited from by organisms, which have not produced them, but which could take them up from the soil soup. This is most likely the mechanism of improved phosphorus (P) and N nutrition from organic sources of plants upon AMF inoculation (Hodge et al. 2001; Feng et al. 2002; Hodge 2003; Hamel 2004), in spite of only very meager evidence for organic matter mineralization potential of the AMF themselves (Joner et al. 2000). Likewise, signaling pathways can be parasitized such as shown recently for strigolactones signaling between roots and AMF hyphae, which is in fact also employed in the seeds of *Striga* plants to trigger germination in the proximity of the host plant roots (Bouwmeester et al. 2007).

Carbon is also in the center point of **mutually beneficial** interactions within the mycorrhizosphere. Apart from the interactions between plants and AMF, interactions between AMF and soil microbes have been described, which apparently bring benefits to both partners involved. In this context, the AMF can be seen as a source of plant-derived C bypassing the battlefields close to the roots and transporting the C fast and efficiently into soil further away from the roots (Heinemeyer et al. 2006; Denef et al. 2007). Hyphal exudates are then preferentially utilized by specific microbial communities on the surface/close vicinity of the AMF mycelium (Perotto and Bonfante 1997; Toljander et al. 2007). In return, the microbes associated with the AMF provide the fungi with exclusive access to otherwise unavailable nutrients such as N and P, made available by microbial-mediated N mineralization, N fixation and P solubilization (Frey-Klett et al. 2007). Endocellular mode of living of some prokaryotes within the AMF cytoplasma can also be seen as physical protection of the bacteria from competition/grazing in the soil. The benefits for the fungus are not quite obvious, however, since the previously reported N-fixation capacity of the endobacteria has been subsequently questioned (Minerdi et al. 2001; Bianciotto and Bonfante 2002; Bonfante 2003; Jargeat et al. 2004). Higher level interactions between the host plants, AMF, and AMF-associated microbes may also be responsible for some of the reported bioprotection effects conferred to the AMF as well as to the associated plants by the AMF. Namely, some prokaryotes such as fluorescent pseudomonads and actinomycetes, capable of producing a broad range of antibiotics and chitinolytic enzymes have been found to be closely associated with the AMF (Table 1 and references therein). This is likely one of the mechanisms of suppression of certain saprophytic or parasitic soil fungi by the AMF (Pozo et al. 2002; Harrier and Watson 2004; Toussaint et al. 2008), which may be beneficial for both the AMF (suppression of mycoparasitic *Trichoderma*) and for the mycorrhizal plants (biocontrol of *Fusarium* and other pathogens) (McAllister et al. 1994; Barea et al. 2005; Jaderlund et al. 2008; Toussaint et al. 2008; Martínez-Medina et al. 2009).

The last mentioned interactions are already involving ***more than two organisms***, thus increasing the level of interaction complexity (see also Table 2). Such interactions appear to be rather common in the mycorrhizosphere, and this also brings with it difficulties in deciphering and exploitation of such interwoven biotic relationships (Buée et al. 2009a). For example, although no direct interactions such as grazing, antibiosis etc. between AMF and nematodes have been described, they may interact indirectly through the effects on plants, or on the AMF-associated microorganisms (Dong and Zhang 2006; Bonkowski et al. 2009), although the experimental evidence is still rather inconsistent (Siddiqui and Akhtar 2009; Wurst et al. 2009; Zhang et al. 2009). Animals like amoebas, bacteria-feeding nematodes, collembolans, and earthworms play an important role in cycling of mineral nutrients in the mycorrhizosphere, affecting also nutrient availability to the AMF (Beare et al. 1995; Bardgett and Chan 1999; Klironomos and Hart 2001; Wilkinson 2008; Bonkowski et al. 2009; Eisenhauer et al. 2009; Milleret et al. 2009).

Many interactions between two individual organisms, either belonging to the same species or not, include exchange of signals (communication, dialogue, crosstalk). The information (signal) is usually carried by chemical ***effectors*** and this also assumes existence of a ***sensing mechanism*** within the recipient organism. Some examples are given below. It is at least theoretically possible that carrier of the information between two organisms can also be light (intensity, color, direction), temperature gradients, electrical field and other sorts of physical effectors. Although these signals have been described for some animals and other organisms in other environments, the amount of information for mycorrhizosphere is up to date very limited (Nagahashi and Douds 2003).

For some interactions within the mycorrhizosphere, chemical effectors have been discovered and the signaling pathways decoded. The effectors may act as attractans or repellents (Jousset et al. 2009); certain effectors are responsible for population control, and for concerted action of single-celled organisms like bacteria. In some cases, the gradients of effectors are being sensed for spatially directed response (e.g. chemotaxis or chemotropism). Therefore, the effectors are usually chemically unstable compounds or compounds like gases, which diffuse quickly through the environment following a steep concentration gradient. Some specific effectors involved in processes like quorum sensing or the symbiosis-dialogue signals (Manefield and Whiteley 2007; DeAngelis et al. 2008; Badri and Vivanco 2009; Cai et al. 2009; Faure et al. 2009) may be produced on purpose, whereas others like CO_2 may be simply waste- or by-products of metabolism (Sage 2002). Low concentrations and chemical instability of most of the specific signals, however, make the hunt for their discovery very difficult, especially when involving non-culturable organisms like the AMF. Only recently, the plant-derived signal molecule family responsible for primary mycelium branching of the AMF has been described (Akiyama et al. 2005), although its existence has been postulated long ago (Giovannetti and Sbrana 1998; Buée et al. 2000; Bécard et al. 2004). Chemical nature of other effectors involved in establishment of AMF-plant symbiosis is mostly unknown, although the genetic basis of these processes is currently being decoded (Frenzel et al. 2005; Drissner et al. 2007; Navazio et al.

2007; Parniske 2008). For example, unknown signal triggers chemotropic growth of AMF hyphal root tip towards the roots (Sbrana and Giovannetti 2005). Various indications for involvement of some flavonoids in spore germination, growth of AMF hyphae, as well as development of intraradical colonization, are spread in the literature (Gianinazzi-Pearson et al. 1989; Ishii et al. 1997; Aikawa et al. 2000; Antunes et al. 2006; Shaw et al. 2006; Scervino et al. 2007). Therefore, it is possible that flavonoids play some role in AMF ontogeny, but experimental evidence on the identity of affected processes is still inconsistent. Identity of the elusive "myc-factor", postulated to follow up the strigolactones sensing in the plant-AMF signaling cascade (Kosuta et al. 2003; Olah et al. 2005) also remains unknown (Parniske 2008). At this place, it is interesting to mention similar efforts invested decades ago into looking for a putative "M-factor", involved in the interactions of partners in ectomycorrhizal symbiosis (Melin 1954; Melin and Das 1954). In analogy to the recent discussions on molecular effectors in arbuscular mycorrhizal symbiosis, existence of the "M-factor" has also been postulated on the basis of growth stimulation of fungal hyphae by the products of the root metabolism. However, the chemical substance, which could be unequivocally identified as "M-factor", the universal effector molecule in ectomycorrhizal symbiosis, was never discovered. Instead, complex interaction of numerous root metabolites at trace concentrations is supposed to play the role.

Identity of factors attracting other organisms towards the AMF hyphae is mostly unknown, although the phenomenon of specific stimulation of some bacteria (e.g. Enterobacteriaceae) by AMF hyphal exudates has been described (Toljander et al. 2007). In the other direction, some volatile compounds like the 2-methylisoborneol produced by some actinomycetes appears to be responsible for the stimulation of AMF spore germination (Carpenter-Boggs et al. 1995). Various compounds produced by a bacterium *Paenibacillus validus* in liquid culture were capable of stimulating AMF hyphal growth and even induction of sporulation in absence of plant roots (Hildebrandt et al. 2006).

Some of the specific biotic interactions in the mycorrhizosphere include not only distance signaling, but also a ***physical contact*** between the partners, involving compounds like polysaccharides, lectins etc. (Rodriguez-Navarro et al. 2007). For example, the mechanisms of physical association of AMF hyphae with bacteria have been studied. It has been reported that the strength of adhesion of pseudomonads and rhizobia to AMF hyphae was bacterial-strain specific and that it involved production of cellulose-containing material by the bacteria (Bianciotto et al. 1996; Perotto and Bonfante 1997). Later on, however, it has been shown that the adhesion strengths of the different bacterial strains with AMF hyphae and root surfaces were similar (Bianciotto and Bonfante 2002), so the conundrum of specific hyphal adhesion remained largely unresolved. Fibrillar structures were described based on microscopy observations of the physical interactions between *Burkolderia* and AMF spores and hyphae, but the biochemical identity of these structures was not described (Levy et al. 2003) (Table 2).

Two more topics, both highly relevant for the functioning of the AMF and both involving biotic interactions, will be briefly handled here. First, this will

be interactions between AMF, soil biota, and the soil organic matter, and, second, the interaction between mycorrhizal hyphae themselves.

The pool of ***soil organic matter***, formed by root exudates, root depositions and other kinds of biotic litter, is transformed by various soil organisms such as bacteria, fungi, and earthworms, and biologically active compounds are released during the process of transformation. Among them, vitamins and plant growth regulators are particularly important. For example, addition of chitin to soil usually promoted hyphal growth and sporulation of the AMF (Gryndler et al. 2003). In spite of this, direct products of chitin degradation had no effect on *in-vitro* growth of AMF hyphae (Gryndler and Jansa, 1997). The chitin addition, however, greatly stimulated actinomycetes community in the soil (Peterson et al. 1965; Gryndler et al. 2003), and their activity is likely to have affected the AMF development (Ames 1989; Carpenter-Boggs et al. 1995). Obviously, not only the specific bioactive compounds may be important for the development of the mycelium of AMF but some more complex fractions of organic matter produced by soil microorganisms might act as components responsible for preferential growth of these fungi. This has been demonstrated in an experiment simulating the input of litter material into the non-sterile soil by the addition of small amount of cellulose (Gryndler et al. 2009). In this case, the growth of AMF hyphae in aqueous soil extract correlated significantly with the abundance of several pyrolysis products of the extracted soil samples. Microbially produced trimethoxybenzoic acid-containing organic matter has been demonstrated to show strongest positive association with AMF hyphal growth. This indicates that AMF are affected by a particular fraction of the soil organic matter. If specificity of these interactions between different species of AMF and particular fractions of soil organic matter shall be discovered, it will indicate that the chemistry of soil organic matter should be considered as an important factor determining the composition of the community of AMF in the given soil. This may have important consequences for strategies of choosing the appropriate AMF isolates for inoculation of a particular soil.

In this context, it also appears important to mention other experiments addressing the mechanisms of stimulation of AMF hyphal growth by different compounds isolated from various sea and freshwater algae (Kuwada et al. 1999; Kuwada et al. 2005; Kuwada et al. 2006a; Kuwada et al. 2006b). Fractionation of the biomass extracts of the algae allowed identification of low-weight compounds like 5′-deoxy-5′-methylamino-adenosine and mannitol, and some polysaccharides, as the active compounds involved in AMF hyphal stimulation. Biological activity of seaweed and other algal biomass may be very important in plant cultures, where this material or its derivatives are exploited as organic soil amendments.

Of particular interest are also the mechanisms, which would regulate co-existence of ***mycorrhizal mycelium*** stretches in a limited space, of the same colony, of the same species (but belonging to different colonies, e.g. originating from different plant individuals) or of different mycorrhizal species (being it AMF only or AMF and ectomycorrhizal fungi). Only limited evidence for interaction between hyphae of different AMF species is available from *in-vitro* experiments, showing that some competition for space may occur upon high hyphal densities (Cano and Bago 2005). However, what are the mechanisms of growth regulation of AMF mycelium

network, induction of hyphal branching, specific proliferation in heterogeneous soil patches etc., being researched for plant roots (Osmont et al. 2007), remains all to be discovered for the AMF (Harris 2008). Numerical models are currently being established for the growth of mycelium network of the AMF (Schnepf et al. 2008). However, these do not take into account possible biotic interactions between neighboring hyphae, which might be very important for efficient spatial exploration of heterogeneous mycorrhizosphere, as documented in some studies (Jansa et al. 2003). Although mechanisms of such interaction remain unknown, it is well documented that the rate of hyphal anastomoses and following nuclear exchange depends on the AMF genotype and genetic similarity between the two fusing hyphal stretches (Giovannetti et al. 2001; Croll et al. 2009), which assumes some sort of self-recognition mechanism at the hyphal level. These topics represent a true scientific challenge still waiting for its breakthrough.

4   Ecological Consequences

Ecological consequences of the interactions between plants and the AMF for plant nutrition, growth, competition and fitness, as well as for soil structuring have been often addressed and there is a large body of knowledge available on these issues (Smith and Read 2008). Other topics like interplant C transfer channeled by the AMF hyphae, involvement of AMF in plant water economy, as well as induction of systemic resistance against aboveground pathogens/herbivores, are objectives of current research (Simard and Durall 2004; Pozo and Azcon-Aguilar 2007; Voets et al. 2008). Importance of the interactions between AMF and other soil biota for plant growth, nutrition, and pathogen/disease resistance, as well as for the soil properties and the ecosystem functions are comparatively less understood than the two-sided interactions between plants and the AMF. However, it appears that the AMF-soil biota interactions, if accounted for, could explain some of the inconsistencies in the research reports on AMF and organic matter mineralization, pathogen resistance, and ecosystem stability (Hodge et al. 2001; Hamel 2004; Fitter et al. 2005; Jones et al. 2005; Artursson et al. 2006; Mallik and Williams 2008).

As shown above, some mycorrhizosphere organisms may ***support the germination*** of AMF spores ***and growth*** of their primary mycelium, extent of root colonization and development of AMF secondary mycelium, as well as the formation of new spores. This may enhance the rate of nutritional benefits conferred to the plants and the improvements of soil structure. On the other hand, some other organisms may preferentially or occasionally feed on the AMF hyphae and/or spores, or they may disturb AMF hyphal networks mechanically, thus decreasing the mycorrhiza-mediated effects on the plants (Milleret et al. 2009). This may be important for ecosystem stability as the benefits to dominant plants or to mycorrhizal cheaters might be limited by these mechanisms (Zobel et al. 1997; Moora and Zobel 1998; Douglas 2008; Facelli et al. 2009). It appears that some microbes closely associated with the AMF mycelium may enhance mycorrhizal functioning through contribution

of specific enzymatic pathways. For example, some bacteria (e.g. endocellular *Burkholderia*, mycorrhizosphere-associated *Azospirillum*, *Azotobacter*, and some cyanobacteria) may **fix atmospheric dinitrogen**, some other microbes may involve in **degradation of organic matter**, which may result in **increased availability of nutrients** such as N and P, as well as in formation of **bioactive compounds** affecting development of the AMF and/or the plants (Minerdi et al. 2001; Mallik and Williams 2008; Richardson et al. 2009). Contradictory results are available in the literature, however. For example, previous reports on beneficial effect of *Azospirillum* on the extent of AMF colonization development in cassava could not be confirmed for cereals (Russo et al. 2005). Part of the story might be the differences in other biotic interactions in the rhizosphere of the different plant species such as those between *Azospirillum* and *Streptomyces* (Elshanshoury 1995).

It is also interesting to mention here some interactions between AMF and **photoautotrophic soil microorganisms** such as cyanobacteria or algae, although very little is known about them. However, it seems that the cyanobacteria and algae may produce many biologically active compounds affecting the AMF (Kuwada et al. 2006a), as well as fix atmospheric dinitrogen and make it available to other organisms. For example, if used in the inoculation experiment with wetland rice, cyanobacteria positively interacted with other co-inoculants (AMF, *Azospirillum* and the P-solubilizing bacteria) in stimulation of plant growth and nutritional status, as well as in sustaining substrate fertility (Chinnusamy et al. 2006).

The capacity of AMF to transfer substantial amounts of N to the plants is well documented now (Mäder et al. 2000; Azcón et al. 2008), even from organic N sources (Hodge et al. 2001; Hodge 2003). However, the enzymatic activities required for **mineralization** of complex organic nutrient sources are likely to be contributed rather by associated microbes than by the AMF hyphae themselves, as is probably the case for organic P sources, too (Joner et al. 2000; Barea et al. 2002; Feng et al. 2003; Barea et al. 2005; Frey-Klett et al. 2007). The biochemical and molecular evidence for N acquisition by and translocation within AMF hyphae (Toussaint et al. 2004; Govindarajulu et al. 2005; Jin et al. 2005) does not solve the conundrum on who is responsible for release of N from organic compounds in soils. Thus the separation of AMF- and associated microbe-mediated enzymatic activities remain difficult to achieve, especially under unsterile soil conditions.

Involvement of AMF in the interaction between **plants and their pathogens** has attracted lots of attention since several decades and a wealth of recent reviews is available on this topic (Jeffries et al. 2003; Harrier and Watson 2004; Selosse et al. 2004; Bennett et al. 2006; Singh and Vyas 2009). Major interest of the research has been on the bioprotection effects of the AMF against soil-borne pathogens such as *Verticillium*, *Fusarium*, *Gaeumannomyces*, nematodes etc. Different mechanisms have been postulated behind these effects such as competition for root colonization space, modulation of root exudation, induced changes in composition and activity of microbial communities (including production of allelopathic and antimicrobial compounds), and induction of systemic resistance (Barea et al. 2005; Frey-Klett et al. 2007; Siasou et al. 2009). The last mechanism is also relevant for (largely) indirect interactions between AMF and herbivorous insects (Gehring and Bennett

2009). Great variation of effects between AMF and insects has been described. For example, generalist insects are usually adversely affected upon establishment of symbiosis between plants and the AMF, whereas specialist insects may benefit (Hartley and Gange 2009). However, many results appear to be ecosystem and context specific and drawing general rules is difficult (Bonkowski et al. 2009; Gehring and Bennett 2009; Hartley and Gange 2009).

Arbuscular mycorrhizal fungi are channeling substantial portion of belowground plant-derived C through the mycorrhizosphere (Pearson and Jakobsen 1993; Heinemeyer et al. 2006; Denef et al. 2007) and thus may also be viewed as important contributors to the diversification of soil *food webs* (Klironomos and Hart 2001; Bonkowski et al. 2009). This view is further reinforced by recent stable-isotopic evidence that the belowground C inputs are particularly important for growth and functioning of soil biota (Albers et al. 2006; Pollierer et al. 2007; Bonkowski et al. 2009), although N-rich leaf-derived organic matter may transiently dominate the food webs (Elfstrand et al. 2008). Thus the AMF are an important factor affecting ecosystem stability and resilience (Fitter and Garbaye 1994; Klironomos and Hart 2001; Barea et al. 2005; Fitter et al. 2005).

5   Research Outlook

Extraordinary diversity of genomes and functions in the mycorrhizosphere and its spatio-temporal dynamics makes it difficult to describe, understand, and model it in its full complexity. Coupling traditional microscopy, chemistry and biochemistry approaches with mathematical modeling offers intriguing options and promises novel insights (Baggs 2006; Neumann et al. 2009), although the conclusions of these studies will still require careful validation in simple model systems. Current methodical advancements in the "-omics" disciplines offer a range of powerful opportunities to gain insight into the dark ecosystem beneath our feet without the need of culturing the microbes. It appears that at least three aspects of our understanding could be substantially improved by using the latest technologies: 1. High throughput, 2. quantification without cultivation, PCR and cloning biases, and 3. ecosystem dynamics. For example, the 454-pyrosequencing emerges very recently as a powerful tool with multiple advantages as compared with the traditional cloning and Sangers-sequencing, in addressing the composition and diversity of mycorrhizal and microbial communities in different ecosystems (Buée et al. 2009b; Öpik et al. 2009).

Employment of radio- and stable isotopes will gain further importance in quantification of pathways of elemental transfers, which would remain hidden otherwise (Smith et al. 2009) Use of stable isotopes in stable-isotope-probing of active microorganisms is extremely valuable, though still scarcely used, approach to identify the relevant players in the food chains (Vandenkoornhuyse et al. 2007). However, care must be taken when interpreting movements of elements such C, oxygen, and hydrogen through the ecosystems, as assessed by isotopic methods. This is because

alternative movement pathways of the isotope (e.g. due to state transition such as liquid-gas) may bias the interpretation of the results. This sort of limitation might be a reason for contradictory results on interplant C transfer as mediated by the AMF (Fitter et al. 1998; Simard and Durall 2004; Selosse et al. 2006; Whitfield 2007; Voets et al. 2008), since the labeled C may move quickly through the system as respired CO_2 and then be fixed either during the photosynthesis or during dark CO_2 fixation in virtually every organism.

Two more challenges shall be mentioned here: (1) *Chemistry of interactions* – between the AMF themselves, as well as between the AMF and other (micro-) organisms. The progress in this field is slow and erratic, since it depends on technology with rather high detection limits and facing the multitude of chemistries, unlike the molecular biology, working with rather uniform nucleic acids and almost invariably benefiting from the magnification power of PCR amplification; and (2) *Interplay between AMF, microorganisms, and their abiotic environment*. The abiotic soil component itself (both the mineral component and the soil organic matter) gains too little attention in many studies, although it is becoming clear that physico-chemical soil properties do modulate the biotic interactions to a large extent (Pendleton et al. 2004; Gryndler et al. 2009; Jansa et al. 2009). Soil environment is namely creating the niches for microorganisms and is both the main factor affecting the behavior of soil biota as well as the product of their activity. Studies in this direction are now accomplished by rapidly developing analytical methods, including pyrolysis-gas chromatography-mass spectrometry (Gryndler et al. 2009). This method allows the analysis of whole soil, without extraction and fractionation, so that no part of the soil remains neglected. Advanced visualization technologies such as fluorescence tagging of specific organisms and/or using transgenic microorganisms as biosensors would allow rare non-destructive insights into the mycorrhizosphere dynamics (Neumann et al. 2009). Last but not least, carefully designed ecological gradient analyses in combination with the above technologies will help answering some burning question about whether the soil microbes have any biogeography, and what factors determine their composition, diversity, and functioning across ecosystems, continents, and biomes (Fitter 2005; Öpik et al. 2006; Chaudhary et al. 2009).

6 Conclusions

Number of novel methodologies and their combinations promise novel insights into composition and functioning of mycorrhizosphere. Although traditional cultivation-based soil microbiology clearly failed in covering most of the relevant microbes, it allowed mining for bioactive compounds like antibiotics, and identification of promising microbial synergists for bioremediation and agricultural applications (Johansson et al. 2004; Barea et al. 2005). It is attainable that many of the rhizosphere interactions involve compounds of a great industrial and medical potential (adhesives, recalcitrant polymers, antibiotics, antivirotics), which might, however,

be impossible to find and tame with traditional approaches. Therefore, innovations such as cultivation-independent screening for biological activities are required (Ferrer et al. 2005). The establishment of linkages between identity and function in a real mycorrhizosphere, however, remains a major challenge, even with metagenome sequencing (Tringe et al. 2005; von Mering et al. 2007) or stable isotope probing (Vandenkoornhuyse et al. 2007; Haichar et al. 2008).

In general, soil is greatly under-appreciated part of the ecosystems, often considered dirty and valueless (Montgomery 2007; Buée et al. 2009a). Yet it is a nonrenewable resource with vital importance for production of human food, animal fodder, fibers, wood and other industrial materials. It is limited and endangered by human activities (Montgomery 2007). Thus understanding the importance of soil biota and the feedbacks between above- and belowground communities (Wardle 2006; van der Putten et al. 2009) as well as co-operation between different microbes in the mycorrhizosphere (Barea et al. 2005) may be critical for designing sustainable production systems of the future.

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Part IV
Influence of the Symbiosis on the Host and its Surrounding: Host Response to Abiotic Stress

Chapter 11
Host Response to Osmotic Stresses: Stomatal Behaviour and Water Use Efficiency of Arbuscular Mycorrhizal Plants

Juan Manuel Ruiz-Lozano and Ricardo Aroca

Abstract Arbuscular mycorrhizal (AM) symbiosis can protect the host plants against the detrimental effects of the water deficit caused by osmotic stresses such as drought and salinity. Stomatal conductance (gs) and water use efficiency (WUE) are among the most studied water relations parameters in the mycorrhizal literature, since they are considered critical to the long-term performance of host plants in semiarid environments. Mycorrhizal effects on gs have been observed in about 50% of experiments involving AM and nonAM plants of similar size. In fact, gs rates usually are higher in AM than in nonAM plants, which implies that AM plants have a lower resistance to vapour transfer from inside the leaves to the atmosphere. AM and nonAM plants have also shown different critical points or thresholds of stomatal behaviour during drought episodes. The higher gs rates in AM plants have been associated with lower xylem-sap abscisic acid (ABA) and lower ABA fluxes to leaves in AM plants. On the other hand, it has been suggested that extraradical hyphae or increased root branching may allow mycorrhizal roots to better explore a particular soil volume, extending soil water depletion zones and giving a mycorrhizal root system more access to available water. In addition, it has been estimated that about half of the promotion of gs by AM fungi can be attributable to the soil colonization by AM fungi. Nevertheless, these results can vary when the host plant shows a water conservative strategy. Moreover, different AM fungal species have been shown to modulate also differently the physiological response, including gs, of host plant to drought. The AM influence on gs can also be modulated by environmental conditions such as irradiance, air temperature or leaf temperature. There are also several reports in the literature showing an increase of plant WUE by the AM symbiosis either under well watered or under osmotic stress conditions. The effects of AM symbiosis on WUE depend on the fungal species involved, without a correlation with the percentage of root infection. These effects have been rather

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related to higher net photosynthetic rate and optimal quantum yield of photosystem II in AM plants than in nonAM ones and with enhanced activities of carbon assimilatory enzymes such as Rubisco. In any case, specific studies dealing with the effect of AM symbiosis on leaf morphology are needed in order to ascertain how these parameters influence the WUE of the host plant.

Keywords Abscisic acid • Arbuscular mycorrhiza • Osmotic stress • Stomatal conductance • Water use efficiency

Abbreviations

ABA   Abscisic acid
AM   Arbuscular mycorrhiza
AMF   Arbuscular mycorrhizal fungus
gs Stomatal conductance
LAR   Leaf area ratio
NAR   Net assimilation rate
QY   Optimal quantum yield of photosystem II
SLW   Specific leaf weight
Ψ   Water potential
WUE   Water use efficiency

1   Introduction

Plants are constantly confronted with environmental constraints of both biotic and abiotic origin. Abiotic stresses such as drought, salinity and extreme temperatures are the most common environmental stress factors experienced by terrestrial plants (Seki et al. 2003). All of those stresses share a common osmotic component since they diminish water uptake by roots and cause a dehydration of plant tissues. Thus we refer to them as osmotic stresses (Zhu et al. 1997). The dehydration caused by those stresses is a consequence of the imbalance between the water lost in the leaves and the water taken up by roots (Aroca et al. 2001).

The water deficit caused by osmotic stresses interferes with both normal development and growth and has a major adverse effect on plant survival and productivity. Indeed, water deficit accounts for over 70% of yield losses during crop production (Kramer and Boyer 1997; Bray 2004). Global climate change is increasing the risk of water deficit in the near future. Nowadays, there is broad consensus that climate change is occurring and that stresses from climatic extremes will continue, and possibly increase, and thus impose water deficit and significant difficulties to plant growth in many parts of the world. In fact, problems of water deficit have recently spread to regions where drought was negligible in the past. These difficulties will be

particularly pronounced in currently semi-arid agricultural zones and/or under conditions of irrigation that often exacerbate soil salinization (Araus et al. 2003; Denby and Gehring 2005).

Most terrestrial plants can establish a symbiotic association with a group of soil fungi called arbuscular mycorrhizal (AM) fungi. The AM symbiosis is present in all natural ecosystems, even in those affected by adverse environmental conditions (Varma 2008). Several eco-physiological studies investigating the role of AM symbiosis in protection against drought stress have demonstrated that the symbiosis often results in altered rates of water movement into, through and out of the host plants, with consequences on tissue hydration and plant physiology (for reviews see Augé 2001, 2004; Ruiz-Lozano 2003). Thus, it is accepted that AM symbiosis can protect host plants against the detrimental effects of water deficit and that the contribution of the AM symbiosis to plant drought tolerance results from a combination of physical, nutritional and cellular effects (Ruiz-Lozano 2003). Studies carried out so far have suggested several mechanisms by which the AM symbiosis can alleviate drought stress in host plants. The most important are: direct uptake and transfer of water through the fungal hyphae to the host plant (Hardie 1985; Ruiz-Lozano and Azcón 1995; Marulanda et al. 2003), better osmotic adjustment of AM plants (Augé et al. 1992a; Ruiz-Lozano et al. 1995a; Kubikova et al. 2001), enhancement of plant gas exchange and WUE (Augé et al. 1992a; Ruiz-Lozano et al. 1995a,b, Goicoechea et al. 1997; Green et al. 1998) and protection against the oxidative damage generated by drought (Ruiz-Lozano et al. 1996, 2001; Porcel et al. 2003; Porcel and Ruiz-Lozano 2004). In addition, changes in soil water retention properties have also been suggested (Augé et al. 2001), although the real significance of soil on mycorrhiza-induced plant drought tolerance is still doubtful according to other subsequent studies (Augé et al. 2004b).

It is well known that in higher plants stomata control leaf gas exchange, providing paths for CO_2 intake and governing the efflux of water vapour to the atmosphere during transpiration. Adequate regulation of stomata may allow keeping the rate of evaporation to a minimum while the rate of CO_2 assimilation is maintained, which in turn, enhances the plant water use efficiency (WUE). There is consistency in published data that mycorrhizal symbiosis usually promotes transpiration and stomatal opening, affecting thus the stomatal conductance (gs) and the WUE of the host plant (Augé 2000). In this chapter we will focus on AM alterations of plant stomatal behaviour and of plant WUE under drought and saline stress conditions.

2 Stomatal Behaviour of Arbuscular Mycorrhizal Plants Under Osmotic Stress

Water deficit causes several alterations on plant physiology, one of the most common being stomatal closure (Rood et al. 2003; Aroca et al. 2003; Loreto and Centritto 2008). Small changes in leaf water status can have relatively large effects on critical physiological processes such as photosynthesis and water transport. Changes in gs

and transpiration are typically balanced by changes in leaf hydraulic conductance, keeping changes in water potential gradient across the leaf to a minimum (Franks 2006). As Augé (2001) noticed in one of the most comprehensive reviews on AM symbiosis and drought stress tolerance, mycorrhizal effects on *gs* have been unpredictable, occurring in about 50% of experiments involving AM and nonAM plants of similar size. Currently, it is not clear how AM effects on stomatal behaviour vary among AM fungi or host plants. Indeed, the literature shows that *gs* of some *Citrus* species are little affected by AM colonization, while soybean, sunflower, lettuce or rose plants show frequent alteration of stomatal behaviour by AM symbiosis (Augé 2001). Where *gs* rates differ in AM and nonAM plants, they usually are higher in AM than in nonAM plants, which implies that AM plants have a lower resistance to vapour transfer from inside the leaves to the atmosphere when exposed to the same water conditions. AM-induced increases in *gs* are often subtle, but promotion of *gs* by twofold in AM plants, have also been recorded (Ruiz-Lozano et al. 1995a; Augé et al. 2004a). These higher *gs* rates in AM plants have been associated with lower xylem-sap abscisic acid (ABA) and lower ABA fluxes to leaves in AM plants (Ebel et al. 1997). In contrast, it seemed that xylem sap concentrations of calcium or zeatin riboside equivalents were not affected by the AM symbiosis and had no influence on *gs* (Duan et al. 1996). On the other hand, it has been suggested that extraradical hyphae (Allen 1991) or increased root branching (Kothari et al. 1990) may allow mycorrhizal roots to better explore a particular soil volume, extending soil water depletion zones and giving a mycorrhizal root system more access to available water. Therefore, AM and nonAM root systems exposed to the same stress and constrained to similar soil volumes may not necessarily suffer similar strain, that would explain the higher *gs* rates in AM plants (Duan et al. 1996).

Recently, Augé et al. (2008) have studied if the changes in *gs* induced by the AM symbiosis are accompanied by changes in leaf hydraulic conductance and in gradients of leaf water potential. They observed that under amply watered conditions the fungus *Glomus intraradices* enhanced by 27% *gs* of squash plants, but leaf hydraulic conductance did not increase in these plants and, consequently, the water potential gradient across the leaf was higher in AM leaves. Authors concluded that the higher gradients of water potential in leaves of AM plants were consistent with the higher rates of gas exchange found in AM plants and that, presumably, are necessary to supply the carbon needs of the fungal symbiont.

It is also noticeable that AM and nonAM plants have shown different critical points or thresholds of stomatal behaviour during drought episodes. Leaf water potential (Ψ) was about 0.2 MPa lower in *Glomus fasciculatum*-colonized wheat plants than in similar-sized nonAM plants when stomata began to close (Allen and Boosalis 1983), and leaf Ψ at stomatal closure was about 0.7 MPa lower in AM rose plants than in similar-sized nonAM rose plants (Augé et al. 1986). Stomatal conductance in AM plants has also remained unaffected by declines in available soil moisture longer than in nonAM plants (Osundina 1995; Duan et al. 1996). Furthermore, AM plants showed higher *gs* than nonAM plants when only a portion of a root system was dried and before drying affected leaf water status and these effects occurred independently of plant size or phosphorus nutrition through

nonhydraulic signals (Augé et al. 1992b, 1994, 1995). In this context, it has been shown that when AM and nonAM plants were exposed to similar soil water deficits, *gs* of AM and nonAM plants were still different, suggesting also that the AM influence on *gs* is of biochemical nature (Augé 2000). Moreover, the questions whether intact root systems are required to observe an effect of AM fungi on transpiration, or whether there is some residual AM influence on foliage that continues to affect transpiration of leaves detached from root systems have been studied by Green et al. (1998). The results of such study showed that there was a residual influence of mycorrhizal symbiosis in rose leaves, but not in cowpea leaves and that exogenous ABA, calcium or phosphorus had little influence on the transpiration rate of AM and nonAM leaves. It has also been shown that partial drying of a root system can lead to reduced *gs*, even when the rest of the root system is moist enough to fully supply shoot requirements for water (Davies et al. 1994). However, the AM symbiosis can affect the inhibition of *gs* that occurs when only a portion of the root system is dried and before drying affects leaf water potential, suggesting an AM effect on nonhydraulic root-to-shoot communication of soil drying (Augé and Duan 1991; Ebel et al. 1996). Thus, it has been proposed that root dehydration alters root metabolism leading to the production of a nonhydraulic, chemical signal that moves to leaves where it induces stomatal closure. Root-to-shoot regulation of stomatal behaviour during soil drying may occur via a multiple chemical signal, including cytokinins and ABA (Druge and Schonbeck 1992; Goicoechea et al. 1997). In fact, Goicoechea et al. 1997 noted that higher *gs* and transpiration of mycorrhizal alfalfa plants was associated with altered ABA/cytokinins ratios in their leaves. Thus, the AM symbiosis can alter the production of ABA and cytokinins in drying roots in order to regulate stomatal opening and *gs*.

2.1 Influence of the Host Plant Life-Style and of AMF Origin

The induction of host *gs* by the AM symbiosis has been observed both under amply watered conditions and under drought stress conditions (Augé 2001). However, the greater impact on plant physiology can be found under drought stress conditions. For instance, Khalvati et al. (2005) found that under drought stress conditions the stimulatory effects of AM symbiosis on *gs* were more evident that under well-watered conditions. Nevertheless, these results can vary when the host plant shows a water conservative strategy as is the case of *Rosmarinus officinalis*. *R. officinalis* is a perennial Mediterranean plant well adapted to dry conditions that avoids drought stress by reducing its transpiration rate, as well as its *gs* (Munné-Bosch et al. 1998). The behaviour of these plants against drought stress was studied by Sánchez-Blanco et al. (2004). Authors showed that, both under well-watered and under drought stress conditions, AM symbiosis enhanced root hydraulic conductivity and plant water status, but these effects did not correlate with *gs* since no significant differences in *gs* between AM and nonAM plants were found (Fig. 1). On the contrary, significant increases of *gs* were found in two aridland woody plants such
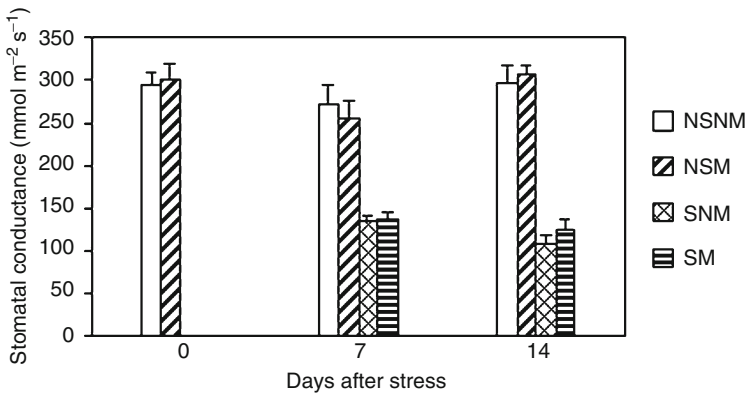
Fig. 1 Stomatal conductance for *Rosmarinus officinalis* plants under four different treatments (NSNM – non-stressed non-mycorrhizal; NSM – non-stressed mycorrhizal; SNM – stressed non mycorrhizal; SM – stressed mycorrhizal) at three different moments of the experimental period (0, 7 and 14 days after the stress) (Taken from Sánchez-Blanco et al. (2004) with kind permission from Elsevier)

as *Olea europaea* and *Rhamnus lycioides* inoculated with the AM fungus *Glomus intraradices* (Caravaca et al. 2003; Querejeta et al. 2003). In spite of the common enhancement of *gs* in both plants, WUE was significantly enhanced only in *Olea europaea*, but not in *Rhamnus lycioides* (Caravaca et al. 2003; Querejeta et al. 2003). Authors related this differential behaviour with the own plant lifestyle. *Olea* is a long-lived, slow-growing evergreen tree with a conservative water use strategy, while *Rhamnus* is a drought-deciduous shrub with a shorter lifespan.

Finally, reduced *gs* under drought stress conditions in AM *Anthyllis cytisoides* plants has been observed by Goicoechea et al. (2004). *A. cytisoides* is a deciduous shrub plant that exhibits a slow growth rate and behaves as a drought avoider. It exhibits a resting period and one of its strategies to withstand prolonged water deficit is defoliation (Haase et al. 2000). In their study, Goicoechea et al. (2004) observed that drought stress induced a more pronounced decline in *gs* in AM plants than in nonAM plants. Moreover, when plants were recovered from drought, young leaves from previously stressed mycorrhizal plants continued showing lower *gs* values than those from nonAM plants, concomitantly with a higher rate of leaf abscission. Leaf shedding can be a beneficial adaptation that reduces water loss and redistributes resources in order to favour plant survival under drought stress (Munné-Bosch and Alegre 2004). Thus, authors considered that this behavior could minimize both water loss by transpiration and whole plant respiration, preventing mycorrhizal plants from suffering desiccation. They concluded that, in this way, AM symbiosis conferred a greater responsiveness of *A. cytisoides* to drought.

Despite their low host specificity, AMF are known to vary widely in their ability to modulate host plant physiology (van der Heijden 2004). Moreover, different AMF species have been shown to modulate also differently the physiological response, including *gs*, of host plant to drought (Ruiz-Lozano et al. 1995a, b). Thus,

the question whether native, drought-adapted AMF improve host plant performance to a greater extent than nonnative AMF was addressed by Querejeta et al. (2006). They conducted a field experiment in which seedlings of two native wild shrub species were inoculated with either native or nonnative *Glomus* species before outplanting in an agricultural land. Their goal was to evaluate the nutritional and/or nonnutritional basis for any differential growth advantage conferred to the host plants by the drought-adapted native AMF under semiarid conditions. The results obtained showed that native AMF enhanced *gs* values (inferred from oxygen isotopic measurements according to Scheidegger et al. 2000) to a greater extent than nonnative AMF in both shrub species.

2.2 Influence of Combined Salt Stress and of Environmental Conditions

The hypothesis that AM effects on stomatal conductance under drought stress would be more pronounced under saline conditions has been also tested (Cho et al. 2006). The study was conducted on sorghum plants and the results showed one more time that *gs* was higher in AM than in nonAM plants. The average *gs* of AM plants during the drying episode was higher than that of nonAM plants by 32% with drought stress alone, by 38% with NaCl/drought stress and by 51% with osmotic/drought stress (Fig. 2). However, considering the whole-plant response to drought stress, authors concluded that the presence of excess salt in soils widened the difference in drought responses between AM and nonAM plants only occasionally. In twice as many instances, salinity stress tended to nullify an AM-induced change in drought response.
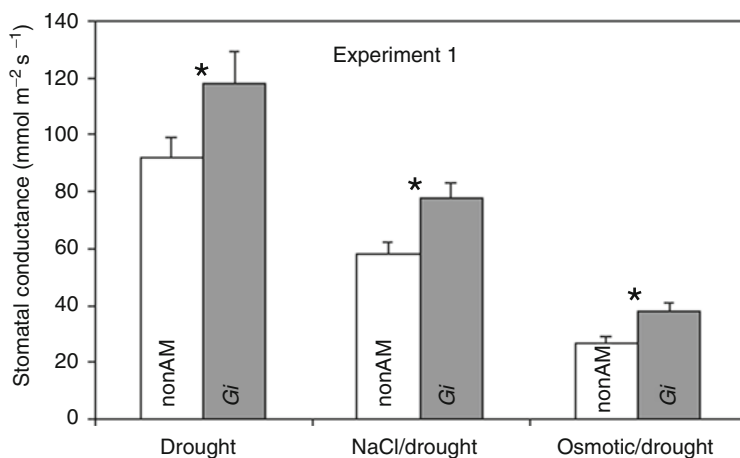
Fig. 2 Average stomatal conductance within treatments during the drying episode. Asterisk denotes significant differences (Taken from Cho et al. (2006) with kind permission from Elsevier)
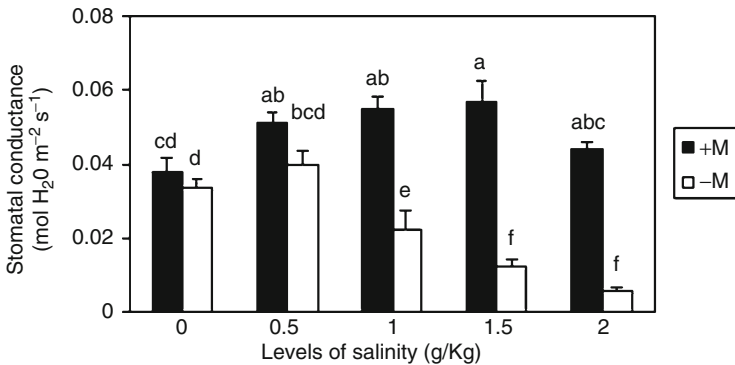
Fig. 3 Influence of *Glomus mosseae* on stomatal conductance in the leaves of maize plants inoculated (+M) or not (−M) with *G. mosseae* at five NaCl levels (Taken from Sheng et al. (2008) with kind permission from Springer-Verlag and Business Media)

In any case, the positive effects of the AM symbiosis on *gs* under salinity stress alone have also been reported. For instance, in a study with maize plants, Sheng et al. (2008) showed that mycorrhization enhanced by 14–853% stomatal conductance under increasing salt levels (Fig. 3).

In order to better understand the AM influence on stomatal behaviour of host plants, Augé et al. (2004a) examined whether the magnitude of AM effects on *gs* was related to environmental conditions: irradiance, air temperature or leaf temperature. These authors showed that *gs* of AM plants was markedly higher than *gs* of similarly sized nonAM plants (Fig. 4). AM promotion of *gs* was minimal at the lowest irradiances and lowest air and leaf temperatures, but was substantial at intermediate irradiance and temperatures. AM promotion was again low or absent at the highest irradiances and temperatures. Thus, author concluded that AM symbiosis is more likely to promote *gs* when plants are growing in moderate environments.

2.3 Influence of Soil Colonization by AMF

The influence of AM symbiosis on plant water relations and on moisture retention properties of soils has also been analyzed (Augé 2004; Augé et al. 2004b). Augé and co-workers examined the possibility that mycorrhizal soil may directly influence plant water relations. Using wild-type (myc⁺) and noncolonizing (myc⁻) bean mutants planted into soils previously produced using AM or nonAM sorghum plants, they partitioned mycorrhizal influence on *gs* and drought resistance into soil and root components, testing whether effects of AM fungi occurred mostly via mycorrhization of roots, mycorrhization of soil or both. The studies conducted showed that mycorrhizal effects on drought resistance were attributable to an effect on plant itself rather than to an effect of mycorrhizal soil. In contrast, about half of the considerable promotion of *gs* by two AM fungi was attributable to soil colonization and about half to root
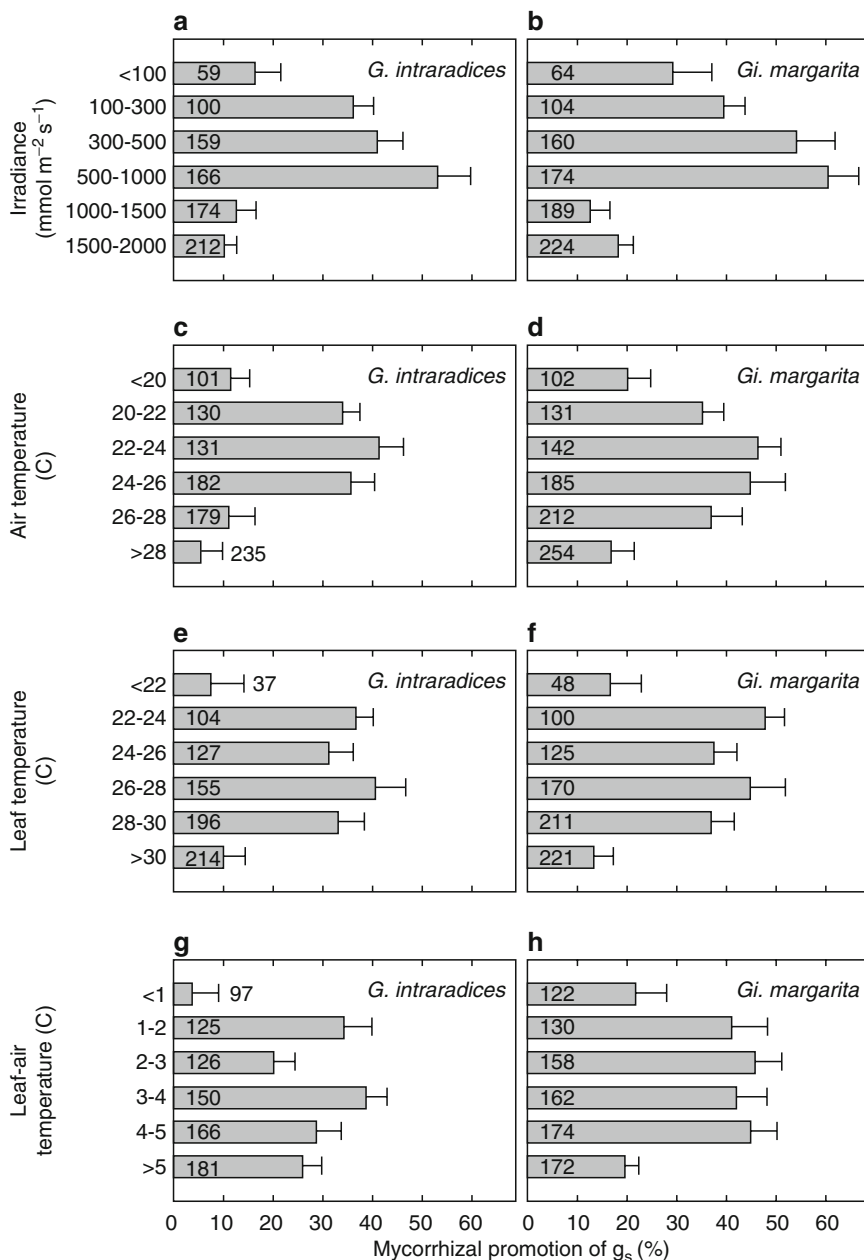
Fig. 4 Mycorrhizal promotion of stomatal conductance (gs) as a function of irradiance and temperature. All measurements were ordered by irradiance and depicted at six irradiance levels (**a**, **b**). Bars represent means (+SE) of each irradiance range. All measurements were similarly re-ordered for the subsequent three pairs of panels; ordered by air temperature (**c**, **d**), ordered by leaf temperature (**e**, **f**), and ordered by leaf-air temperature difference (**g**, **h**). Numbers within histogram bars are the means of absolute *gs* for each sub-range (Taken from Augé et al. (2004a) with kind permission from Springer-Verlag and Business Media)
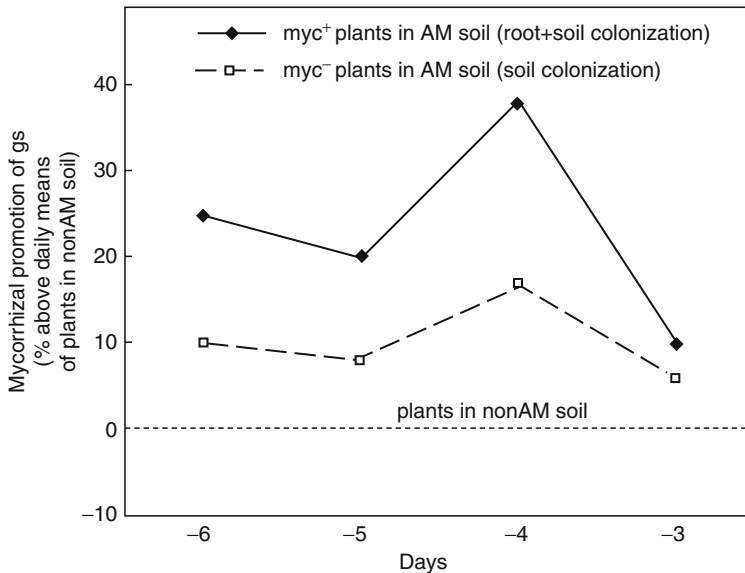
Fig. 5 Mycorrhizal promotion of *gs*, showing AM influence partitioned into soil and root components using wild type (myc$^+$) and non-colonizing mutant (myc$^-$) bean plants. A value of 0% promotion denotes that daily average *gs* of AM plants was similar to that of nonAM plants; nonAM plants for each genotype are depicted by the dotted line (Taken from Augé 2004 with kind permission from Agricultural Institute of Canada)

colonization. Moreover, merely growing in a mycorrhizal soil resulted in promotion of *gs* of nonAM plants under both amply watered and droughted conditions (Fig. 5). That means that AM soil can affect the physiology of nonAM plants when grown in that soil. To explain this surprising result, authors suggested a nonhydraulic basis for the AM influence on the nonAM plant and proposed that the biochemical effects of the different soil organisms that interact with AM fungi in the rhizosphere (including P solubilizers, N$_2$ fixer, producers of hormones or siderophores, etc) may contribute to the effects of AM soil on *gs* of host and nonhost plants (Augé et al. 2004b).

In order to better understand why merely growing in a mycorrhizal soil resulted in promotion of *gs* of nonAM plants, Augé et al. (2007) further studied a possible relationship between the degree of soil mycorrhization (estimated as soil hyphal density) and variations in plant *gs* before and during drought. The objectives of the study were to learn if the amount of soil hyphae would also contribute to variation in stomatal behaviour and soil drying and to test if soil colonization would make stronger contributions than root colonization to these variables. Results obtained showed that both hypotheses were true. Firstly, the significant soil hyphae path coefficient occurred in each *gs* model (Fig. 6) and were negative, indicating that less hyphae in soil were related to higher gs values. Secondly, soil colonization had

Sorghum
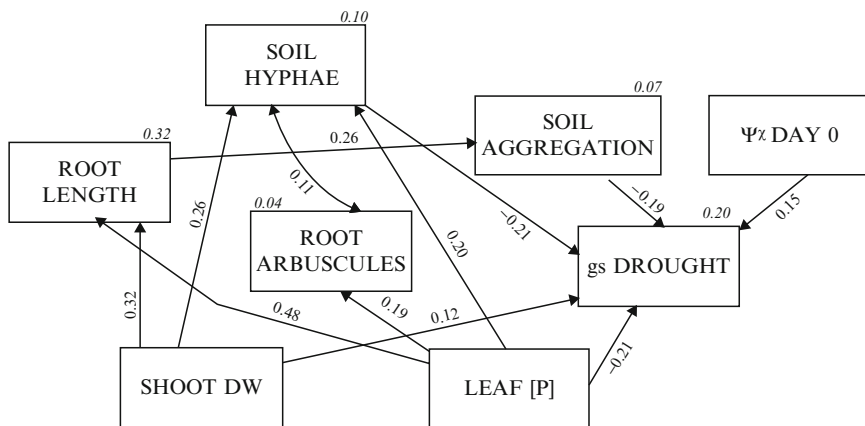
Squash
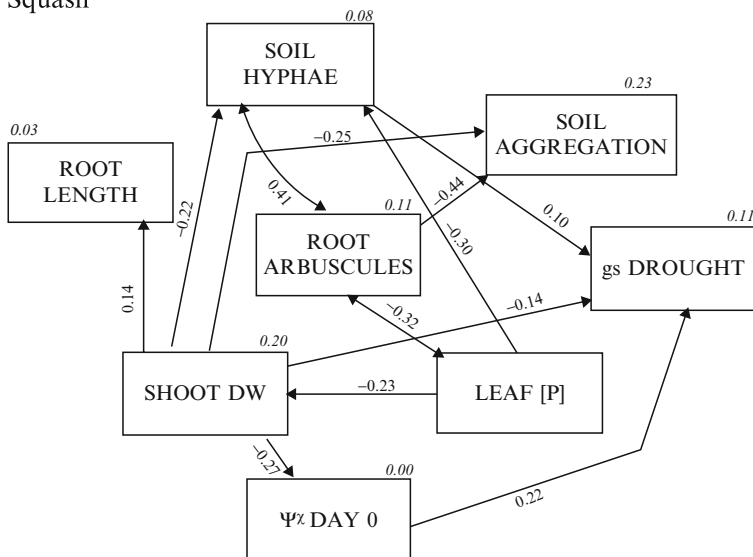
Fig. 6 Path diagrams for sorghum and squash depicting the hypothesized causal relationships among independent and dependent variables for *gs* during the first 9 d of drought. Each single-headed arrow signifies a direct causal relationship in the direction of the arrow. Double-headed arrows indicate a correlation between two variables. Indirect causal effects occur if one variable is linked to another via other, intermediate variables. Numbers on arrows are path coefficients (standardized partial regression coefficients derived from the regression of each response variable on those variables directly linked to it) indicating the relative strength of each path leading to a given response variable. Values ≥0.20 represent significant contributions. Italicized numbers above variable boxes are estimates of the proportion of total variance explained (squared multiple correlations) for each dependent variable (Taken from Augé et al. (2007), with kind permission from Elsevier)

more influence on *gs* values than did root colonization. Thus, authors concluded a role for mycorrhization of soil itself in contributing to the regulation of stomatal behaviour of host plants (Augé et al. 2007).

3 Water Use Efficiency of Arbuscular Mycorrhizal Plants Under Osmotic Stress

The term water use efficiency (WUE) refers to the ability to limit water loss while maintaining net carbon uptake in the leaves. Although in general the WUE is calculated as the ratio of carbon fixed into dry weight to the total amount of water lost by evapotranspiration, there are several ways to measure or estimate WUE, each of those having its own components and their interpretation (Tambussi et al. 2007). In summary, there are two main ways of measuring plant WUE. The first method addresses the WUE of the photosynthetic organ, that is the leaf, and it is referred as instantaneous WUE (WUE_i). WUE_i is measured by infrared gas analyzers and it is the ratio between net photosynthesis rate (CO_2 gained by the leaf) and leaf transpiration rate. The second way to measure WUE is to calculate the ratio between the dry biomass produced during a period of time and the total amount of water consumed by the plant in the same time frame. This second type of measuring WUE is called biomass WUE (WUE_b), and it is integrative of the whole plant processes and not only related to the photosynthetic efficiency of the leaf, hence more realistic to what happens in the field. For a review on the topic see Tambussi et al. (2007).

There are several reports in the literature showing an increase of plant WUE by the AM symbiosis either under well watered or osmotic stress conditions (Ruiz-Lozano et al. 1995a; Kaya et al. 2003; Khan et al. 2008). At the same time, these reports were referred to both WUE_b (Kaya et al. 2003; Khan et al. 2008) or to WUE_i (Ruiz-Lozano et al. 1995a). However, other authors have found no effect in the plant WUE neither calculated as WUE_i (Klingeman et al. 2005; Querejeta et al. 2007) nor as WUE_b (Bryla and Duniway 1998). In any case, the effect on WUE by AM symbiosis depends on the fungus species involved without correlation with the percentage of root infection (Ruiz-Lozano et al. 1995a).

There are several physiological and biochemical parameters involved in the regulation of WUE besides stomatal conductance as is described above. WUE can be determined obviously by leaf photosynthetic efficiency, but also by leaf morphological traits as specific leaf weight (SLW) or leaf area ratio (LAR). In the following sections we will discuss how AM symbiosis may alter these parameters.

3.1 Influence of AMF ont Leaf Photosynthetic Efficiency

In order to increase WUE without growth penalties, plants should either increase their net CO_2 assimilation rate (NAR) without diminish their *gs* or decrease their *gs*

but keep their NAR. Both strategies involve an increase of the leaf photosynthetic efficiency. Hence, Wu and Xia (2006) found in tangerine trees a higher net photosynthesis and stomatal conductance during drought stress when plants were inoculated with the AM fungus *Glomus versiforme*. Therefore, in this case the photosynthetic efficiency was kept constant by AM fungus inoculation. However, in other several studies, leaf photosynthetic efficiency increase during drought or salt stresses by AM inoculation (Ruiz-Lozano et al. 1995a; Goicoechea et al. 1997; Sánchez-Blanco et al. 2004; Khalvati et al. 2005; Sheng et al. 2008). Thus, Khalvati et al. (2005) found a higher net photosynthesis rate in barley plants inoculated with the AM fungus *G. intraradices* than in non inoculated ones although both groups of plants showed similar *gs*. Ruiz-Lozano et al. (1995a) compared the effects of different AM fungi on net photosynthesis rate and *gs* of lettuce plants under drought stress. These authors found that almost all the fungi raised both parameters, but when the ratio between net photosynthesis and *gs* was calculated only a few fungi increased such ratio, and even the inoculation with the AM fungus *G. etunicatum* decreased this ratio (Fig. 7). Curiously, the fungus that showed the better ratio (*G. ocultum*) was the fungus that had the lower positive effect on lettuce growth under drought conditions (see Ruiz-Lozano et al. 1995a). At the same time, *G. etunicatum* was the second fungus among which was tested in enhancing lettuce growth under drought conditions (Ruiz-Lozano et al. 1995a). These results pointed out the difficulty in establishing a correlation between WUE and growth (Blum 2005), and that each AMF species has its own mechanism in increasing host plant tolerance to drought.

One of the parameters more widely used to estimate leaf photosynthetic efficiency is the optimal quantum yield of the photosystem II (QY; Baker 2008). When this parameter has been determined in *Rosmarinus officinalis* plants inoculated with *G. deserticola* under drought conditions (Sánchez-Blanco et al. 2004) or in maize plants inoculated with *G. mosseae* under salt conditions (Sheng et al. 2008), it showed higher values compared to plants not inoculated. At the same time, values
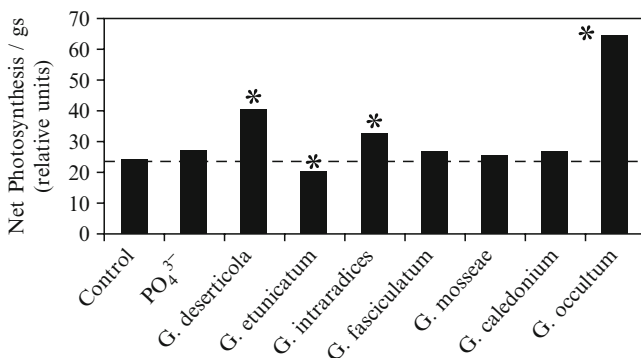
Fig. 7 Ratio between leaf net photosynthesis and leaf stomatal conductance (*gs*) of lettuce plants subjected to drought non inoculated (Control), PO_4^{3-} added, or inoculated with different *Glomus* species. * indicates significant differences with control plants. Adapted from Ruiz-Lozano et al. (1995a)

of QY correlated with higher values of shoot dry weight and net photosynthesis (Sánchez-Blanco et al. 2004; Sheng et al. 2008). Therefore, QY could be used as a simple parameter for a first screening in order to know how AM symbiosis is improving host plant tolerance to osmotic stresses.

Photosynthetic efficiency also depends on the activity of carbon assimilatory enzymes such as Rubisco (Masumoto et al. 2005). Valentine et al. (2006) found that grapevines inoculated with one AM fungus showed higher WUE and Rubisco activity than non inoculated ones during a drought period, establishing a relationship between WUE and Rubisco activity. Unfortunately, no more studies dealing with AM symbiosis effects on carbon assimilatory enzymes during an osmotic stress are available, and therefore this would be a matter of study for the next years.

3.2   Influence of AMF on Leaf Morphology Parameters

Higher values of the ratio between total leaf weight and total leaf area (called specific leaf weight; SLW) have been related in some reports to higher WUE under drought conditions (Craufurd et al. 1999; Songsri et al. 2009). Theses results are explained because since the leaves become thicker there should be more photosynthetic machinery per leaf area to gain CO_2 with less water lost by the stomata (Tambussi et al. 2007). There are very few reports where SLW has been analyzed in AM plants under osmotic stress conditions. Thus, Estrada-Luna et al. (2000) found that guava plantlets colonized by a mixture of AM fungi have higher growth rate and higher SLW during acclimatization to *ex vitro* conditions than non colonized plantlets. However, earlier Berta et al. (1995) had been found that transplanted plum trees inoculated with either *G. intraradices* or *G. mosseae* had higher growth rate but also lower SLW than non colonized trees. Therefore, the relationship between SLW and the improvement of WUE by the AM symbiosis needs to be further studied in the future, since SLW is a cheap and easy parameter to be measured.

In several reports a positive correlation between leaf area ratio (LAR; the ratio between the total leaf area and the total plant weight) and WUE have been found (Ghannoum et al. 2001; Ge et al. 2003). Plant relative growth rate (plant weight increment per plant weight unit) is determined by the net assimilation rate (the increase in plant weight per leaf area unit) and by LAR (Poorter and Remkes 1990). As happens for SLW, very few studies have addressed how AM symbiosis alters the host plant LAR. Thus, Lovelock et al. (1996) found an increase of LAR in a tropical tree (*Beislchmiedia pendula*) when it was inoculated with an AM fungus, being such increase in LAR the main reason for the increase at the same time of the tree relative growth rate. At the same time, Bray et al. (2003) found that increases in LAR by AM symbiosis depended on the different AM isolates. Unfortunately, the above studies were carried out without controlling the plant water regime. Therefore, specific studies dealing with the effect of AM symbiosis on leaf morphology, more

precisely analyzing SLW and LAR parameters, are needed in order to ascertain how these parameters influence the WUE of the host plant.

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Chapter 12
Metal Tolerant Mycorrhizal Plants: A Review from the Perspective on Industrial Waste in Temperate Region

Katarzyna Turnau, Przemysław Ryszka, and Grzegorz Wojtczak

Abstract The chapter summarizes research carried out on the role of mycorrhizal fungi in phytoremediation of heavy-metal-rich wastes in temperate regions. Symbiotic fungi are an important component of soil microbiota, especially under harsh conditions. Properly developed mutual symbiosis enhances the survival of plants in polluted areas by improving nutrient acquisition and water relations. In addition, mycorrhizal fungi were found to play an important role in heavy metal detoxification and the establishment of vegetation in strongly polluted areas. Fungal strains isolated from old zinc wastes also decrease heavy metal uptake by plants growing on metal rich substrata, limiting the risk of increasing the levels of these elements in the food chain. The effectiveness of the bioremediation techniques depends on the appropriate selection of both the plant and the fungal partners. Plants conventionally introduced in such places disappear relatively soon, while those appearing during natural succession are better adapted to harsh conditions. Symbiotic partners selected on the basis of such research are often the best choice for future phytoremediation technologies. Moreover, mycorrhizas of different types are also helpful in substratum toxicity monitoring. Further improvements can be obtained by optimization of diverse microbiota including various groups of rhizospheric bacteria and shoot endophytes.

Keywords Heavy metals • Industrial wastes • Phytoremediation • Phytostabilisation • Phytoextraction

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1 Introduction

Phytoremediation of metal contaminated areas is attracting increasing interest as a cheaper alternative to chemical methods, more friendly for environment and non-destructive to soil biota. Efficiency of this technology strongly depends on the characteristics of the given site, type of heavy metals in the substratum, climatic conditions, use of native and indigenous plants and microbiota. Our research was focused on post-flotation, heavy metal rich wastes in Southern Poland. Intensive exploitation of the metasomatic ores, found in triassic dolomites, started in the twelfth century (Szuwarzyński 2000; Strzyszcz 2003). The following eight centuries brought increased heavy metal extraction efficiency, but at the same time the risk of wind and water erosion accelerated dramatically, resulting in immediate need of phytostabilisation of the waste. Presently, the ores are subjected to flotation process. Waste material is composed of two fractions. The solid part is used to form tailing ponds into which the liquid phase is dumped. Modern flotation technologies resulted in lower content of heavy metals in the waste, but the material is much more susceptible to wind and water erosion. The toxicity of the waste substratum itself is relatively low as its pH is ranging from 7 to 8, but the waste particles can cause serious atmospheric pollution and subsequently can increase soil toxicity in the surrounding area. The dusts originating from the area often contain above the threshold levels for Zn, Pb, Tl and Cd (Dmowski 2000). Biological reclamation faces several serious difficulties, e.g. plant growth inhibition (Strzyszcz 2003). The density of particles within the sediment is much higher than in natural soils making it impermeable for water and air. This is disadvantageous during both dry and wet periods: even increased precipitation cannot assure appropriate infiltration, while too low porosity disables water recharge by capillary rise from the deeper layers. At the same time such material easily undergoes water or wind erosion (Strzyszcz 2003) and the heap slopes tend to slide down making stabilization efforts even harder. These processes accelerate when the exploitation is finished. When additional watering is stopped the situation changes dramatically. Chemical composition of the waste material is another factor complicating the reclamation of such places, namely low levels of basic ions like Na^+, K^+, Mg^{2+} and Cl^-, almost complete lack of organic matter and significant deficiency of N and P. On the contrary, the carbonate content of the waste substratum exceeds 75% and is usually accompanied by high levels of Ca^{2+} and sulphate ions. Additionally, the substratum contains high levels of heavy metals although alkaline pH of waste substrata highly reduces the availability of potentially toxic elements to plants. The major concern is to keep these metals in place and avoid their transfer into areas with lower pH values.

2 Plant Reaction to Heavy Metals

The major toxic effects of transition metals appear to result from: (i) generation of reactive oxygen species (ROS), e.g. by the Fenton reaction (e.g. Gallego et al. 1996; Keightley et al. 2004; Kieffer et al. 2008); (ii) damaging cellular components and

interfering with metabolic processes; (iii) binding heavy metals to SH-groups of enzymes and inactivation of their catalytic domains. Individual organisms respond to heavy metals by: (i) prevention of heavy metal uptake (exclusion); (ii) absorption of heavy metals and attenuation of the toxic effect by chelation, covalent bonding, dilution, compartmentalization, extrusion, etc. (amelioration); (iii) production of physico-chemical barriers to protect crucial organs from toxicity (avoidance) (Baker 1987). The toxic effect of metals that entered the cells can be counteracted by: (i) complex organic molecules such as metallothioneins and phytochelatins that are synthesized by the organisms (Cobbett and Goldbrough 2002; Hall 2002); (ii) heavy metal transporters, a broad group of different proteins such a CPx-ATPases for Cu or Cd, ABC- transporters for Cd-transport into the vacuole, ZIP-transporters (ZRT-, IRT-related proteins for Fe or Zn) and Nramp transporters (Hall 2002). Recent advance in proteomic studies discovered also the induction of an array of proteins that are expressed under stressed condition such as chitinases (Van Keulen et al. 2008), the heat shock protein HSP60 (Rios-Arana et al. 2005), proteins of the sulfur metabolism pathway (Roth et al. 2006) and cysteine synthase (Yang et al. 2007).

Not only individual plants, but also whole communities and populations respond to heavy metals. This response involves: (i) succession that includes changes in species composition and diversity and (ii) natural selection that leads to communities of higher tolerance to stress (Baker 1987; Fitter and Hay 1987; Salisbury and Ross 1992). Such processes take long time (Bradshaw and McNeilly 1991; Hoffmann and Parsons 1991). Succession occurs faster than selection and uses a broader range of metabolic processes. Plant populations are capable of the so called "phenotypic plasticity" meaning a range of variation in phenotypes expressed by a single genotype under different environmental conditions (Hoffmann and Parsons 1991). The phenotypic plasticity involves diverse aspects of plant life such as closure of stomata, development of roots, shoots and leaves, flowering and metabolic rate. Depending on genetic variation in species/population, natural selection changes the plasticity level under heavy metal stress and results in selection of resistant genetic variants. Studies by Wierzbicka and Panufnik (1998), Wierzbicka and Potocka (2002), Załęcka and Wierzbicka (2002), Pielichowska and Wierzbicka (2004), Baranowska-Morek and Wierzbicka (2004) and Olko et al. (2008) are the best examples of research of such processes that were observed in plants colonizing 100-years-old wastes rich in heavy metals in Poland.

3   Revegetation Technologies Non Assisted by Microorganisms

So far, there are no large scale documented applications of restoration techniques including microorganisms in Central and Eastern Europe. The slopes are often covered with a 20 cm layer of humus, or other material such as spoils from hard coal mining (Aldag and Strzyszcz 1980; Strzyszcz 1983) or material excavated during mining. Such material can be relatively rich and theoretically can be used to grow plants. Clean soil is usually avoided as it is very expensive if the area is big.

Such material is often sliding down the steep slopes and needs to be stabilized in various ways. The introduction of such a layer allows for easier establishment of the vegetation by blocking the factors limiting plant growth and it may diminish the transfer of heavy metals into the plant material. Several experiments were carried out to evaluate the influence of diverse soil amendments to improve the physico-chemical properties of the waste substratum. Among them were bentonite, bituminous emulsion, gigtar and vinacete (Trafas 1996). At least in the first few years following the application, the results were encouraging.

Among trees, the most commonly introduced are birches, pines and poplars. Especially the last ones, belonging to phreatophytes, under moderate climate are genetically predisposed to rooting at up to 12 m (Negri et al. 2003). Roots of this species can be important in improvement of the water supply due to so called "hydraulic lift", the process of water movement from relatively moist deeper layers and its release into the shallower layer (Richards and Caldwell 1987; Caldwell et al. 1998). The role of hydraulic lift in moderate environment was discussed by Dawson (1993). It is expected that this phenomenon can result in improved rhizosphere processes, especially activity and life span of fine roots and associated microorganisms (Lacombe et al. 2009). This might be also the mechanism by which the mobilisation of nutriens can be improved (Caldwell et al. 1998).

Comparatively well establishing on metal rich industrial wastes are shrubs such as *Hippophaë rhamnoides*, *Eleagnus angustifolius*, *Robinia pseudoacacia* and *Physocarpus opulifolius*. Most often used are grasses including: *Festuca ovina*, *F. rubra, Phleum pratense, Poa pratensis, Lolium perenne, Dactylis glomerata, Arrhenatherum elatius, Bromus inermis* and *Festuca pratensis*. The grasses are often supplemented with *Medicago sativa* and *Trifolium repens*. According to long term observations carried out on zinc wastes' populations of introduced non-woody species, their number dramatically decreases with time and almost none of them is left on the 20–30 years old wastes. Much more stable are plants that appear on the wastes spontaneously, but, it takes a long time till they establish and form stable communities (Ryszka and Turnau 2007).

4 Why Mycorrhizal Fungi Are Important in Restoration of Metal Rich Wastes

In natural soil, nutrients are most abundant in the surface layer. The industrial waste substratum, if not fertilized or covered with the humus layer, is originally uniformly poor. The nutrient distribution becomes patchy when decaying plant or animal material such as feces are deposited. Also dead microorganisms (bacteria and fungal hyphae) can be an important source of nutrients (Tibbett 2000). Plant roots have to reach these patches, at the same time competing for nutrients with other organisms. While plants growing in nutrient-rich soils do not need efficient root systems, the so-called "hidden half" of the plant has to be built very efficiently to survive under industrial waste condition. In this nutrient-poor environment the mycorrhizal

association proves to be very useful. Mycorrhizal fungi enhance the root absorption area up to 47-fold (Smith and Read 1997) and provide access to nutrients and water otherwise not accessible for plants (Cui and Nobel 1992; George et al. 1992; Nadian et al. 1997). The fungi also enhance the substratum structure by formation of the hyphal net (Jasper et al. 1989; Smith et al. 1998; Jeffries et al. 2003). In general fungi are known to be able to accumulate significant amounts of heavy metals (Gadd 1993) varying from a few percentage to 20% of dry mass (Tobin et al. 1984) suggesting that microbial biomass may affect the mobility of metals in the soil system. Processes such as metabolism dependent (bioaccumulation) or independent (biosorption) can be involved in removal of metals from the wastes (Gadd 1993). In the second case, both living and dead biomass can be active (Volesky and Holan 1995). Cell wall components of fungal mycelium contain free amino, hydroxyl, carboxyl and other groups (Gadd 1993), that can be very efficient in binding heavy metals. The ability to chelate and retain heavy metals within cell walls is so high that some saprobic fungi producing large biomass are used as commercial biosorbants (Mullen et al. 1992; Kapoor and Virarghavan 1995; Morley and Gadd 1995). The similar role of arbuscular mycorrhizal fungi was demonstrated (Joner et al. 2000; Gonzales-Chavez et al. 2002, 2004). Janouskova et al. (2006) showed that extramatrical mycelium of AMF is capable to accumulate ten to twenty times more Cd per unit biomass than tobacco roots. Abuscular mycorrhizal fungi (AMF) produce glomalin (glomalin-related protein, GRSP), first believed to be a hydrophobin, later identified as likely a 60-kDa heat shock protein homolog (Gadkar and Rillig 2006; Rillig et al. 2007) that seems to be efficient in sequestering Cu, Cd, Zn (Gonzales-Chavez et al. 2004, 2009; Cornejo et al. 2008) and also Fe, Pb, Mn (Chern et al. 2007; Vodnik et al. 2008). Immunolocalization of glomalin in the AMF mycelium and spores has shown the presence of glomalin mainly in the inner cell wall layer L2 and L3 while it is less abundant in the outer layer L1 (Purin and Rillig 2008). This result might be, however, an artifact due to chemical fixation of the material and release of the glomalin in a similar way as heavy metals are released during chemical fixation (as explained by Turnau and Kottke 2005). Interestingly, this substance is also present outside the fungal structures and being deposited in the environment (Driver et al. 2005), similarly to compounds produced by saprobic fungi and active in formation of nanoparticles. Manceau et al. (2008), in his pioneer studies on the distribution of Zn and Cu in roots and mycorrhizal hyphae, used techniques such as micro-extended X-ray absorption fine structure (μEXAFS) spectroscopy with the addition of micro-X-ray fluorescence (μXRF). Besides GRSP also phyllosilicate were shown to chelate Zn. The sequestration and spatial distribution of Zn was studied with μSXRF (micro synchrotron based X-ray fluorescence) and μEXAFS by Sarret et al. (2003) and these techniques showed that while in roots Zn is mainly associated with malate, in the mycelium it is chelated by phyllosilicate that is most probably found on the surface of the mycelium and not inside. Both results need further studies.

According to Joner and Leyval (1997) the efficiency of protection against heavy metals depends on the given AMF isolate, but generally the transfer from the soil into the plant is restricted by metal immobilization in extraradical mycelium.

These authors have also shown that no inhibition of mycelium growth was observed even at 20 mg of NH_4NO_3-extractable Cd kg^{-1} of substratum. MeT-like sequences were identified in the arbuscular fungus *Gigaspora rosea* (Stommel et al. 2001) although the metal sequestration capacity and actual MeT-like nature was not determined till recently. The identification and functional characterization of an MeT-encoding gene from *G. margarita* was demonstrated by Lanfranco et al. (2002) and in addition the differences in gene expression in symbiotic and pre-symbiotic stages were shown.

Heavy metal distribution within mycorrhizal roots compared to nonmycorrhizal were investigated by various groups using techniques such as EELS, EDAX, SIMS, LAMMA and PIXE. In general, the cortex where the fungal structures are formed was selectively enriched with Fe, Ni and Zn (Turnau et al. 1993; Kaldorf et al. 1999; Orłowska et al. 2008). Ultrastructural localization of metals in extramatrical mycelium and spores (Gonzales-Guerrero et al. 2008) confirmed that heavy metals were mainly localized within cell wall and in the vacuoles. Cu mainly accumulated in the spore vacuoles while Cd in vacuoles of the mycelium. (Ferrol et al. 2009) studying extraradical spores suggested that Cu might be accumulated within some spores in high concentrations, and this might be a novel mechanism to store excess of this element.

Mycorrhizal fungal activity also influences qualitatively and quantitatively the microbial populations of the mycorrhizosphere. They are accompanied by bacteria such as legume symbiotic nodular bacteria, plant growth promoting rhizobacteria (PGPR), mycorrhiza helper bacteria (MHB) and saprobic fungi. As these organisms influence plants either by interactions with abiotic and biotic components of the soil, or by stimulating plant growth through the production of vitamins and hormones, they should be included in the optimisation of the restoration processes. Unfortunately, so far very little has been done in this respect.

5   Role of Arbuscular Mycorrhizal Fungi in Revegetation of Metal Rich Wastes

Mycorrhizal symbiosis is an important component of industrial waste habitats as the conditions are extremely harsh for plant and microbial growth. Inappropriate management of such areas results in low availability of mycorrhizal inoculum within the deposited substratum, originally devoid of AMF propagules. The first possible source of mycorrhizal inoculum is the soil or humus transferred from another area to cover the waste material. Fertilization aiming to improve the growth of freshly sown grasses is detrimental to most or all members of Glomeromycota. Although the transferred soil contains a seed bank including AM hosts, they are usually unable to survive and die shortly after germination. The exceptions are *Molinia caerulea* (Poaceae) and *Anthyllis vulneraria* (Fabaceae). These plants are accompanied by various rhizosphere microorganisms such as AMF, diazotrophs and rhizobacteria (Reinhold-Hurek and Hurek 1998; Hamelin et al. 2002) and they are well adapted to dry and nutrient poor substrata. These two species are able to

form stable patches of vegetation, although their area is not increasing fast due to the prevalence of vegetative reproduction under this particular situation. Under restoration practices commercial cultivars of grasses are usually sown on the introduced soil in favour of high biomass production at high fertilizer input. Being independent upon mycorrhizal symbiosis, such plants are able to grow there as long as the introduced soil is not removed by water erosion. Such grasses disappear either leaving uncolonized areas or spontaneous plant colonizers completely displace them (unpublished data).

Zn-Pb heaps are an ideal object of plant natural succession studies. The oldest parts are located at its basis and its age decreases with height enabling further estimations of age of the plant communities. The first plants appearing on the bare surfaces of wastes were nonmycorrhizal members of Caryophyllaceae (*Cerastium* spp.*, Silene vulgaris*) and Brassicaceae (*Cardaminopsis arenosa*). They were, however, incapable of forming a dense cover of the waste substratum. These were followed by facultative mycorrhizal species. Grasses such as *Molinia caerulea, Agrostis gigantea, Bromus inermis, Calamagrostis epigejos, Corynephorus canescens, Dactylis glomerata, Festuca tenuifolia* and *F. trachyphylla*, all turned out to be very expansive and promising in terms of phytostabilisation; similarly to other members of this particular plant family they were also shown to be strongly colonised by mycorrhizal fungi when collected from the zinc wastes (Ryszka and Turnau 2007), with some species (e.g. *Festuca tenuifolia*) exhibiting twofold to fivefold higher difference in mycorrhizal colonization and arbuscular richness when compared to unpolluted sites. Nevertheless, concerning plant growth, mycorrhizal dependence was not equal among all grass species. In fact, only 45% exhibited increased growth following mycorrhization, while 38% did not react at all and a negative effect was observed in the remaining 17%. This results are in accordance with Newsham and Watkinson (1998) who showed that grasses can respond differently to mycorrhizal symbiosis. The observed negative effect can be attributed to increased transfer of carbon compounds from the host to the fungus, low photosynthetic activity and increased activity of the mycelium. Such situation was already shown in the case of *Lolium perenne* (Buwalda and Goh 1982). It is not always possible to observe a direct positive influence of AMF symbiosis. Some effects of symbiosis are visible only after a long time, in subsequent generations or may concern seedlings survival rates.

It is noteworthy that exceptionally low levels of mycorrhizal colonization in some grasses were observed in individuals in places flooded by fresh waste liquid from broken pipes. This shows that the waste material is harmful when deposited, although, due to the neutral pH of the substratum, the toxicity is not extremely high. The main source of stress under these conditions is rather extreme water and nutrient deficiency. This was clearly shown in the case of zinc mound located in Bolesław (Southern Poland), where a part of the waste was covered with material excavated from metal ores, but containing similar metal content to post-flotation substratum. The unprocessed substratum contained much more N and P and had much higher water holding capacity. Therefore, a much better development of plants and higher viability of AMF spores (Ryszka and Turnau 2007) was observed.

Several decades of spontaneous succession led to the creation of communities consisting of very rare and interesting species such as *Biscutella laevigata, Silene vulgaris, Gypsophila fastigiata, Cerastium arvense* and *Armeria maritima subsp. halleri* (Grodzińska et al. 2000). The last species is considered to be a metallophyte (Szafer 1959). Among the listed species, the most astonishing was documenting of mycorrhizal symbiosis in *Biscutella laevigata* (Orłowska et al. 2002) since this plant belongs to Brassicaceae (Cruciferae) family, widely accepted to consist of non-mycorrhizal species. Well developed arbuscules were found within the thin lateral roots, while the main (thick) root remained uncolonized. According to DeMars and Boerner (1996), members of this particular plant family can possibly be colonised by AM fungi, but for unknown reason the arbuscules which are the most important criterion of a functional mycorrhiza do not develop under glasshouse conditions. This hypothesis was indeed confirmed by Orłowska et al. (2002) who during a 2-year cultivation assay was not able to observe arbuscules. On the other hand, arbuscules were found in *B. laevigata* growing in the wild, both in Tatra Mountains (Orłowska et al. 2002) and Alps (H. Bothe personal communication).

Other members of plant communities appearing on the Zn-waste consist of strongly mycorrhizal species. In the younger waste areas *Festuca ovina, Plantago lanceolata*, and *Viola tricolor* are the most common plants; they may serve as a source of mycorrhizal inoculum. Other species like *Thymus pulegioides* and *T. serpyllum* form dense tufts on bare ground, while members of Fabaceae possess the ability to form triple symbiosis with both AMF and nitrogen-fixing bacteria. Other plant species, less common or visible but still worth noticing are *Gentianella germanica* and *Linum catharticum*. Both strongly mycorrhizal and considered as extremely rare in the wild. It is also worth pointing out that within a given plant community on industrial waste one can find species originating from entirely different environments. *Molinia caerulea, Carex flacca, Sanguisorba officinalis, Valeriana officinalis, Phragmites australis* – all are almost exclusively found in swamp-like habitats. Those plants are mostly mycorrhizal and often resistant to heavy metals due to increased content of Si in their tissues (Kabata-Pendias and Pendias 2001). They can easily survive periodical changes of water level. Another group belongs to those typical for xerothermic grassland species: *Poa compressa, Scabiosa ochroleuca, Sedum acre, Dianthus carthusianorum, Linum catharticum, Thymus pulegioides* – most developing mycorrhizal associations.

In terms of practical applications, perennial plants are important for restoration as they can cover the area in a relatively short time, although it is limited by low viability of seeds produced on zinc wastes.

Fungal symbionts can be used furthermore in biomonitoring of ecosystem soil quality and effectiveness of restoration practices (Turnau and Haselwandter 2002; Leyval et al. 2002; Orłowska et al. 2002). Such applications require an appropriate plant host and *Plantago lanceolata* seems to be a good choice. This plant is not only widespread, but also tolerant to increased levels of Pb (Wu and Antonovics 1976), Sb (Baroni et al. 2000) and PAHs (Bakker et al. 1999). *P. lanceolata* is mycorrhizal in greenhouse conditions (Walker and Vestberg 1994) and can be propagated vegetatively (Wu and Antonovics 1976). Mycorrhizal parameters like relative mycorrhizal

colonisation, relative arbuscular formation and arbuscule richness can be used to assess the differences between restored and the non-restored areas, under the condition that the same fungal strain is used. Additionally, the fungal strains used must not be adapted to conditions present on industrial wastes. The main limitation of such approach is therefore a narrow group of fungal strains that can be used in the analyses. This can be easily overcome with *P. lanceolata* as a host as it can host a broad spectrum of AM species. With this system it was possible to indicate the toxicity of various sites of Polish industrial wastes (Orłowska et al. 2005b). Our results closely corresponded to those obtained by means of chemical analysis of heavy metal "availability" (i.e. metals extraction in $Ca(NO_3)_2$ and NH_4NO_3). A slightly different system for testing soil toxicity also employing AMF was developed by Leyval and was based on *Glomus mosseae* spore germination rates, but it seems that both approaches lead to similar results. Soil quality can be also accessed via examination of several morphological changes in the mycelium such as thick septa, thickenings of the mycelium and changes in shape of arbuscules (Orłowska et al. 2005b).

6   Abundance and Diversity of Arbuscular Mycorrhizal Fungi in the Wastes

The number of spores in the substratum of different age vary from 0 to 100 spores per 100 g in revegetated areas. The restoration practices, including scarification and planting trees or covering the original substratum with material excavated from the part of mine that has a higher nutrient content (N and P) results at least in doubling the number of spores and the percentage of alive spores (E. Orłowska personal communication).

The diversity of AMF on metal rich substrata in Europe is mostly limited to species belonging to genus *Glomus*. The diversity of AMF on industrial wastes seems to be very dependent on the number of conditions including substratum characteristics and the presence of carriers that could transfer the spores into the waste. In a recent study by Gonzales-Chavez et al. (2009) on a Cd rich slag heap in Mexico, spores of *Gigaspora, Glomus. Scutellospora* and *Acaulospora* were found. Spores and even sporocarps were found in vegetal residues and in animal feces. As suggested by the authors, the propagules were efficiently spread by mesofauna. The slag was spontaneously colonized by invasive plant species that were almost all colonized by AMF. This seems to be, however, an extraordinary situation. While studying wastes from Poland the spread of fungal inoculum is very slow, although, when the propagules are already on the waste, spreading might be enhanced by animals such as ants. AMF diversity in Polish wastes was studied in the Boleslaw calamine area and on the zinc wastes of ZG Trzebionka. Morphological analysis of spores from 100-year-old calamines has shown the presence of *G. aggregatum, G. constrictum, G. fasciculatum, G. pansihalos* and *Entrophospora* sp. (Pawlowska et al. 1996). Nested polymerase chain reaction (PCR) with taxon-specific primers was used to identify the species *G. moseae, G. intraradices, G. claroideum, G. gerdemannii, Glomus occultum* (Turnau et al. 2001) and morphological analysis added *G. fasciculatum* and *G. aggregatum* to the list of AMF species of

zinc wastes of ZG Trzebionka (Turnau et al. 2001). Several, but not all of these fungi are by now available in monocultures. The roots of *Fragaria vesca* from Trzebionka waste were used to evaluate which fungus is the most effective in mycorrhizal colonisation. The most frequent fungus was *G. gerdemannii*, which, however, was not obtained in trap cultures. Slightly less frequent were *G. claroideum* and *G. occultum* while the least common were *G. intraradices* and *G. mosseae*. The easiest to get in trap cultures was *G. claroideum*. These studies were followed by the localisation of heavy metals using rhodizoniate (Turnau 1998; Turnau et al. 2001). This stain was used to evaluate the differences between fungal species in their ability to absorb metals. The experiment has shown that fungi isolated from industrial wastes such as *G. intraradices* and *G. clarum* reduce metal uptake into the roots and shoots of *P. lanceolata*, while compared to strains originating from soils of low HM content (Orłowska et al. 2005a). In addition, in spores of a *G. intraradices* isolated from zinc wastes of ZG Trzebionka, characteristic depositions within periplasmic space, between the inner layer of the wall and plazmolemma, were observed. The accumulation of heavy metals within these depositions were confirmed by EDS analysis coupled with SEM. The method was also used to estimate the percentage of the mycelia that show increased metal accumulation. In the case of *Euphorbia cyparissias* (Turnau 1998) about 80% of the intraradical mycelium showed an increased content of heavy metals. However, at the same time, the number of arbuscules was slightly lower than in mycelium containing lower levels of these elements. On the contrary, *Fragaria vesca* roots from the same industrial wastes that were selected for molecular studies on fungal identity, when stained with rhodizoniate, have shown that increased metal accumulation is visible only in case of *G. mosseae* that is one of the least efficient colonizers (below 10%). These results clearly show that there are differences not only between fungi but also between plant species in their preference concerning symbiotic associations. Under laboratory conditions, for practical reasons, we usually inoculate the plants with one species, while under field conditions the plants are inhabited by several different strains or species. Studying the metal uptake by plants is one of those examples that has to be carried out first with the use of the single species.

7 Metal Accumulation in Plants Growing on Industrial Wastes

The response of the plant to soil pollution is known to vary depending on the AMF isolate and species (Streitwolf-Engel et al. 1997; van der Heijden et al. 1998). AMF were already reported to both decrease and increase HM uptake by plants (Leyval et al. 1997; Khan et al. 2000). Therefore, the selection of appropriate plant and fungal genotypes, can provide a potential for decreasing health hazards during crop production and improve sustainable agriculture and phytoremediation technologies, including phytoextraction (Turnau and Mesjasz-Przybylowicz 2003; Jurkiewicz et al. 2004). To demonstrate the importance of the selection of the appropriate plant variety, 15 cultivars of *Zea mays* were cultivated on Zn-Pb waste substratum with *Glomus intraradices*. Heavy metal content in plant material varied significantly between the

varieties. A different experiment, in which *Plantago lanceolata* was grown in pots filled with Zn-Pb substratum demonstrated the influence of various AMF strains on Cd, Zn and Pb uptake (Orłowska et al. 2005a). Heavy metal uptake clearly depended on the fungal strain used. Plants inoculated with AMF strains originating from soils not affected by heavy metals had higher metal concentrations in tissues than plants inoculated with strains from polluted areas. Similar results have been obtained by Sudova et al. (2008) while comparing selected fungal isolates and plant clones from contaminated and noncontaminated substrates. Also results by Redon et al. (2008) confirmed that fungi from polluted soils increase metal tolerance of plants by increasing plant biomass and reduce metal toxicity. Fungi isolated from polluted sites were also found to be effective to reduce shoot Cd concentration. In *Medicago truncatula* also the interactions with rhizobacteria were influencing metal transfer. As suggested by Lingua et al. (2008), metal uptake can differ depending on the use of particular fungal strains and particular plant genotypes involved in the plant-fungal interaction. These findings are very important as plants growing on wastes often contain high levels of metals in aboveground tissues, and this can create the risk of metal transfer into the food chain, as the industrial wastes are often inhabited by a broad range of wild animals. The introduction of proper fungi can decrease metal uptake into the shoots, while the available pool of metals could be stored within the root system.

The success of the introduction of the selected fungus into the non-polluted or moderately polluted soils could be low due to the presence of native fungi that are better adapted to the particular soil characteristics. However, zinc wastes at the beginning of the succession are often devoid of AMF and inoculation seems to be reasonable. Still, the practical questions related to the application of the AMF await further experiments and studies. There were so far several experiments showing weak points of the technology. Among them the use of annual plants has been shown not to be the best solution; promising perennial plants are already known but they require optimization of propagation techniques in order to obtain suitable amounts of material. The metal content of the waste is not the most important problem for the revegetation, other issues such as poor water holding capacity, water/wind erosion and mineral deficiency have to be addressed. The water holding capacity can be improved e.g. by the use of water-binding gels ("hydrogels") applied simultaneously with the inoculum, which prevents the inoculum from being removed by the wind. The application of inorganic fertilizers should be replaced by compost or manure. The use of sewage sludge might be a good choice, but may led to increased toxicity (e.g. Cu). Therefore, the dosage needs to be optimized in order to sustain the development of the belowground microbial consortia.

8   Introduction of Plants from Xerothermic Grasslands into the Metal Rich Wastes: New Approach

The establishment of an appropriate plant cover of reasonable diversity in order to reduce erosion and further contamination of areas surrounding the wastes requires further efforts (Turnau et al. 2008). On the basis of long-term observations it was

observed that among plants that spontaneously colonize heavy metal rich wastes are species that normally occur on calcareous or/and sandy xerothermic grasslands, such as *Potentilla arenaria, Hieracium pilosella, Anthyllis vulneraria*. In 2003, first attempts were carried out to experimentally introduce *Anemone sylvestris* and *Primula veris* into the Zn-Pb wastes in Poland. Seedlings of these plants accompanied by microbiota and original soil from xerothermic grasslands were introduced into the wastes, and were found to establish successfully. During further studies, almost 20 xerothermic grassland species were first grown in the laboratory on industrial substratum and their survival rates were monitored. These experiment showed that most of the plants introduced as seeds are not able to germinate and develop on industrial wastes. The seedlings have to be cultivated on a mixture of clean soil and industrial waste substratum and equipped with mycorrhizal fungi – this is the most important condition for mycorrhizal species. Non-inoculated plants moved to field conditions of the waste die within a few days or weeks. The inoculum used in this experiment originated either from the xerothermic grasslands or from industrial wastes. Plants colonized by mycorrhizal fungi established well on the experimental plots (Turnau et al. 2008). The results suggest that inocula from xerothermic grasslands are well suited for improving plant growth on industrial wastes. Although in several cases the photosynthetic activity of plants from the waste was lower than in plants growing at natural sites, almost all plants survived and produced seeds. In all experiments the plant vitality was estimated on the basis of chlorophyll *a* fluorescence. This method was useful to show differences between the various waste substrata, different inocula and coexisting plant species. The interactions between mycorrhizal and non-mycorrhizal plants were studied under greenhouse conditions and at least no negative effect of this coexistence was observed (Turnau et al. 2008). The next step of this experiment was to check whether the plants growing on industrial wastes are accumulating heavy metals that would create a health risk for potential grazers. This was done using shoots of plants that survived already the third vegetation season, using Total Reflection X-ray Fluorescence (TXRF). The data were compared to plants that were collected in xerothermic grasslands (Turnau et al. unpublished). Among all introduced plants, three grass species (*Melica transsilvanica, Bromus inermis, Elymus hispidus*) and one legume (*Anthyllis vulneraria*) turned out to be the most suitable for phytostabilisation. The highest metal accumulation among all plants was found in *Verbascum thapsus*, one of few species that efficiently started to produce seeds that germinated successfully. Higher levels of heavy metals (Zn, Y, As, Pb, Cu) in plants grown on the waste were usually accompanied by higher Ca, suggesting a possible role of this element in detoxification mechanisms.

9   Attenuation of Stress by Mycorrhizal Fungi and Monitoring

The possible mechanisms involved are: (i) dilution of HM concentration by increased growth (Liu et al. 2005); (ii) barrier effect of mycorrhizal fungi; (iii) metal sequestration within fungal mycelium due to phytochelatin and/or melathionin

production; (iv) precipitation of metals on the surface of extraradical mycelium; (v) altering the metabolism e.g. by increased production of proline in stress conditions (Bassi and Sharma 1993; Costa and Morel 1994; Schat and Vooijs 1997; Chen and Dickman 2005) that was shown to be a potent scavenger of ROS. As suggested by Rodriguez and Redman (2005), symbiotic fungi could potentially activate the biosynthesis of proline. None of these mechanisms were so far proven with molecular techniques. Recently, the molecular responses of AM plants to Cd at proteomic level were approached by Aloui et al. (2009). Down-regulation of several Cd stress responsive proteins was found. Out of 26 mycorrhiza-related proteins, only six displayed differences following Cd application and as suggested by the authors "a part of symbiotic program may be recruited to counteract Cd toxicity through the mycorrhiza dependent synthesis of proteins having functions putatively involved in alleviating oxidative damages". Fester and Hause (2005) suggested that increased levels of antioxidant enzymes and nonenzymatic antioxidants found in mycorrhizal plants is probably linked to arbuscule senescence, and this may protect against oxidative damage resulting from Cd presence. The effect of AMF can be further improved by introduction of organic matter into the nutrient-poor soil and saprobic organisms (Azcón et al. 2009a, b).

10   Use of Photosynthesis to Show Adaptation of the Plant to Survive

The cost of maintenance of AM symbiosis, similarly to symbiosis of legume plants with rhizobia, can be as high as 16% of photosynthetic carbon. As recently calculated by Kaschuk et al. (2009), rhizobia and AMF improve photosynthesis by 28% and 14% respectively and by 51% if they act together and the rate of photosynthesis increases more than the C cost of these symbioses. The authors proposed that the sink stimulation represent an adaptation mechanism that allow the plant to take advantage of the microsymbionts. This is the result of removing the limitation of rubisco activity and electron transport rates through increased leaf N and P mass fraction and removal of the triose-P utilization limitation of photosynthesis (literature cited in Kaschuk et al. 2009). Microsymbionts can become even more important under heavy metal stress, when several physiological processes such as plant growth, photosynthesis and finally the yield are reduced (Jamal et al. 2006). Excess metals affects the absorption of nutrients (Barbosa et al 2007), reduce pigment content, alter chloroplast morphology. For monitoring plants on the industrial wastes, both under field and laboratory conditions, a fast, simple and non-invasive method to measure plant vitality is needed. This can be met by the Handy PEA and analyses of the OJIP chlorophyll *a* fluorescence transients (Strasser et al. 2004), suitable also for screening the beneficial role of symbiosis (Tsimilli-Michael et al. 2000; Tsimilli-Michael and Strasser 2008; Zubek et al. 2009).

11 Use of Hyperaccumulators in Phytoremediation of Areas Surrounding Industrial Tailings

The discovery of hyperaccumulating plants lead to the use of plants in remediation and to establishment of the phytoremediation as a new technique. Presently, over 400 species are known to be able to accumulate heavy metals at levels several times higher than non-accumulators (Baker et al. 2000; Verbruggen et al. 2009). It is a convenient and economically feasible technique to extract precious metals such as nickel; However, the use of hyperaccumulators in places such as industrial wastes, where the content of metals is very high would take a very long time. The options and possibilities of phytoextraction have been recently reviewed by Ernst (2005). Microbes such as mycorrhizal fungi were also found to be useful in phytoextraction of nickel (Turnau and Mesjasz-Przybylowicz 2003; Wu et al. 2007; Lopes de Andrade et al. 2008).

12 Conclusions

Phytoremediation of heavy metal contaminated areas is a very complex aim and it cannot be reached only by using a single plant species (what is often the case of large industrial wastes). To stimulate and to build a sustainable plant community, a selected array of various plant species is needed that can stimulate the wide range of soil biota. Industrial wastes are often limited by insufficient microbial populations (Haferburg and Kothe 2007). As shown above there are many possibilities to select appropriate strains of fungi, however, for each particular waste it seems to be necessary to optimize both, AMF and plant species/varieties, clones to create sustainable plant communities. There are still many additional possibilities to improve plant growth by adding inoculation with N-fixing bacteria, actinomycetes, cyanobacteria or shoot endophytes as recently reviewed by Lafferty Doty (2008). More work is therefore needed on plant and microbe selection, optimization of irrigation and amendments, evaluation of root capability and estimation of the risk connected with various ranges of heavy metal uptake into the shoots. More care should be also paid to mixed application of herbs and trees. While trees could influence water uptake from deeper layers of substratum, herbs building abundant AMF could respond by higher substratum aggregation (Hallett et al. 2009).

Phytostabilization should be the way to protect the wastes from erosion and in the meantime further technologies concerning the recovery of metals from solids should be developed, as reviewed by Krebs et al. (1997).

Optimizing practical applications of phytoremediation may result not only in stabilization of the ground but can also create an interesting area that supports the survival of rare and interesting plants. Still, this kind of depositions should be always carefully monitored and should never be considered as safe and stable.

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Part V
**Evolutionary and Diversity Perspectives
of Mycorrhizal Association**

Chapter 13
Effect of Differences Among Crop Species and Cultivars on the Arbuscular Mycorrhizal Symbiosis

Victoria Estaún, Cinta Calvet, and Amèlia Camprubí

Abstract The persistence of the arbuscular mycorrhizal symbiosis, is maintained in crop plants after, in many cases, decades of breeding new varieties with no consideration for the symbiosis presence or role. Differences in the responsiveness and dependence of diverse genotypes of legumes, cereals and tree crops to the symbiosis have been found, however no clear pattern for the variation has been shown. Facultative mycotrophs might have been selected in crop species because they would have a competitive advantage, being able to uptake phosphorus as efficiently whatever the mycorrhizal status of the field was. The use of field trials might have preserved the symbiotic capacity in the varieties obtained up till now. Plant–host metabolic and genomic findings should be taken into consideration in the new breeding technologies to work towards a holistic approach to breeding crop cultivars that makes full use of the arbuscular mycorrhizal symbiosis.

Keywords Mycorrhizal dependency • Legumes • Cereals • Fruit trees • *Citrus* • *Prunus* • Olive trees • Breeding • Genotype

Abbreviations

AMF   Arbuscular Mycorrhizal Fungi
MD   Mycorrhizal Dependency
GM   Genetically modified

Arbuscular mycorrhiza fungi (AMF) are by far the most important root-fungus association found in plants, they occur in nearly all agricultural and natural soils, colonising roots of many plant species (Smith and Read 1997).

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Although the most commonly found response to the AMF colonisation is an increase in plant growth demonstrated in many different plants and environments for the past 50 years thus giving credit to the term symbiosis when referring to this association, a wide variation on the response of different plants have been shown. The interaction between AMF and the plant host goes from a beneficial relation, where AMF plants improve their growth and reproduction success to a parasitism where colonised plants show a reduction in growth (Mojo and Hendrix 1986) due to the carbon drain imposed by the fungus (Fitter 2006). In order to determine the significance of the relationship between host plant and fungus the parameter Mycorrhizal Dependency (MD) was proposed by Gerdemann in 1975, and defined as the degree to which a plant species is dependent on the mycorrhizal association to produce its maximum growth or yield at a given level of soil fertility. The knowledge of the MD of a host species should be critical for predicting the host response to AM inoculations at a particular fertility level. However the mycorrhizal dependency of a given plant will be not only determined by the plant species (host) but also by the fungal partner and the environment. Due to all these parameters it is difficult to determine similarities, differences and variations on the behaviour of different plant species and even cultivars with regard to the mycorrhizal symbiosis. In this chapter we will attempt a survey of the knowledge, however fragmentary and incomplete, that we have on the response of field crop varieties to mycorrhiza, with a special emphasis on legumes, cereals, and tree crops.

1 Legume Crops and Mycorrhiza

When assessing natural habitats a lot of work has been done on natural grassland and prairie systems (Hartnett et al. 1993; Wilson et al. 2001; Bever et al. 2003) and it seems now clear that when considering legumes versus grasses, legumes are more dependent on the mycorrhizal symbiosis (Wilson and Hartnett 1998) and therefore respond better to the inoculation when attempting to restore a degraded ecosystem (Estaún et al. 2007). Wild legume species are closely related to many of the most important staple crops in the world, bean, soyabean and other pulses such as lentils, peas and chickpeas. All these crops have been found mycorrhizal (Bala and Singh 1985; Ortas 2003). Common bean (*Phaseolus vulgaris*) originated in America and cultivars can be grouped according to their origin, Mesoamerican or small navy beans and Andean, kidney or cranberry beans. Common beans are now extensively cultivated and are adapted to a wide range of environmental conditions resulting in the evolution of an extensive genetic variation for nutrient efficiency. Hacisalihoglu et al. (2005) found a wide variation in the responsiveness of bean cultivars from very responsive to no response, the cultivars that were more responsive to AMF were Calima (Andean origin) and DOR 364 (Mesoamerican origin) thus no evident correlation between responsiveness and origin of the bean was found. Estaún et al. (1987) working with peas found that there were differences in AMF root colonisation and growth response even between cultivars of the same parentage.

Kleikamp and Joergensen (2006) working with two iso-lines, one of them nonmycorrhizal and non-nodulating, found that mycorrhiza increased plant growth and harvest index, although the percentage increase over the non-symbiotic line depended on the field experiment site. The use of non-symbiotic pea mutants has been a tool used to study the genes involved in the symbiosis establishment and functioning (Duc et al. 1989; Marsch and Schultze 2001; Morandi et al. 2002). Mutations therefore can enhance or hinder the establishment and/or the efficacy of the symbiosis. This fact has been of concern because transgenic crops are widely spread and little is known on their interaction with the AMF. Both soya bean and alfalfa are two legume crops that have been genetically modified (GM) to incorporate and express a bacterial variant of a gene (cp4-epsps) encoding 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS). The herbicide Glyphosate acts inhibiting the wild type EPSPS activity, but not that of the cp4-epsps variant, thus allowing for easy chemical weed control of the transgenic crop. As EPSPS is involved in the synthesis of phenolic compounds and phenolic compounds have been associated in the signalling, infection and establishment stages of both AMF and rhizobial symbiosis, it was a concern that this pathway or the resulting compounds could be altered in the GM cultivars and as a result the AMF/rhizobial symbiosis could be affected. Powell et al. (2007) addressed this issue in soya bean and compared six transgenic mutants expressing the cp4-epsps gene, and three non GM cultivars expressing the wild type gene, the cultivars selection was done including parental cultivars of the GM cultivars so the comparison among the cultiva**r**s was done basically for the absence or presence of the transgenic gene cp4-epsps. The results showed that although there were differences among cultivars these did not relate to the absence or presence of the transgene. Meghvansi et al. (2008) found, also in soyabean, that although there were differences in the response to AMF of 4 genotypes studied, the combination of a particular strain of *Bradyrhyzobium japonicum* with a *Glomus intraradices* isolated from a cultivated soil, gave better results in all genotypes, than other combinations with different AMF. *Glomus intraradices* type fungi are often isolated from cultivated soils and they result, frequently, in positive plant growth effects, highlighting the importance of the identity of the fungal partner as differences in the ensuing efficacy have been found. The interaction between N_2 fixing rhizobia and mycorrhiza has been recently reviewed (Saxena et al. 2002). In many instances the interaction between the two microorganisms is positive and even synergic; however there are examples where an incompatibility has been shown between some strains of rhizobia and some AM fungal partners. Xavier and Germida (2002) found how different combinations of rhizobia strains and isolates of *Glomus mosseae* and *Glomus clarum* gave very different results on lentil plants growth, underlining again the importance of no oversimplifying the data because different fungi can and do produce entirely different results. Jemo et al. (2006) studied the interactions between P uptake, AMF colonisation and plant growth of 4 different genotypes in cowpea (*Vigna unguiculata*). Differences among the genotypes in growth, N_2 fixation and P acquisition were observed under low soil P conditions. Two of the studied genotypes, efficient at P uptake, differed in the mechanisms: one genotype responded efficiently to AMF colonisation while in the other genotype root morphological and

physiological characteristics were more important at increasing P-uptake. Similarly Shoeneberger et al. (1989) attributed to differences in the root system architecture the higher dependency of clover (*Trifolium subterraneum*) to the inoculation with *Gigaspora margarita* compared to *Lotus pedunculatus* in an acidic forest soil. The most sensitive indicator of plant response differences to mycorrhizal colonisation in white clover (*Trifolium repens*) was found to be the host plant root morphology, with a strong effect of the parental lineage of the 43 near-isogenic lines inoculated with *Glomus mosseae* (Eason et al. 2001).

Within the last decade *Medicago truncatula* has become an important model plant for legumes (Cook 1999), and, as expected, the AMF symbiosis has received a lot of attention, attempting to unravel specific alterations due to the establishment of the symbiosis, focusing mainly on differential gene expression and protein profiles (Franken and Requena 2001; Liu et al. 2007; Massoumou et al. 2007). This approach has lead to the identification of 568 genes differentially regulated in *Medicago truncatula* roots colonised with AMF (Grunwald et al. 2004). These genes are induced during the establishment of both endosymbiosis, rhizobia and endomycorrhizal (Manthey et al. 2004). Sinvany et al. (2002) showed how a tobacco homologue of the gene enod40 induced in *M. truncatula* by mycorrhizal root colonisation is expressed in tobacco mycorrhizal roots. As the knowledge in the gene and protein expression and the metabolic alterations of mycorrhizal plants compared to non-mycorrhizal plants are unravelled we shall be able to fine tune the symbiosis to our advantage. But now, we need to evaluate the efficacy of the symbiosis in different plant-systems to prepare for a predictable increase in fertiliser prices and thus a higher pressure in the plants nutrient efficiency.

2 Cereal Crops

Grasslands are important communities widespread from the arctic to the tropic. The domestication of some of these grasses, cultivated for the edible components of their seeds started about 10,000 years ago, and has resulted in very important food crops: the cereals. Maize, wheat, barley and rice, are the most widespread cereal crops and between them, accounted for 87% of all grain production, worldwide, and 43% of all food calories in 2004 (FAOSTAT 2006). Most of the grasses are divided into two physiological groups, using the C3 and C4 photosynthetic pathways for carbon fixation. It has been shown that C3 grasses are less dependent than C4 grasses to mycorrhizal colonisation, among the three most widespread cereal crops only corn belongs to the C4 physiological group while wheat, barley and rice are C3 grasses. Wheat cultivars shown to be non-susceptible to AM colonisation (Azcon and Ocampo 1981) established the symbiosis after a change in the inoculation procedure (Vierheilig and Ocampo 1991). From all the literature published, most wheat cultivars can establish the AM symbiosis, whether the symbiosis has an effect on plant growth is another issue. Hetrick et al. (1993) in a survey of wheat land races found that old accessions (before 1910) where more responsive to the AMF symbiosis than

new accessions. Kirk et al. (2008) studying wheat tried ten different cultivars: five old (released from 1886 to 1969) and five modern cultivars (released from 1991 to 2006). They found no differences in colonisation or yield that could be associated to the year of release of the cultivars, although there was a trend with a decrease in root colonisation from the most recent cultivars to the oldest cultivars in the field with higher phosphate. This indicates that the modern cultivars retain the ability to form extensive symbiosis in the high input conditions that are prevalent in Europe and North America. Comparing wheat and barley Baon et al. (1992) found that although there were no differences in root colonisation of the cereal crops grown in the same soil, AMF root colonisation was positively correlated with efficiency of P uptake in barley but not in wheat. Efficiency of P uptake is one of the parameters that Sawers et al. (2008) propose to evaluate the responsiveness of cereal crops to AMF. In their paper they divide responsiveness in dependence and non-dependence effects. Dependent effects would relate to the inability of a certain host or cultivar to grow without the symbiosis under certain environmental constrains (low P level, high salinity), while non-dependent factors would relate to variations in the interaction plant-fungus, i.e. variation of the efficiency of nutrient uptake between AM plants and non AM plants. If the classic studies of Hetrick et al. (1993, 1995) are interpreted under this new light, the differences observed between old and new lines of wheat cultivars are largely determined by the ability of the new lines to take up phosphate without AMF, therefore the new lines present a reduction in dependence but they are still responsive and compatible with the symbiosis. Similarly Zhu et al. (2001) compared two old and four modern wheat cultivars response to the inoculation with *G. intraradices* in pots in a growing chamber and found a decrease in shoot growth for all cultivars when inoculated with the fungus, although all plants showed an increased phosphorus uptake. The authors suggest that the decrease in growth was partly due to low irradiance in the growth chamber and also partly to the pot size used in the experiments that limited the soil volume available for exploration to the fungal mycelia. The fact that all cultivars showed an increased uptake in P, when colonised by *G. intraradices*, indicates that the cultivars studied were responsive to the AMF colonisation, and in this study the mycorrhizal responsiveness decreased with the year of release of the cultivar, thus supporting the theory that modern breeding techniques might decrease the responsiveness of the new cultivars to the symbiosis. Zhu et al. (2003) compared an old barley land race originated from Africa ('Sahara') with a modern improved cultivar ('Clipper') and found that the plant rooting characteristics might have a determinant effect on the response to the symbiosis, the root/shoot ratio and the P allocation ratios were higher for the 'Sahara' cultivar, characteristic of a species adapted to low nutrient soils (Marschner 1995). Mycorrhizal phosphorus responsiveness increased with the pot size for the old cultivar and was not affected in the modern cultivar, probably because of the smaller and finer root system of the later. There were no differences in plant growth attributable to the symbiosis, however there was a decrease in the root/shoot ratio and the P concentration was higher in all mycorrhizal plants. The lack of growth effect indicates that the carbon allocation to root growth for the non-mycorrhizal plants or to the AMF symbiosis development was equal but was more economical for the AMF

plants when considering P uptake. The results of the experiments on wheat and barley described here show that for these C3 cereal crops, the artificial selection pressure inherent to breeding has acted mostly on the dependence factors of the AMF responsiveness, based on plant characteristics alone, reducing their weight, but has little affected other responsiveness factors based on the plant fungus interaction.

Rice, although also a C3 cereal crop, is in a completely different situation. Rice, a very important food crop in the tropics, has received little attention in mycorrhiza research up until now; this is probably because plants growing in marshy environments, like the semi-aquatic rice, were previously considered to be nonmycorrhizal (Bagyaraj et al. 1979). Later studies showed the occurrence of arbuscular mycorrhizal fungi in many aquatic plants, including those which are completely submerged (Chaubal et al. 1982; Clayton and Bagyaray 1984). The situation of rice in mycorrhiza research has however changed in the last 5 years, because it is the only mycorrhizal plant that has had its genome completely sequenced and has whole-genome profiling tools available. Therefore, much work is being done at the moment on the gene expression and transcriptome analysis of mycorrhizal rice (Güimil et al. 2005). Despite all these molecular analysis little is known about the responsiveness of the different rice cultivars to mycorrhizal colonisation. There are several reports of AMF inoculations in wetland rice culture under greenhouse conditions, but very little information on responses under field conditions, and even less comparing wetland and upland rice varieties. Some studies under pot culture conditions revealed that AMF increased grain and straw yields of wetland rice (Sivaprasad et al. 1990) and increased the grain yield and P and Zn content in plants (Secilia and Bagyaraj 1994). Gupta and Ali (1993) reported a significant increase in the grain yield by AMF colonisation in wetland rice under both pot and field conditions. Gao et al. (2007) have studied 6 different upland rice varieties inoculated with two different fungi (*Glomus mosseae* and *Glomus etunicatum*). They found that all varieties except one showed a growth increase with *G. mosseae* inoculation and that *G. etunicatum* was effective at increasing plant growth in four varieties. When analysing Zn uptake, *G. mosseae* increased Zn uptake in all studied varieties whilst *G. etunicatum* was effective in only four. There was not a relationship between increased growth and increased Zn uptake. This data indicate that in rice the selective pressure of breeding is also acting differently on dependent and non-dependent responsiveness factors. Because rice is a model plant it should be easier to attempt to determine which genes might be involved in the selection of these traits.

Corn (*Zea mays*) was domesticated in America, probably in Mexico, and is now grown in virtually all countries under very different environments. It is a C4 plant therefore originally adapted to high light radiance and warm temperatures, although plant breeding and selection have produced genotypes that can be grown under many different situations. It has been a concern that breeding for high input systems could have selected for non mycorrhizal genotypes, and therefore it may be difficult to select cultivars adapted to low input, organic agriculture from this core germplasm pool. Kaeppler et al. (2000) studied 28 inbred maize lines developed for the USA Corn Belt and found that all lines were able to establish the symbiosis, and all benefited from the AMF when grown at low soil P content. The extent of the

response to the symbiosis at low P soil contents, measured as shoot weight, varied from 106% to 819%, showing a substantial variation among genotypes for AMF responsiveness. The addition of high doses of fertilizer doubled the weight of the plants grown at low P level with AMF. High P level and mycorrhizal inoculation produced overall a slight decrease in shoot growth, although the symbiosis was established. Wright et al. (2005) analysed the response to P fertilisation and AMF colonisation of two maize cultivars: one, 'River', used widely in high input systems in Europe, the other, 'H511', in subsistence agricultural systems in Africa. Both cultivars were extensively colonised by *G. intraradices* at all the P levels studied. There were expected differences as the higher root/shoot ratio of H511, characteristic of plants adapted to grow in low nutrient environments. Other results were unexpected as the strong growth promotion by AMF of the high input cultivar 'River' and a higher dependency on the mycorrhizal symbiosis at low and intermediate P levels when compared to the African cultivar. At high P levels both cultivars grew well and established the symbiosis although there was no benefit measured in plant growth. However when analysing P content and specific P uptake mycorrhizal plants were always better than non-mycorrhizal plants, despite differences between genotypes. From these results the high input system cultivar is more responsive to the AMF symbiosis, than the African cultivar, indicating that the gene pool for AMF response is not selected out in high input breeding programs. These studies however have been done in controlled conditions, in containers, where the limited soil volume can have a determining effect on the symbiosis result. An et al. (2009) studied the root colonisation of 255 maize genotypes, including inbred lines, hybrids and landraces originated from different locations (Canada, USA and Japan) that were grown in a field for two consecutive years. The results show a significant correlation between country of origin and root colonisation, however all the genotypes assayed established the colonisation.

When assessing the variation of the mycorrhizal symbiosis in both legume and cereal crops we are evaluating some of the older plants domesticated for food by humans. The selection of these plants has been mostly done in open field trials, where mycorrhiza were present, therefore it is to be expected that the trait for mycorrhization has not been lost, in fact in some of the experimental data show that new varieties are still making full use of this symbiosis and increasing, among other desirable aspects, the P uptake efficiency ability. The knowledge of the existing genetic variation of different cultivars response to the mycorrhiza symbiosis is a powerful breeding resource that can allow the selection of desirable traits for the optimisation of this symbiosis for agricultural use.

3   Tree Crops

Horticultural crops in general and tree crops in particular have received comparatively less attention when assessing the responsiveness of different genotypes to the symbiosis.

   Olive trees are among the older tree crops domesticated (Warnock 2007) and many different genotypes have developed around the Mediterranean in the traditional growing areas where the wild olive (*Olea europaea* subsp. *sylvestris*) was first planted as a crop, about 6,000 years ago, originating a wide array of locally selected cultivars (Erre et al 2009).

   The interest in olive trees has rekindled during the last decade of the XXth century because of health benefits associated to its use and new plantations have been established, in new areas where olives had never been grown before, such as California (USA), Argentina, Chile, Australia and even in areas of Brazil. Consequently to this interest land races/cultivars have been studied to assess different characteristics, from the agronomy of the crop to the oil quality. Olive trees are able to grow under very harsh environment conditions, and as the pressure to use marginal or low-quality lands for crop production increases, the use of olive trees is reassessed, not only for crop production but also for non-productive purposes like landscaping, and control of soil erosion, for which they are a good alternative to herbaceous crops as they provide a full-year and permanent biomass cover (Barranco and Rallo 2000). Olive trees form the symbiosis (Roldán-Fajardo and Barea 1985), and a differential growth response of several olive cultivars to the inoculation with AMF has been shown (Vitagliano and Citernesi 1999; Calvente et al. 2004; Estaún et al. 2009), where the responsiveness to mycorrhizal inoculation has been found to depend on both the AMF species and the plant genotype (Fig. 1). Olive nursery trees are reproduced from leafy cuttings, which are rooted in a soilless substrate and then transplanted to the field. Prior inoculation, either at the propagation stage (Binet et al. 2007) or at the nursery/hardening stage might prove essential if the plants are to be established in a soil with low nutrient conditions and low AMF propagule levels (Estaún et al. 2003).

Fig. 1 Differential effects of AM fungi, *Glomus mosseae* and *Glomus intraradices* in commercial olive cultivars: 'Picual' and 'Arbequina'

Although grapevines are also old crops and as such many different cultivars have evolved, vineyards in Europe and North America are established using *Vitis vinifera* cultivars grafted on rootstocks resistant to the phylloxera pest, developed since 1890. These rootstocks are basically combinations of *Vitis rupestris*, *Vitis berlandieri* and *Vitis riparia*, american grapevines resistant/tolerant to the insect. Therefore however old vine growing might be, rootstocks are relatively new and, unlike the situation with olive trees, where almost every town in the Mediterranean has its own special cultivar, rootstocks used for vineyards are essentially the same, globally. Schreiner (2003) surveyed the mycorrhizal root colonisation of 10 different rootstocks in an experimental vineyard and found that all rootstocks established the symbiosis with the AMF existing in the soil.

Nogales et al. (2008), in greenhouse conditions, showed that rootstock and fungus genotype modulated the response of the grapevine to the symbiosis, although the response was always an increase in growth (Fig. 2).

The parental heritage of the rootstocks does not seem to relate to the root colonisation or to the mycorrhizal responsiveness of the plants evaluated in the different studies (Schreiner 2003). The factors governing mycorrhiza responsiveness seem to be well preserved within the *Vitis* genus, and the ultimate level of responsiveness might be modulated by the scion identity and by the whole plant source-sink relationships (Schreiner 2007). In many wine growing areas vineyards are replanted and in this situation there can be a stunting growth or decreased survival in the new plants due to both biotic and abiotic constrains. Calvet et al. (2007) and Camprubí et al. (2008) showed that mycorrhization of plants can address these conditions whether due to abiotic factors such as highly calcareous soil or biotic constrains like the root rot fungus *Armillaria mellea*. Grapevine plants can be inoculated at the nursery stage
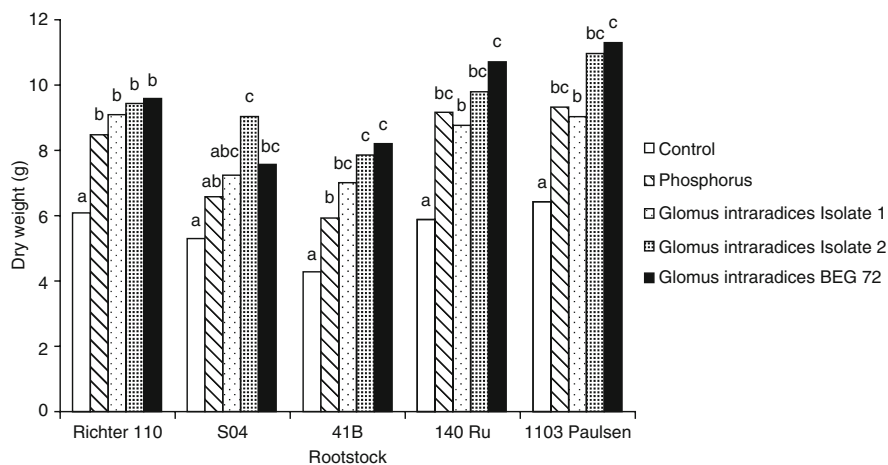
Fig. 2 Effects of inoculation with different isolates of *Glomus intraradices* on the growth of grapevine rootstocks (Nogales et al. 2008)

(Aguin et al. 2004; Krishna et al. 2005), integrated in the production system, assuring the quality of the plantlet and helping its establishment in problem areas.

The *Citrus* group of plants is extremely numerous, a pool of complex genetic diversity, evolved mostly in the last three centuries. Most citrus trees establish the AM symbiosis (Reed and Fremont 1935). *Citrus* are considered highly dependent on AM fungi (Menge et al. 1978; Nemec 1979; Levy et al. 1983; Ishii and Kadoya 1994). *Citrus* crop trees, are grafted on selected rootstocks to improve disease tolerance, mainly to the tristeza virus, or tolerance to soil constrains like lime, salt or clay. Studies and surveys done at different times and in different growing areas (Menge et al. 1978; Graham and Syversten 1985; Camprubí and Calvet 1996; Youpensuk et al. 2009) on the variation of the AMF responsiveness of different rootstocks have achieved coincident results (Table 1). In all the surveys cultivars of C*itrus aurantium* were highly mycorrhiza dependent, followed by Cleopatra mandarin, at the low range there was the sweet orange (C*itrus sinensis*) and as virtually non dependent the trifoliate orange (*Poncirus trifoliata*) and its hybrid the Citrange. Menge et al. (1978) reported a correlation between the plant's ability to take up

Table 1 *Citrus* rootstocks ranked according to their AM dependence

Rootstock name	Genera species	Dependence to AMF	Reference
Rough lemon	*C. jambhiri*	Very high	Menge et al. (1978)
Lime	*C. aurantifolia*	Very high	Youpensuk et al. (2009)
Sour orange	*C. aurantium*	High	Menge et al. (1978)
			Graham and Syversten (1985)
			Camprubí and Calvet (1996)
Ocean (Tangerine)	*C. reticulata*	High	Youpensuk et al. (2009)
Fremon (Tangerine)	*C. reticulata*	High	Youpensuk et al. (2009)
Pomelo/grapefruit	*C. maxima*	High	Youpensuk et al. (2009)
Sainamphung (Tangerine)	*C. reticulata*	Medium	Youpensuk et al. (2009)
Alemow	*C. macrophylla*	Medium	Menge et al. (1978)
Cleopatra (mandarine)	*C. reshnii*	Medium	Graham and Syversten (1985)
			Camprubí and Calvet (1996)
			Youpensuk et al. (2009)
Sweet orange	*C. sinensis*	Low	Menge et al. (1978)
			Youpensuk et al. (2009)
Swingle citrumelo	*Citrumelo*	Low	Graham and Syversten (1985)
			Camprubí and Calvet (1996)
Carrizo (citrange)	*Poncirus trifoliata x C. sinensis*	Low	Graham and Syversten (1985)
Troyer (citrange)	*Poncirus trifoliata x C. sinensis*	Very low	Menge et al. (1978)
			Camprubí and Calvet (1996)
			Youpensuk et al. (2009)
Trifoliate orange	*Poncirus trifoliata*	Very low	Graham and Syversten (1985)

phosphorus and the dependence to the mycorrhizal symbiosis. Graham and Syversten (1985) and Youpensuk et al. (2009) found that the non-dependent rootstocks, like citrange, sweet orange and the trifoliate orange, when establishing the symbiosis, had a higher leaf P content than non mycorrhizal plants. This illustrates a positive response of the non-dependent cultivars to the AM symbiosis, favouring P uptake, the increased nutrient uptake will have an effect on the scion performance. As a result this trait, linked to a positive response to the symbiosis, has not been lost in the breeding effort.

Other temperate fruit trees, like soft and stone fruit trees, establish the AM symbiosis, and measurable growth responses have been reported in peach (Gilmore 1971), peach-almond hybrids (Estaún et al. 1994) and apple (Granger et al. 1983). Mycorrhizal plants of this group have been shown to benefit from the symbiosis under physiological stress such as nutrient deficient soils (Lindermann 1988), drought conditions (Nelson 1987; Estaún et al. 1997), salinity (Pfeiffer and Bloss 1988) or when plants are attacked by root diseases like the rootknot nematode *Meloidogyne javanica* (Hernández-Dorrego et al. 1998), the lesion nematode *Pratylenchus vulnus* (Calvet et al. 1995) and the root rot fungi *Rosselinia necatrix* and *Armillaria mellea* (Calvet et al. 2006). The *Prunus* family, with around 430 species, is spread throughout the northern temperate regions of the globe and includes several species producing edible drupes (plums, cherries, peaches, apricots and almonds), with a large economic impact. Consequently this group has been the subject of an intense effort in the breeding of new varieties and new rootstocks.

Despite the beneficial effects of the mycorrhizal symbiosis shown in several *Prunus* species there is not much information on the mycorrhizal dependency of the most commonly used rootstocks (Schubert et al. 1996; Monticelli et al. 2000), and information on new released rootstocks is either inexistent or unavailable. Calvet et al. (2004) evaluated 18 different *Prunus* rootstocks for the aptitude to form the AM symbiosis and the compatibility with three different fungi: *G. etunicatum*, *G. mosseae* and *G. intraradices*, colonisation with both *G. etunicatum* and *G. mosseae* was low, and in some rootstocks almost nil. These results confirm those of Monticelli et al. (2000) that evaluated the effect of AMF inoculation on three micropropagated *Prunus* rootstocks using *G. mosseae* and *Scutellospora calospora* as the fungal partners, and found a low root colonisation with both fungi at this early stage of the plant development. Calvet et al. (2004) found that *G. intraradices* was by far the most infective fungus, colonising significantly more root length than any of the other fungi studied. In the same study a similar colonisation response was found for a group of rootstocks belonging to the *Prunus insititia* group that became heavily colonised when inoculated with *G. intraradices*, whilst the root colonisation of a wider subgroup including peach-almond hybrids and peaches fluctuated between 10% and 65%. The selections evaluated by Calvet et al. (2004) of *P. insititia* are fairly new, their genetic manipulation has been limited as compared to other European, Japanese and American plums, and the trait for establishing the symbiosis has been well preserved. Early mycorrhizal inoculation can be of special interest to nurseries producing both cuttings and micropropagated material that are usually free of mycorrhiza (Vestberg and Estaún 1994), the production of

mycorrhizal plants may represent an advantage when these young rootstocks are transplanted into the field where the symbiosis can help the plants to withstand stressful conditions (Estaún et al. 1999). However it is important to determine the best combination plant-fungus-substrate to succeed in the mycorrhization process (Estaún et al. 1994). Due to the economic importance of the *Prunus* family and together with a small and well characterised genome, an ambitious breeding program is being developed based in molecular markers development and gene transfer techniques, using in vitro evaluation of agronomic traits to overcome constrains due to a long juvenile period, large plant size and differences in trait expression between juvenile and mature trees characteristics of this genera (Martinez-Gomez et al. 2005). The proposed breeding tools have advantages but we must also be aware of the potential draw backs. Due to the lack of comprehensive field trials, where not only mycorrhiza but other microorganisms interacting with the plants are present, the selected plants might not be the best possible choice. Camprubí et al. (1993) evaluated the response of three plum rootstocks: Marianna (*Prunus cerasifera* x *Prunus munsoniana*), Myrabolan (*Prunus cerasifera*) and San Julian (*P. insititia*) for tolerance to the lesion nematode *Pratylenchus vulnus.* If the AM symbiosis was disregarded, the most tolerant rootstock was San Julian, but if the inoculation with AMF was taken into account the best performer was Marianna, explaining the fact that Marianna has been found tolerant to *P. vulnus* under field conditions in California (Culver et al. 1989).

4   Conclusions

The persistence of the AMF symbiosis, is maintained in crop plants after, in many cases, decades of breeding new varieties with no consideration for the symbiosis presence or role. Some cultivars have "evolved" from dependent, as defined by Janos (2007) and Sawers et al. (2008) to non-dependent or facultatively mycotrophic. Paradoxically new varieties bred in conditions of heavy P input have been found to be even more dependent on the symbiosis than older varieties, when grown in nutrient deficient soils. Smith et al. (2009) have recently reviewed the relevance of the "hidden P uptake" of mycorrhizal non-dependent plants via the extensive external hyphae of the AMF, and the specific transporters up-regulated at the establishment of the symbiosis (Paszkowski et al. 2002). From this standpoint facultative mycotrophs might have been selected in crop species because they would have a competitive advantage, being able to uptake phosphorus as efficiently whatever the mycorrhizal status of the field was. The use of field trials to test new varieties and the different functionalities of the symbiosis would have preserved the symbiotic capacity in the varieties obtained up till now. The use of novel methodologies, with marker assisted breeding, clonal propagation and in vitro testing of specific characteristics needs to be complemented with these new findings on metabolic and fungal-plant genomic interactions to achieve superior cultivars that can make full use of the advantages bestowed by the AM symbiosis. However the assessment of the plant performance on the field is also a capital step that should not be sided.

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Chapter 14
Mycorrhizal Symbiosis and Plant Reproduction

Roger T. Koide

Abstract Reproduction is an essential function of all organisms and, for many crop species, reproductive structures are the principle edible parts. Still, relatively little is known about the effects of the mycorrhizal symbiosis on host plant reproduction. Common limitations to reproduction include nutrient deficiency, herbivory and disease, and mycorrhizal fungi can influence each of these. Several aspects of sexual reproduction may be influenced by colonization of mycorrhizal fungi including the timing of reproductive events, the number of inflorescences per plant, the number of flowers per inflorescence, the amount of pollen per flower, the proportion of flowers producing fruits, and the number of seeds per fruit. Seed quality can also be strongly influenced by colonization of mycorrhizal fungi, resulting in variation in seedling vigor and resultant competitive ability. Because infection by mycorrhizal fungi can influence interactions among plants, it may lead to variation among individuals in their contributions to the next generation and, therefore, may control the genetic structures of populations and communities.

Keywords Community structure • Density • Disease • Fecundity • Fitness • Herbivory • Offspring quality • Pollen • Population structure

1 Introduction

Most plant biologists are aware that mycorrhizal symbioses are common, but some may not fully appreciate the many significant effects of the symbiosis on the most basic of all biological functions, which is reproduction. Many reviews have summarized the general effects of infection by mycorrhizal fungi on nutrient acquisition

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and vegetative growth (Abbott and Robson 1982; Abbott and Robson 1984, Cooper 1984; Gianinazzi-Pearson and Gianinazzi 1983; Hayman 1983; Koide 1991b; Read 1991; Smith and Gianinazzi-Pearson 1988; Gerdemann 1968; Bolan 1991; Read and Perez-Moreno 2003). Fewer have concerned themselves with other aspects of the symbiosis such as molecular and cellular interactions between host and fungus (Smith and Gianinazzi-Pearson 1988; Bonfante and Perotto 1995; Harrison 1999), the effects of the symbiosis on phytopathology (Newsham et al. 1995; Azcon-Aguilar and Barea 1996), or its influence on biological communities (Read 1991; Gehring and Whitham 1994). Fewer still have had much to say about the effects of mycorrhizas on plant reproduction. Yet reproductive structures (including flowers, fruits and seeds), rather than vegetative tissues, are the principle edible parts of many crop species. Moreover, reproduction determines fitness, and it is variation in fitness, not variation in nutrient uptake or size, upon which nature acts in the selection of new traits.

There are several types of mycorrhiza involving different fungal and host taxa (Smith and Read 1997). The effects of infection by mycorrhizal fungi on reproduction of ectomycorrhizal and ericaceous mycorrhizal host species are not often documented, probably because of their relatively slow-growing and long-lived nature. One exception is a report by Powell and Bates (1981) in which blueberry (*Vaccinium corymbosum*) fruit yields were increased by infection by mycorrhizal fungi. However, they did not determine the effect of mycorrhizal infection on reproduction over the entire lifespan of the blueberry. Annual plant species, for which the determination of lifetime reproduction is a relatively simple task, provide a useful model for the study of mycorrhizal effects on plant reproduction. Because arbuscular mycorrhizal (AM) fungi infect many annuals hosts, the vast majority of the literature on this subject concerns AM host species. Of necessity, therefore, this review emphasizes the AM symbiosis.

Plants reproduce either sexually or asexually. For species that exhibit asexual reproduction, the iteration of vegetative parts is frequently equivalent to reproduction. An exception is the apomictic production of seed. It is not surprising that when vegetative growth of strawberry was limited by phosphorus (P), infection by AM fungi increased stolon production (Khanizadeh et al. 1995). However, even when P availability is very high, infection by mycorrhizal fungi may still increase vegetative reproduction. For example, mycorrhizal infection increased tiller production in *Agropyron smithii* (Miller et al. 1987) and tuber production in *Solanum tuberosum* (Douds et al. 2007), possibly by altering host hormone balance (Miller et al. 1987). For most terrestrial plant species, however, it is sexual reproduction (seed production) that determines their evolutionary success. One might even argue that for such species vegetative growth is only necessary to provide the structure and resources necessary for seed production. In this review I concentrate on sexual reproduction.

For plants that are obligate mycotrophs, infection by mycorrhizal fungi is, by definition, essential for reproduction. Due to their achlorophyllous habit, plants such as *Monotropa*, *Pyrola*, or orchid protocorms rely on mycorrhizal fungi to supply the reduced carbon necessary for growth and are thus referred to as

mycoheterotrophic plants (Leake 1994). Even photosynthetic plants may be obligate or near-obligate mycotrophs. For example, nonmycorrhizal roots of *Hyacinthoides non-scripta* (Merryweather and Fitter 1996) or *Allium cepa* (Dodd et al. 1983) are apparently incapable of acquiring sufficient P to maintain life in soils of low P availability. However, I focus on the effects of infection by mycorrhizal fungi on reproduction of facultative mycotrophs because the majority of mycorrhizal plant species appear to fall into this category.

There are inherent morphological limitations to the maximum number of seeds that can be produced by an individual plant, but it is usually the supply of limiting resources, or the degree of herbivory or disease, that limit seed production (Hendrix 1988; Lee 1988). Thus, one can easily appreciate that factors affecting either the supply of limiting resources, or the degree of herbivory or disease, would have large effects on sexual reproduction. Infections by mycorrhizal fungi may alter nutrient uptake, disease resistance and herbivory and, hence, may influence seed production.

2   Mycorrhizal Effects Mediated by Enhanced Nutrient Uptake

At least in some cases, infection by mycorrhizal fungi does not result in a net increase in the uptake of nutrients due to a compensatory reduction in nutrient uptake by the root system itself (Smith et al. 2009). However, when mycorrhizal infection does increase the availability of a limiting resource or if it increases the allocation of limiting resources to reproductive structures, it can have a direct, positive impact on host reproduction. In cases where P limited vegetative growth, infection by mycorrhizal fungi increased reproduction in many plant species including *Abutilon theophrasti* (Koide et al. 1994; Stanley et al. 1993), wild and cultivated species of *Avena* (Koide et al. 1988b), wild and cultivated species of *Lycopersicon*, now *Solanum* (Bryla and Koide 1990), *Hordeum vulgare* (Clarke and Mosse 1981; Jensen 1982; Powell 1981), *Glycine max* (Vejsadova et al. 1993), *Triticum aestivum* (Karagiannidis and Hadjisavva-Zinoviadi 1998), *Kummerowia striata* (Nakatsubo 1997) and *Capsicum annuum* (Dodd et al. 1983).

A positive vegetative response to mycorrhizal infection will generally lead to a positive reproductive response. This relationship exists because of the inherent architectural constraints on flower production; more flowers generally require more branches, which contain more leaves and require more roots. However, the effects of infection by mycorrhizal fungi on vegetative growth may differ quantitatively from their effects on reproduction (Bryla and Koide 1990; Koide et al. 1988b; Stanley et al. 1993; Karagiannidis and Hadjisavva-Zinoviadi 1998; Nakatsubo 1997), both because the extent of P deficiency (the P deficit, Koide 1991b) for vegetative growth and reproduction may differ, and because some of the nutrient requirement for reproduction may be met by reallocation from vegetative structures. In any case, predictions of the magnitude of the mycorrhizal effect on reproduction may not be possible from an examination of vegetative growth alone.

The P deficit experienced by a plant is determined by the difference between the demand for P by the plant, and the supply of P to the root system (Koide 1991b). When infection by mycorrhizal fungi increases seed production by increasing P uptake, the maximum response is determined by the magnitude of the P deficit. Variation among host species in their P deficits for reproduction may thus lead to variation in reproductive response to infection by mycorrhizal fungi. For example, owing to its inherently higher root:shoot ratio, wild oats (*Avena fatua*) probably experience a lower P deficit than cultivated oats (*Avena sativa*) in a given soil. Not surprisingly, infection by mycorrhizal fungi increased seed production less in wild oats than in cultivated oats (Koide et al. 1988b).

Significant variation may also occur among species of mycorrhizal fungi in their ability to influence host plant seed production in *Vigna unguiculata* (Muthukumar and Udaiyan 2002), seed nutrient concentration and individual seed weight in *Glycine max* (Bethlenfalvay et al. 1997) and vegetative reproduction in *Prunella vulgaris* (Streitwolf-Engel et al. 2001). This may be due, at least partly, to variation among species of mycorrhizal fungi in their capacity to influence P uptake (Koide 2000).

2.1 Temporal Variation in Response to Infection by Mycorrhizal Fungi

Because both the P supply and the P demand may vary through time, it is not surprising to find variation in response to infection by mycorrhizal fungi as a plant develops. Fitter (1991) has explained that for much of the existence of a faculta- tively mycotrophic plant, demand for P may be quite low. Some plants may only occasionally require assistance from mycorrhizal fungi to meet their demand for P. Reproduction may be one such occasion for many species. A plant, therefore, may not need to maintain consistently high levels of infection by mycorrhizal fungi to benefit from it. For some species, fractional total infections (hyphae, vesicles and arbuscules) or fractional arbuscular infections exhibit maxima imme- diately prior to or during reproduction, such as for *Triticum aestivum* (Boswell et al. 1998; Dodd and Jeffries 1986), *Secale cereale* and *Hordeum vulgare* (Dodd and Jeffries 1986), and the positive effect of infection by mycorrhizal fungi on P uptake appears to correspond to those times (Dunne and Fitter 1989; McGonigle and Fitter 1988).

Because flowering in the weedy annual *Abutilon theophrasti* is indeterminate, the reproductive phase may account for 70% of the plant's life cycle (Sattin et al. 1992). This long reproductive period makes it possible to study temporal variation in reproductive response to mycorrhizal infection. Because infection by mycor- rhizal fungi is often greatest just before reproduction in annual plants, we hypoth- esized that the benefit to seed P content from infection by mycorrhizal fungi would be highest early in the reproductive phase. Indeed, we found that seeds from mycor- rhizal plants contained significantly more P than seeds from nonmycorrhizal plants, but only for seeds produced early in the reproductive phase (Fig. 1a).
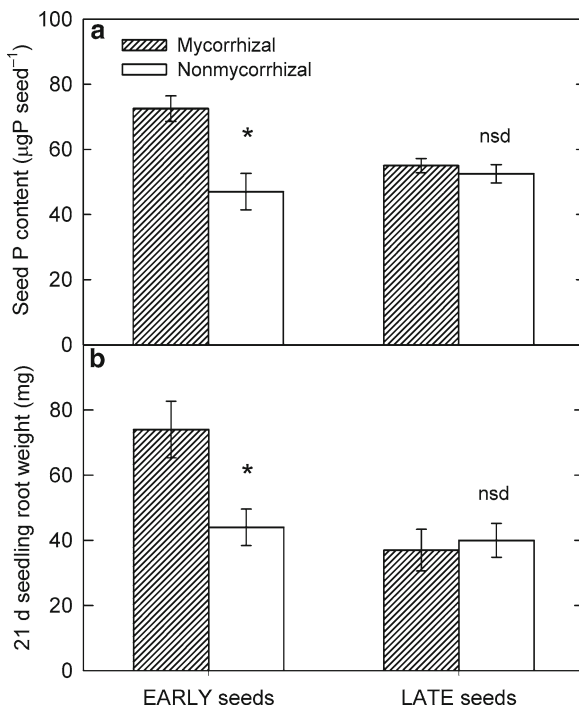
Fig. 1 (**a**) P contents of seeds produced by either mycorrhizal or nonmycorrhizal *Abutilon theophrasti* parents, and collected either early (16 August 1991) or later (1 October 1991) in the season. n = 5. (**b**) Weights of roots of resultant offspring grown for 21 days in a nutrient solution containing 0.5 mol m^{-3} KH$_2$PO$_4$ (n = 16). Error bars represent ±1 s.e.m. * = significantly (P ≤ 0.05) different, nsd = not significantly different (see Shumway and Koide (1994) for details)

   Of course, if infection by mycorrhizal fungi is to increase reproduction of an annual plant, the benefit (in terms of nutrient allocation) must be conferred before or during reproduction. This is not necessarily the case for perennials. For example, maximum arbuscular infection and P uptake in *Ranunculus adoneus*, occur after reproduction (Mullen and Schmidt 1993). A high demand for P occurs in the spring for this species, when vegetative parts are regrown, and when the soil is still too cold to permit adequate P uptake. The spring regrowth must therefore rely on P that was acquired after reproduction in the previous season.

   There is only a single reproductive season for annuals and semelparous perennials, making documentation of the net effect of infection by mycorrhizal fungi on reproduction relatively easy. Iteroparous perennials have potentially two or more seasons during which reproduction occurs. The effect of infection by mycorrhizal fungi on reproduction in one year may not be the same in the next because, among other things, nutrient availability may vary from year to year. Thus, one would need to consider the entire reproductive lifespan of the individual in order to determine the total mycorrhizal effect on plant fitness, a large task for most perennials.

2.2 The Components of Sexual Reproduction

Seed output is a function of one or more reproductive traits including the number of inflorescences per plant, the number of flowers per inflorescence, the proportion of flowers producing fruits, and the number of seeds per fruit. Seed production may also be function of pollen availability, and this is a function of both pollen production and the frequency of pollinator visitation. Pollinator visitation is frequently a function of the qualities of the floral display and the pollinator reward. Pollen production is determined by the number of inflorescences per plant, the number of flowers per inflorescence and pollen production per flower. Theoretically, variation in any of these components caused by infection by mycorrhizal fungi is capable of causing variation in seed output.

Infection by mycorrhizal fungi decreased the time taken to initiate flowering in *Abutilon theophrasti* when plants were grown in a P-deficient soil (Lu and Koide 1994). This was probably related to increased P uptake early in the growth of mycorrhizal plants because the onset of flowering is related to plant size or resource accumulation in many plant species (Schemske et al. 1978; Wyatt 1981). The decrease in time taken to initiate flowering resulted in a lengthening of the flowering duration and, thus, an increase in the number of flowers produced per plant (Fig. 2a).

The proportion of *Abutilon* flowers actually producing fruits also was significantly increased by infection by mycorrhizal fungi, resulting in significantly more fruits per plant compared to nonmycorrhizal plants. As was the case for flower number, however, the differences between mycorrhizal and nonmycorrhizal plants were diminished at higher P availabilities (Fig. 2b). In studies of soybean (*Glycine max*), Busse and Ellis (1985) and Schenck and Smith (1982) also showed that the proportion of flowers producing fruits was increased by infection by mycorrhizal fungi.

Flower production in *Lycopersicon* was primarily a function of the number of inflorescences produced because there was little variation in the number of flowers per inflorescence (Bryla and Koide 1990). This was similar to our results from *Avena sativa* for which the trait most responsive to infection by mycorrhizal fungi was panicle number (Koide et al. 1988b). In addition, for some genotypes of *Lycopersicon*, infection by mycorrhizal fungi had small positive effects on the proportion of flowers producing fruits and, less frequently, the number of seeds per fruit (Bryla and Koide 1990).

The number of seeds per fruit in *Abutilon* also was increased as a consequence of infection by mycorrhizal fungi and, again, this effect was reduced at higher P availabilities (Fig. 2c). The net result of the effects of infection by mycorrhizal fungi on flower number, fruit abortion and seeds per fruit was a 500% increase in seed production at low P availability and 12% increase at high P availability. The various components of reproduction were not equally affected by infection by mycorrhizal fungi, however. Averaging across all P treatments, infection by mycorrhizal fungi increased flower production by 64%, the proportion of flowers producing fruits by 24%, and the number of seeds per fruit by 16%.
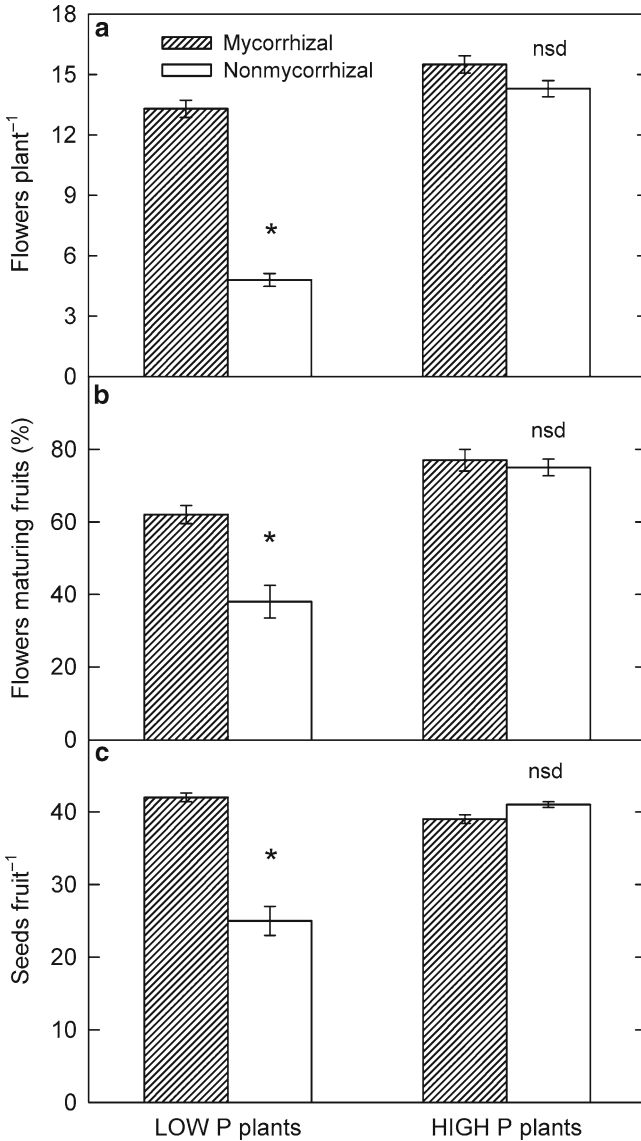
Fig. 2 Means of various reproductive traits of mycorrhizal and nonmycorrhizal *Abutilon theo-phrasti* plants growing in soil. n = 10. (**a**) Flowers per plant. (**b**) The percentage of flowers maturing fruits. (**c**) The number of seeds per fruit. Error bars represent ±1 s.e.m. * = significantly (P ≤ 0.05) different, nsd = not significantly different (see Lu and Koide (1994) for details)

As for *Abutilon*, mycorrhizal infection resulted in an earlier conversion from vegetative to reproductive growth in *Petunia* (Daft and Okusanya 1973). However, mycorrhizal infection may also prolong the vegetative period

(Nakatsubo 1997). The range of effects of mycorrhizal infection on flowering duration is shown in *Lycopersicon esculentum* genotypes (Bryla and Koide 1990). There was no significant effect on flowering duration or on the number of flowers produced per day in some genotypes (Fig. 3a). In others, infection shifted flowering to an earlier period without affecting the flowering duration or total flower production (Fig. 3b). In still others, infection decreased the time taken to initiate flowering, resulting in a protracted flowering period and greater flower production (Fig. 3c). Finally, infection by mycorrhizal fungi of some genotypes had no significant effect on flowering duration, but it still significantly increased the number of flowers produced per day (Fig. 3d), resulting in greater total flower production.

Relatively little is known about the effects of infection by mycorrhizal fungi on the male function of plants. In the field, the size and duration of the floral display for insect-pollinated species may affect reproduction by affecting the number of pollinator visits (Patton and Ford 1983; Schaffer and Schaffer 1979; Schemske 1980a,b). Thus, it is not surprising to find that when mycorrhizal infection increased the floral display, it also increased pollinator visitation frequency in the annuals *Tagetes patula* and *Centaurea cyanus* (Gange and Smith 2005). In *Tagetes erecta*, however, pollinator visitation may be increased by mycorrhizal infection as a consequence of increased nectar availability (Gange and Smith 2005). Moreover, a minimum number of pollen grains must be deposited on the stigma of some species to produce a fruit, and pollination intensity (pollen grains per stigma) is usually positively correlated with the number of seeds per fruit (Lee 1988 and references therein). Therefore, the more pollen produced by a plant, the greater is the likelihood that it will sire seed. Increased pollen production results in greater male fitness.

When a plant that produces hermaphrodite flowers is caused to produce more flowers as a consequence of infection by mycorrhizal fungi, more pollen also is produced per plant. However, even for plants that produce hermaphrodite flowers, the effects of infection by mycorrhizal fungi on the male function may be independent of those on the female function. In some cases, for example, flower production may not be affected by mycorrhizal colonization while pollen production per anther and per flower are increased by mycorrhizal infection, such as in *Lythrum salicaria* (Philip et al. 2001). The mechanism for controlling male and female functions on hermaphrodite species is not well understood. For plants that produce distinct male and female flowers (monoecy or dioecy), independent control of male and female functions is, perhaps, easier to fathom, and there are some examples of this. In maize, infection by mycorrhizal fungi significantly increased the likelihood of male tassel development (Daft and Okusanya 1973). Lau et al. (1995) demonstrated that infection by mycorrhizal fungi of *Cucurbita pepo* (a monoecious species) had no significant effect on the production of male flowers, but did marginally increase (P = 0.097) pollen production per male flower. In *Cucurbita foetidissima*, mycorrhizal infection increased male flowers but did not influence female flowers (Pendleton 2000).

We have shown that infection by mycorrhizal fungi can influence pollen vigor. Pollen produced by mycorrhizal *Lycopersicon esculentum* plants was significantly
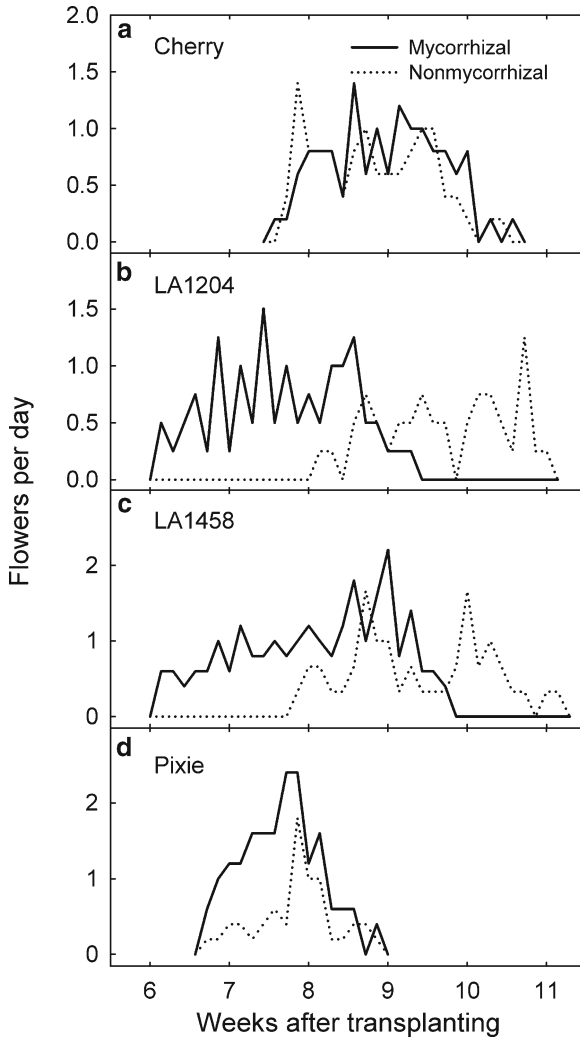
Fig. 3 The number of flowers produced per day per individual plant for *Lycopersicon esculentum* genotypes. (**a**) Large cherry cultivar. (**b**) *Lycopersicon esculentum* var. *cerasiforme* accession LA 1204. (**c**) *Lycopersicon esculentum* var. *cerasiforme* accession LA1458. (**d**) Pixie cultivar. See Bryla and Koide (1990) for details. The Tomato Stock Center, Department of Vegetable Crops, University of California, Davis, made wild accessions available

more likely to sire seed than pollen produced by nonmycorrhizal plants when 50:50 mixtures of the two pollen types were applied to stigmas by hand (Stephenson et al. 1999), possibly because pollen from mycorrhizal plants produced faster-growing pollen tubes. In addition to pollen quality, infection by mycorrhizal fungi may influence seed quality and the vigor of resultant offspring.

2.3   Offspring Quality

The weight and nutrient contents of individual seeds of some species appear to remain constant despite changes in resource availability. For example, Merryweather and Fitter (1996) showed that flowers and seeds of *Hyacinthoides non-scripta* maintained a constant P concentration despite significant reductions in infection by mycorrhizal fungi, apparently at the expense of vegetative parts such as leaves. Similarly, infection by mycorrhizal fungi had no significant effect on individual seed weights of *Lycopersicon esculentum* genotypes (Bryla and Koide 1990). For other species, however, seed weight or nutrient content may be far more plastic. In *Triticum aestivum*, for example, individual seed weight was increased by up to 60% as a consequence of mycorrhizal infection in some soils (Karagiannidis and Hadjisavva-Zinoviadi 1998). In *Abutilon theophrasti*, infection by mycorrhizal fungi had significant but modest positive effects on individual seed weight (4% increase averaged across three soil P availabilities) and seed N content (7% increase), but a very large, positive effect (53% increase) on seed P content (Lu and Koide 1994). Most of the increase in P content of seeds of mycorrhizal plants was due to an increase in phytate-P. Other host species exhibit increased seed P concentrations as a consequence of mycorrhizal infection, including *Triticum aestivum* (Karagiannidis and Hadjisavva-Zinoviadi 1998). Such increases in seed P concentration may even occur in the absence of any positive effects on plant growth, flower production, the number of seeds per fruit or seed weight (Nuortila et al 2004).

There are several possible consequences of increases in the nutrient contents of seeds. In cereals, for example, greater seed N content can increase seedling establishment and, remarkably, the reproductive output of the offspring (Kaufman and Guitard 1967; Ries and Eversen 1973; Schweizer and Ries 1969). In *Abutilon theophrasti*, an increase in seed N content improved competitive ability of the offspring (Parrish and Bazzaz 1985). An increase in seed P content can also positively affect the germination, growth or reproductive output of the offspring such as in *Campanula* (Nuortila et al. 2004), *Pisum* (Austin 1966), in other legumes (Bolland and Paynter 1990), and in *Hordeum* (Zhang et al. 1990). Thus, even when additional P supplied by the mycorrhizal fungus does not result in increased seed number (when seed production is no longer P-limited), significant consequences to the next generation of plants may still result from mycorrhizal infection of the former generation.

Although mycorrhizal *Avena fatua* produced lighter seeds, they nonetheless contained more P than those from nonmycorrhizal plants (Koide and Lu 1992; Lu and Koide 1991). Seeds produced by mycorrhizal plants also resulted in more vigorously growing seedlings (Koide and Lu 1992). There are at least two possible reasons for this positive effect of seed P content on offspring vigor. Seeds of higher P content could simply germinate faster and thus "get a jump" on seeds of lesser P content, or they may simply produce inherently faster-growing plants.

In *Avena fatua*, seeds produced by mycorrhizal plants did not germinate before seeds from nonmycorrhizal plants (Koide and Lu 1992). During germination, however, more endosperm P could be allocated to the offspring from mycorrhizal plants

(Lu and Koide 1991). Moreover, these offspring exhibited higher root and rhizosphere acid phosphatase, ATPase and phytase activities (Lu and Koide 1991). Thus, offspring from mycorrhizal plants were not only better endowed with P upon germination, they also possessed a potentially greater capacity to acquire P from organic P sources in the soil.

To test whether differences in offspring growth were attributable to differences in the ability to acquire P from the soil, we grew offspring from mycorrhizal and nonmycorrhizal *Abutilon* plants in water (containing no nutrients) rather than in soil. The differences between offspring types persisted, indicating that there were inherent differences in growth that were unrelated to nutrient uptake (Koide and Lu 1995). Offspring of mycorrhizal plants simply grew faster than did those of nonmycorrhizal plants. This suggested that the differences were somehow associated with differences in seed P content and not with differences in the ability to acquire P from the environment. Shumway and Koide (1994) found that seeds collected early in the season from mycorrhizal and nonmycorrhizal *Abutilon theophrasti* differed in P content and produced offspring that differed in vigor (Fig. 1). In contrast, seeds collected later in the season did not exhibit significant differences in P, and did not produce offspring differing significantly in vigor. This suggests, again, that seed P content is somehow related to offspring vigor.

Austin (1966) earlier had found in *Pisum* that the addition of P to offspring reduced the differences in growth associated with variation in seed P content. Thus we were surprised to find that additions of P to offspring plants did not reduce or eliminate the differences in vigor between those produced by mycorrhizal and nonmycorrhizal plants (Koide and Lu 1992; Lewis and Koide 1990; Shumway and Koide 1994). One possible explanation is that different forms of P within the seed may have different effects on seedling performance. We found that in *Abutilon*, for example, the ratio of organic to inorganic P was significantly higher in seeds produced by mycorrhizal plants. Indeed, seed P content can be increased by the addition of P to mother plants, or by infection by mycorrhizal fungi of mother plants. However, offspring vigor was significantly affected only by infection by mycorrhizal fungi of mother plants (Table 1).

Mycorrhizal plants produced seeds with significantly more phytate-P than nonmycorrhizal plants. The phytate fraction may be particularly important to seedling growth (Hall and Hodges 1966), but it may even have ramifications for later plant development. For example, Barry and Miller (1989) showed that leaf P concentrations of young *Zea mays* seedlings were correlated with adult plant seed yield. Thus, in some species developmental trajectories established in the very earliest phases of seedling growth may not be easily altered by subsequent additions of nutrients, possibly because the myo-inositol portion of the phytate can be used as a source of energy, and because inositol phospholipids are growth regulators (Stevenson et al. 2000).

All of the mechanisms for the maternal mycorrhizal effect on offspring performance are still not well understood, but the ecological consequences are potentially large. Initial size advantage may be particularly consequential when small differences become magnified by competition (Weiner 1990). We tested this

Table 1 Mean seed P content and shoot and root system weights of resultant offspring produced by hydroponically-grown and soil-grown *Abutilon theophrasti* mothers given various treatments. Different letters within columns and within mother types indicate a significant difference between means according to Fishers protected LSD method. Offspring were planted in a sterile mixture of soil and sand with a bicarbonate-extractable P concentration of 3 μg P g^{-1} and grown in a growth chamber at 25°C. All offspring were irrigated with a one-fifth strength Hoagland nutrient solution lacking P. See Lewis and Koide (1990) for further details

	Seed P (μg P seed^{-1}), n = 45	Offspring shoot weight (mg), n = 19	Offspring root weight (mg), n = 19
Hydroponic (NM) mothers			
P2 (10 μM P)	48.8a	72.6a	42.5a
P3 (30 μM P)	69.6b	75.6a	38.8a
P4 (90 μM P)	105.4c	85.8a	44.2a
	Seed P (μg P seed^{-1}), n = 47	Offspring shoot weight (mg), n = 24	Offspring root weight (mg), n = 24
Soil-grown mothers			
Low P NM. (333 μM P)	37.3a	44.9a	25.8a
High P NM. (1,000 μM P)	54.6b	58.0a	33.6ab
M (0 μM P)	78.4c	86.7b	44.8b

hypothesis by growing swards of *Abutilon theophrasti* plants in eight large wooden boxes each filled with 80 L of field soil that previously had been treated with methyl-bromide/chloropicrin (2:1) at the rate of 560 kg ha^{-1} (Heppell et al. 1998). In each box 200 seeds each from mycorrhizal and nonmycorrhizal mothers were planted in alternate positions in a checkerboard pattern to maximize the likelihood of interaction between the offspring types. We could track individual offspring because each was planted in a separate square demarcated by a grid of monofilament line on the soil surface.

By 20 days fewer offspring of nonmycorrhizal mothers were represented in larger size classes and more were represented in the smaller size classes than were offspring of mycorrhizal mothers. By 47 days, mortality (self-thinning) had occurred, but this was not randomly distributed among the offspring types. Offspring of mycorrhizal mothers had significantly greater survival than offspring of nonmycorrhizal mothers (Fig. 4a), and accounted for nearly all individuals in the larger size classes as well as the majority in the smallest size class. The size distribution suggested that many of the smallest offspring of nonmycorrhizal mothers had died. This was not due competition for N or P or water because offspring of mycorrhizal mothers actually had lower concentrations of N and P (Fig. 4b, c), probably due to greater dilution from growth, and because all offspring plants were watered regularly. We concluded that the major reason for death was competition for light. By 98 days, offspring of mycorrhizal mothers constituted 69% of the survivors. Among the survivors, a significantly greater proportion of offspring of mycorrhizal mothers were reproductive compared to those from nonmycorrhizal mothers (Fig. 4d). Further, of those reproducing, offspring of mycorrhizal mothers
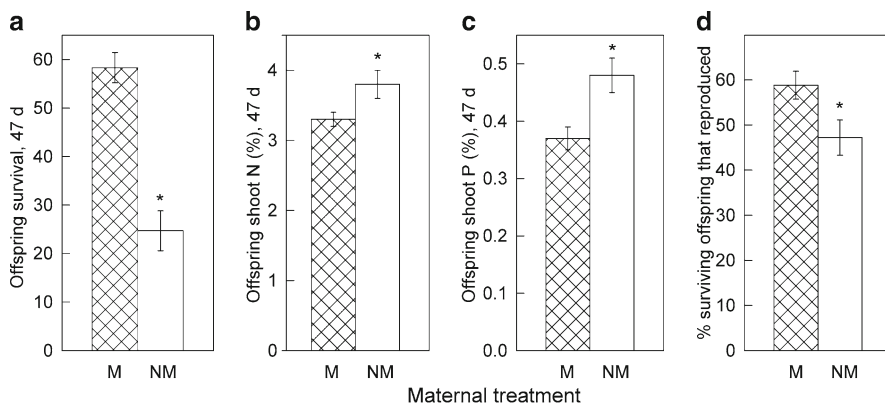
Fig. 4 Characteristics of offspring of mycorrhizal and nonmycorrhizal *Abutilon theophrasti* plants growing in dense, mixed populations (see Heppell et al. (1998) for details). (**a**) Survival after 47 days. (**b**) Shoot N concentration at 47 days. (**c**) Shoot P concentration at 47 days. (**d**) The percentage of surviving offspring that became reproductive. Error bars represent ±1 s.e.m. * = significantly (P ≤ 0.05) different

produced more fruits that contained significantly more seeds than those produced by offspring of nonmycorrhizal mothers. The increase in reproductive output was associated with an increase in plant size. We concluded that infection by mycorrhizal fungi of the parent generation can increase the competitive ability of the offspring, and that competition could magnify inherent differences in growth between offspring of mycorrhizal and nonmycorrhizal mothers, resulting in selective mortality and differential reproductive output.

Our results suggest that infection by mycorrhizal fungi can influence plant fitness in at least two ways. First, infection by mycorrhizal fungi can increase fecundity by influencing a variety of reproductive traits. Second, infection by mycorrhizal fungi may have effects that carry over into the next generation by increasing seed quality and resultant offspring vigor. The consequence of this may be an increase in competitive ability, growth and reproduction of the offspring generation. The results suggest that if an *Abutilon* plant were unable to develop mycorrhizas for reasons such as mutation, its offspring could be at a competitive disadvantage. The natural selection for development of the symbiosis must be large, indeed.

3   Population-Level Phenomena

Except in rare instances, plants must interact with other plants. Intraspecific interactions can exert a strong influence on the reproductive response to infection by mycorrhizal fungi (Allsopp and Stock 1992; Bååth and Hayman 1984; Hartnett et al. 1993; Koide 1991a; Koide et al. 1994; Facelli and Facelli 2002). As plant densities increase, both vegetative and reproductive responses to infection decline

Fig. 5 Vegetative and reproductive traits of individual *Abutilon theophrasti* plants grown at four different densities. Pots had square openings, 11.4 cm on a side. See Koide (1991a) for details. (**a**) Individual shoot dry weight. (**b**) Flowers per individual plant
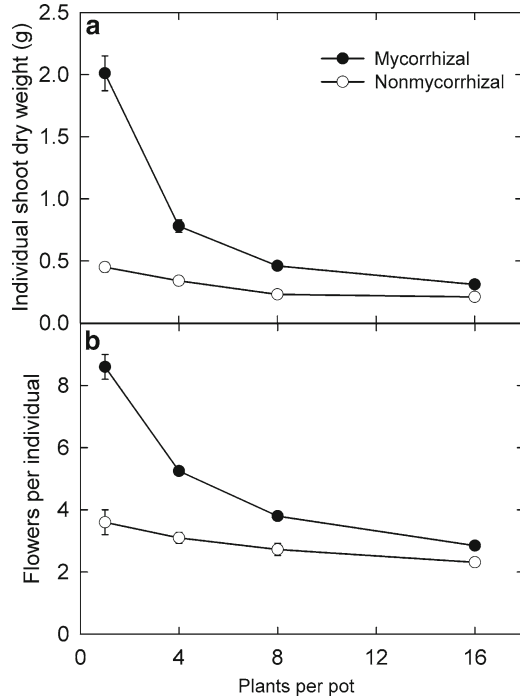
precipitously. This was shown clearly in *Abutilon theophrasti* (Fig. 5). Mycorrhizal fungi may be more capable of increasing nutrient uptake when root densities are low but, as nutrient absorbing organs the fungi may become increasingly superfluous at higher root densities (Allsopp and Stock 1992; Bååth and Hayman 1984; Koide 1991a). Because plant densities vary substantially within and between natural and agricultural ecosystems, the likelihood that different plants respond differently to mycorrhizal infection is high. Moreover, in many modern agricultural ecosystems, plant densities frequently have been established under conditions of optimum fertility. As more growers reduce nutrient inputs in order to reduce costs and environmental degradation, planting densities may need to shift, resulting in a change in the response of the plants to mycorrhizal infection.

Mycorrhizal fungi may also influence host plant populations by influencing the extent of variation among individuals. No two individuals within a population are exactly the same size. Even in populations whose members germinate synchronously, some inequality among individuals will occur as a consequence of genetic differences or microsite environmental variation, and such differences among individuals may be magnified by the process of competition (Wilson 1988). For example, if one individual shades another, the shaded individual may not grow

much while the overtopping individual continues to grow rapidly. In this case, the acquisition of light will initially be disproportionate to the size of the plants. Indeed, the individual on top need not be any larger than the shaded individual beneath, yet the individual on top may capture nearly all the light. Disproportionate acquisition of any limiting resource will result in increasing size inequality among individuals. The consequence of this size variation will often be that the largest, dominant individuals will contribute disproportionately to the next generation of plants and alter the genetic structure of future generations.

Can infection by mycorrhizal fungi influence the development of size or reproductive inequality? On the one hand, infection by mycorrhizal fungi may intensify competition among individuals and thus increase size or reproductive inequality because faster growing individuals may be more responsive to infection by mycorrhizal fungi than slower growing individuals (Koide 1991b). Indeed, infection by mycorrhizal fungi has been shown to increase size inequality among closely planted individuals of *Otholobium hirta* (Allsopp and Stock 1992), *Prunella vulgaris* (Moora and Zobel 1996) and *Trifolium subterraneum* (Facelli and Facelli 2002). This may result in a disproportionate contribution of offspring to the next generation by the dominant individuals. Alternatively, infection by mycorrhizal fungi might allow limiting resources to be shared among individuals (Grime et al. 1987) and thus reduce size or reproductive inequality. Maffia and Janos (1993), for example, have shown that size inequality among members of a population of *Helianthus annuus* was reduced by infection by mycorrhizal fungi. Unfortunately none of the above-mentioned studies examined the consequences to reproduction.

We, therefore, set out to determine the effect of infection by mycorrhizal fungi on reproductive inequality in *Abutilon theophrasti* (Shumway and Koide 1995). Dense plantings of mycorrhizal and nonmycorrhizal *Abutilon* were made in the field and, over the course of time, we followed the density and reproductive inequality in the populations. Mycorrhizal populations developed significantly greater inequality in reproduction than nonmycorrhizal populations. The proportion of individuals reaching reproductive maturity was similar in the two types of populations, but reproductive individuals in mycorrhizal populations had more capsules than those in nonmycorrhizal populations. Because inequality in fecundity can result in a disproportionate genetic representation of the most fecund individuals in the next generation (Weiner 1988), the increasing inequality in fecundity due to infection may have long term consequences for the genetic structure of plant populations. This may be particularly for species that are capable of self-pollination such as *Abutilon theophrasti*.

4 Community-Level Phenomena

While interactions among plants of the same species are common, most natural communities comprise several plant species. Even crop plants growing in monospecific agricultural systems interact with weeds, and multiple-species cropping

systems are becoming more common. Thus, some effort has been made to study the effects of mycorrhizal fungi on competition among plant species (Allen and Allen 1984; Buwalda 1980; Crush 1974; Fitter 1977; Hall 1978; Hetrick et al. 1989; Koide and Li 1991) and on the structure of plant communities (Gange et al. 1993; Grime et al. 1987; Hartnett et al. 1994; Janos 1980, Koide et al. 1988a, Miller 1987; Sanders et al. 1999). Despite the importance of infection by mycorrhizal fungi to interspecific plant competition and to plant community structure, few experiments document the effects of infection on reproduction of competing plants. Sanders and Koide (1993) demonstrated that in artificial communities, the presence of mycorrhizal fungi decreased reproduction in a nonmycotrophic species (*Amaranthus retroflexus*), increased reproduction in a strongly mycotrophic species (*Abutilon theophrasti*), and had no significant effect on the mycotrophic but less responsive *Setaria lutescens*. However, simply because infection has been reported to have a positive influence on the seed output of one species and a negative influence on another species competing with it does not mean that the structure of the community will be influenced in the predicted fashion. After all, seed quality effects may come into play and, in subsequent years, mycorrhizal effects may be diminished if plant densities are large. The existence of these phenomena points to the necessity for long-term studies on the effects of mycorrhizal fungi on plant community dynamics.

5 Mycorrhizal Effects Mediated by Altered Herbivory or Disease

Mortality due to either herbivory or disease can obviously eliminate any reproductive effort. Even if herbivores or pathogens do not kill the plant outright, they can certainly reduce reproduction, either indirectly by reducing the capacity to capture, transport or store resources needed for reproduction, or directly by damage or removal of flowers, fruits or seeds (Hendrix 1988). Any reduction in herbivory or disease caused by infection by mycorrhizal fungi thus has the potential to increase reproduction. Little has been written about the influence of herbivores or pathogens on plant reproduction as influenced by mycorrhizal fungi, so the discussion here is, of necessity, general in nature.

Gehring and Bennett (2009) have recently reviewed interactions among mycorrhizal fungi, insects and plants. They found that mycorrhizal effects on herbivores vary from negative to positive, and very few generalizations could be made. The same is probably true of mycorrhizal effects on plant pathogens, but some have suggested that the primary benefit of mycorrhizal infection in some cases may not be enhanced nutrient uptake but may actually be increased disease resistance (Carey et al 1982; Newsham et al. 1994; Newsham et al. 1995; West et al. 1993a; b). Indeed, some examples exist in which mycorrhizal infection inhibited pathogens or decreased disease without increasing host tissue P concentrations (Caron et al. 1986; Davis and Menge 1981; Thompson and Wildermuth 1989).

Mycorrhizal fungi have the potential to influence herbivory and disease by several mechanisms. Obviously, any increase in nutrient uptake or any alteration of the allocation of nutrients within the plant has the potential to influence the quantity of both energy and nutrients available to pests. Moreover, one might predict that the ability to recover from herbivory or disease would be improved by mycorrhizal infection when plant growth is limited by a resource whose uptake is increased by infection by mycorrhizal fungi.

Mycorrhizal infection may also influence plant secondary metabolism (Guo et al 2006; Venkateswarlu et al 2008), and secondary metabolites are key molecules in the interaction between plants and their pests (Pickett et al. 1999; Mayer 2004). For example, infection by of Douglas-fir by *Laccaria laccata* can increase resistance to fungal pathogens, possibly by inducing greater production of phenolic compounds by the host (Sylvia 1983). Rabin and Pacovsky (1985) reported that the growth rates of larvae of *Spodoptera frugiperda* and *Heliothis zea* were lower when they were fed leaves of arbuscular mycorrhizal soybean compared to leaves of nonmycorrhizal plants given additional P. This could not be explained by differences in the concentrations of carbohydrates, amino acids, or mineral nutrients, and the authors suggested that variation in secondary metabolites may have been important. Furthermore, larvae of *Arctia caja* (Lepidoptera) grew slower, ate less and produced less frass when fed on leaves of mycorrhizal *Plantago*, possibly because the concentrations of the iridoid glycosides aucubin and catalpol (considered to be deterrents to generalist feeders) were higher in leaves of mycorrhizal plants (Gange and West 1994).

Infection by mycorrhizal fungi may also influence herbivory in indirect ways. Decreased herbivory, for example, may occur as a consequence of mycorrhizal infection via promotion of more prostrate shoot growth and more vigorous root growth (Wallace 1981). In other cases, shifts in phenology of host plants may influence herbivory. Mycorrhizal plants frequently flower earlier than nonmycorrhizal plants (Bryla and Koide 1990; Lu and Koide 1994) possibly because resource status determines when flowering is initiated. This small shift in the phenology of flowering resulted in a significant decrease in the level of infestation of seeds of *Abutilon theophrasti* by the beetle *Althaeus folkertsi* (Bruchidae) in mycorrhizal plants (Koide 1998). The adult beetles were present for only a few days at a time when both mycorrhizal and nonmycorrhizal plants were flowering, but because mycorrhizal plants flowered for a greater period of time than nonmycorrhizal plants, the beetle larvae infested proportionally fewer seeds produced by mycorrhizal plants.

One must consider the possibility that infections by mycorrhizal fungi actually make herbivory or disease more likely or more consequential. Borowicz (1997) found that infection by mycorrhizal fungi of *Glycine max* actually increased the pupation rate and survival of the beetle *Epilachna varivestis*. One possible explanation for increased herbivory in mycorrhizal plants is that carbon allocated to mycorrhizal fungi cannot be used for defense (Bazzaz et al. 1987). Jones and Last (1991) have suggested that when carbon is limited, a tradeoff may exist between carbon supplied to mycorrhizal fungi and that for carbon-based defenses. Gehring and Whitham (1991) and Del Vecchio et al. (1993) compared ectomycorrhizal infection

of herbivore-susceptible and herbivore-resistant pinyon pines and found evidence of a tradeoff between defense and mycorrhizal infection. When insects were removed from insect-susceptible trees (with presumably lower levels of defense), the proportion of mycorrhizal roots increased above the level of infection of the insect-resistant trees. One also can envision the case in which nonmycorrhizal plants are less prone to herbivory than AM plants because the latter produce more nutritious leaves. I am unaware of documented examples of this, but there is at least one case in which mycorrhizal infection of *Abutilon* stimulated parasitism by *Cuscuta* (Sanders et al. 1993). Unfortunately the mechanism was never worked out.

6   Negative Effects of Infection by Mycorrhizal Fungi

Although not commonly reported, there are cases in which infection by mycorrhizal fungi have proven to reduce reproduction. Seeds residing in the soil, for example, have reportedly become infected and killed by AM fungi (Taber 1982). Arbuscular mycorrhizal fungi may also directly reduce the growth and reproduction of some plant species. For example, vegetative reproduction was reduced as a consequence of mycorrhizal infection of *Potentilla reptans* and *Fragaria moschata* (Sudová and Vosátka 2008). Negative effects of mycorrhizal fungi may be particularly common among normally nonmycotrophic or weakly mycotrophic plant species growing amongst highly mycotrophic species (Francis and Read 1994; Gange 1998). Perhaps this is why nonmycotrophic plant species often preferentially occur in soils where the mycorrhizal mycelium is poorly developed or severely disturbed (Cuenca and Lovera 1992).

7   Conclusions

Continued studies on the effects of mycorrhizal infection on plant reproduction are important because the nature of the interactions between mycorrhizal fungi and host plants are changing. As a consequence of an increasing concern about water pollution, food safety, food quality, greenhouse gas emissions, energy consumption and the use of genetically modified plants in agriculture, many agricultural practices are changing. For example, organic agriculture is increasing in scope and, in such systems, growers often rely on tillage as an important strategy for weed control. Such regular soil disturbance may cause mycorrhizal infection to be severely reduced and may even change the structure of mycorrhizal fungal communities. This may have significant consequences for the effects of mycorrhizal infection on both vegetative growth and reproduction of host plants. What will the consequences of other changes be to the influence of mycorrhizal fungi on yield or food quality? Climate change will also impact the influence of mycorrhizal fungi on plant reproduction, particularly if mycorrhizal fungi have different temperature optima from

their host plants. And, if fungal isolates differ amongst themselves in temperature optima or moisture requirements, climate change may significantly alter the structure of communities of mycorrhizal fungi. Clearly more research in these areas would be helpful in our quest to better understand the constraints on food production and to better predict natural vegetation dynamics.

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