Soil Biology

Ajit Varma
Gerhard Kost
Ralf Oelmüller *Editors*

Piriformospora indica

Sebacinales and Their Biotechnological Applications

Springer

Soil Biology

Volume 33

Series Editor
Ajit Varma, Amity Institute of Microbial Technology,
Amity University Uttar Pradesh, Noida, UP, India

For further volumes:
http://www.springer.com/series/5138

Ajit Varma • Gerhard Kost • Ralf Oelmüller
Editors

Piriformospora indica

Sebacinales and Their Biotechnological Applications

Springer

Editors
Ajit Varma
Amity Institute of Microbial Technology
Amity University Uttar Pradesh
Noida, Uttar Pradesh
India

Gerhard Kost
FB Biology, Systematic Botany & Mycology
Philipps University of Marburg
Marburg, Germany

Ralf Oelmüller
Inst. Allgemeine Botanik
LS Pflanzenphysiologie
Univ. Jena
Jena, Thüringen
Germany

ISSN 1613-3382
ISBN 978-3-642-33801-4 ISBN 978-3-642-33802-1 (eBook)
DOI 10.1007/978-3-642-33802-1
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013931211

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Foreword

Crop productivity is being challenged by various adverse environmental factors (biotic and abiotic stress factors) worldwide. To meet this challenge we must increase the yield potential of our food crops. Sebacinales are known to be involved in a variety of mutualistic plant–fungal symbioses, which may involve transport of mineral nutrients. The order Sebacinales exclusively harbours beneficial fungi, encircling ectomycorrhizas, orchid mycorrhizas, ericoid mycorrhizas, cavendishioid mycorrhizas and jungermannioid mycorrhizas in liverworts. In special, *Piriformospora indica* (an Orchid Mycorrhizal Fungus) is an interesting endophytic fungus of the order Sebacinales, the fungus is capable of colonizing roots and forming symbiotic relationship with every possible plant on earth. *P. indica* has also been shown to increase both crop yield and plant defence of a variety of crops (barley, tomato, maize, etc.) against various biotic and abiotic stress factors. It has vast potential for improving crop productivity and protection against various stress factors in the era of changing climatic conditions.

I am glad to see this book addressing the potential of Sebacinales in crop improvement and stress tolerance and providing scientific know-how to solve some of the problems. The whole book is very beautifully managed by the editors to provide actual insight to the readers about the potential of *P. Indica* for crop improvement and biotic and abiotic stress tolerance. Various parts including Part I: *P. indica* as Beneficial Root Symbiont; Part II: Interaction with Different Plant Species; Part III: Resistance Against Biotic and Abiotic Stress Factors; Part IV: *P. indica* and Macronutrients for Plants and Part V: Experimental Protocols for *P. indica* Studies covers state-of-the-art articles from well-known scientists around the world. All chapters are very well written and created a scientific interest to the learners/readers and researchers.

Editors of this volume (Drs. Ajit Varma, Gerhard Kost and Ralf Oelmüller) have done an admirable job of assembling a wealth of information on Sebacinales including their new role in crop improvement. They have sought chapters from

well-known authors from all over the world. This comprehensive volume should prove useful for students, scientists and teachers interested in the area of Soil Biology, Microbiology, Plant Pathology, Bio-Fertilizer, Soil Fertility and Plant Health. I would like to congratulate the editors and also the publishers for their excellent job.

Aruna Asaf Ali Marg, New Delhi, India Narendra Tuteja

Preface

The genesis of this book goes back to summer 2010 while formulating Indo-German DFG Workshop on Amity University Campus, Noida, India under the frame of the programme in "Initiation and Intensification of Bilateral Cooperation". Among the eminent plant biologist, mycologists and microbiologists especially working on the members of Sebacinales like Ralf Oelmüller (Jena), Gerhard Kost (Marburg), Anton Hartmann (München) and François Buscot (Halle) realized that at the moment information on different aspects of members of the Sebacinales is highly scattered and normally not available to scientists in general and young scholars in particular. A decision was taken to concise the published information's in a form of a book. The active scientists were invited to contribute their valuable chapters. It is gratifying to note that they were so enthusiastic that chapters were contributed much before the deadline. Unfortunately, due to pre-occupation it got delayed. Ajit Varma takes entire responsibility for the inordinate delay.

The first species of this fungal group with inconspicuous corticoid fruit bodies were described by C.H. Persoon in the eighteenth century and the genus name *Sebacina* was created by brothers Tulasne in 1871. In recent decades, the basidiomycetes of the order Sebacinales have emerged as a fascinating order of mutualistic plant–fungal symbionts. While a few members form mycorrhiza-type interactions, others behave more like beneficial endophytes. They promote plant growth and fitness, confer resistance of the host against abiotic and biotic stress and are involved in nutrient exchange between the two partners (Weiß et al. 2011). The authors demonstrated that Sebacinales are not only extremely versatile in their associations with roots but also almost universally present as symptomless endophytes. The multitude of symbiotic interactions in Sebacinales may have arisen from an ancestral endophytic habit by specialization. Weiß et al. (2011) further established a phylogenetic tree for all known Sebacinales and identified two subgroups, A and B. The most basal clades in both subgroups were endophytic, which indicates that this lifestyle maybe ancestral in the Sebacinales and the starting point for further specification towards mycorrhizal symbiosis. "However, the close relationship of endophytes, in particular with orchid mycorrhizal strains, could also be indicative of the capability of Sebacinales strains to switch between

symbioses or to fall back on endophytic habit if no appropriate mycorrhizal partner is present" (Weiss et al. 2011). There is no question that Sebacinales are not only interesting for basic science but also important contributors to the ecosystems.

Two members of Sebacinales have been developed as model systems to study plant/microbe interactions at the cellular and molecular basis *Sebacina vermifera* and *Piriformospora indica*. The later colonizes the root cortex of many different plant species including important crop plants with beneficial effects to its hosts. Given the capability of *P. indica* to colonize a broad range of hosts, it must be anticipated that the fungus has evolved efficient strategies to overcome plant immunity and to establish a proper environment for nutrient acquisition and reproduction (Khatabi et al. 2012). Qiang et al. (2012) have recently demonstrated that *P. indica* colonizes *Arabidopsis* roots by inducing an endoplasmatic reticulum stress-triggered caspase-dependent cell death. Studies in several laboratories have demonstrated that the degree of root colonization is important for the benefits of the plants in this symbiosis (Khatabi et al. 2012; Jacobs et al. 2011; Camehl et al. 2010; Sherameti et al. 2008) and that a balanced activation of the plant defence mechanism is required to maintain a stable symbiosis and to prevent over-colonization (Camehl et al. 2010). Furthermore, an effective phosphate transport process places an important strategy to enhance the symbiotic interaction between the fungus and the host. In addition to this, recently the role of OXI1 (oxidative signal inducible one) has also been implicated in facilitation of fungal colonization process (Camehl et al. 2011). OXI1 becomes activated by H_2O_2 and phospholipid signalling, and it has been proposed that this pathway may balance growth/developmental processes and defence in response to the beneficial fungus *P. indica*. Two groups have established efficient transformation systems for *P. indica*. Using this technology, Yadav et al. (2010) have shown that a phosphate transporter from *P. indica* plays a role in phosphate transport to the host plant. Also Zucccro et al. (2007) established an efficient transformation system by introducing a reporter gene into the fungal genome. In addition, Zuccaro et al. (2011) have sequenced and annotated the entire genome of *P. indica*. Comparative analysis of the *P. indica* genome with other Basidiomycota and Ascomycota fungi that have diverse lifestyle strategies identified features typically associated with both, biotrophism and saprotrophism. The authors concluded that the tightly controlled expression of the lifestyle-associated gene sets during the onset of the symbiosis argues for a biphasic root colonization strategy, an early biotrophic growth followed by a cell death-associated phase. About 10 % of the fungal genes induced during the biotrophic colonization encoded putative small secreted proteins (Zucccro et al. 2011; Lahrmann and Zucccro 2012).

Since it is proved that Sebacinales are universally present in the ecosystems on the entire world (Weiß et al. 2011), and since their symbiotic interaction with the plants is either symptomless or beneficial for the hosts, they have an enormous potential for agricultural applications. Several groups have already started to study the potential of Sebacinales for improving crop plants, which will be a fascinating tool for the future, in particular if it can be combined with results from basic science.

The book contains 22 chapters, and they are grouped into five parts namely *Piriformospora indica* as Beneficial Root Symbiont; Interaction with Different Plant Species; Resistance Against Biotic and Abiotic Factors; *Piriformospora indica* and Macronutrients for Plants and Experimental Protocols for *Piriformospora indica* Studies followed by subject index.

It has been a pleasure to edit this book, primarily due to the stimulating cooperation of the contributors. We wish to thank Hanna Hensler-Fritton, Jutta Lindenborn and Dieter Czeschlik at Springer Heidelberg for their generous assistance and patience in finalizing the volume. Finally, we give specific thanks to our families—immediate and extended—for their kind support and their incentive to put everything together.

Ajit Varma in particular is very thankful to Dr. Ashok K. Chauhan, Founder President of the Ritnand Balved Education Foundation (an umbrella organization of Amity Institution), New Delhi, for his kind support and constant encouragement.

Special thanks are also due to his student, Ms. Kritika Rastogi.

New Delhi, India — Ajit Varma
Marburg, Germany — Gerhard Kost
Jena, Germany — Ralf Oelmüller

References

Camehl I, Drzewiecki C, Vadassery J, Shahollari B, Sherameti I, Forzani C, Munnik T, Hirt H, Oelmüller R (2011) The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in Arabidopsis. PLoS Pathog 7:e1002051

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Oelmüller R (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and non-beneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol 185:1062–1073

Jacobs S, Zechmann B, Molitor A, Trujillo M, Petutschnig E, Lipka V, Kogel KH, Schäfer P (2011) Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus *Piriformospora indica*. Plant Physiol 156:726–740

Khatabi B, Molitor A, Lindermayr C, Pfiffi S, Durner J, von Wettstein D, Kogel KH, Schäfer P (2012) Ethylene supports colonization of plant roots by the mutualistic fungus *Piriformospora indica*. PLoS One 7:e35502

Lahrmann U, Zuccaro A (2012) Opprimo ergo sum-evasion and suppression in the root endophytic fungus *Piriformospora indica*. Mol Plant Microbe Interact 25:727–737

Qiang X, Zechmann B, Reitz MU, Kogel KH, Schäfer P (2012) The mutualistic fungus *Piriformospora indica* colonizes *Arabidopsis* roots by inducing an endoplasmic reticulum stress-triggered caspase-dependent cell death. Plant Cell 24:794–809

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmüller R (2008) PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 54:428–439

Weiss M, Sýkorová Z, Garnica S, Riess K, Martos F, Krause C, Oberwinkler F, Bauer R, Redecker D (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS One 15(6):e16793

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. J Biol Chem 285:26532–26544

Zuccaro A, Lahrmann U, Güldener U, Langen G, Pfiffi S, Biedenkopf D, Wong P, Samans B, Grimm C, Basiewicz M, Murat C, Martin F, Kogel KH (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. PLoS Pathog 7:e1002290

Contents

Part I
Piriformospora indica as Beneficial Root Symbiont

Chapter 1
Introduction to Sebacinales

Ajit Varma, Pooja Chordia, Madhunita Bakshi, and Ralf Oelmüller

1.1 Introduction

The Sebacinales is an order of fungi in the class Agaricomycetes and division Basidiomycetes. Within the Basidiomycetes, the vast majority of known mycorrhizal species are Homobasidiomycetes. It was therefore surprising when molecular and ultrastructural studies revealed a broad diversity of mycorrhizal associations involving members of the heterobasidiomycetous Sebacinaceae, fungi which, due to their inconspicuous basidiomes, have been often overlooked.

1.1.1 Mycorrhizal Symbiosis

The term "mycorrhiza" literally means "fungus root". This term is derived from the Greek "mykes" which means mushroom or fungus and "rhiza" the root. The term "mycorrhiza" was first used in 1885 by A. B. Frank to describe the modified root structures of forest trees and has since been extended to cover a range of mutualistic, symbiotic associations between fungi and plant roots.

The mycorrhizal symbiosis is an intimate association which exists between plant root system and certain groups of soil fungi. Nearly all plant life is dependent on the mycorrhizal association, which began some 465 million years ago and likely to have played role in colonisation of land by plants. Mycorrhizal fungi grow through

A. Varma (✉) • P. Chordia • M. Bakshi
Amity Institute of Microbial Technology, Amity University Uttar Pradesh, E-3 Block, IV Floor, Amity University Campus, Sector -125, Noida 201303, U.P, India
e-mail: ajitvarma@amity.edu; pooja29jain@gmail.com; bakshi.madhunita@gmail.com

R. Oelmüller
Friedrich-Schiller-University Jena Institute of General Botany and Plant Physiology, Dornurger Str, 159 07743 Jena, Germany
e-mail: b7oera@rz.uni-jena.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_1,

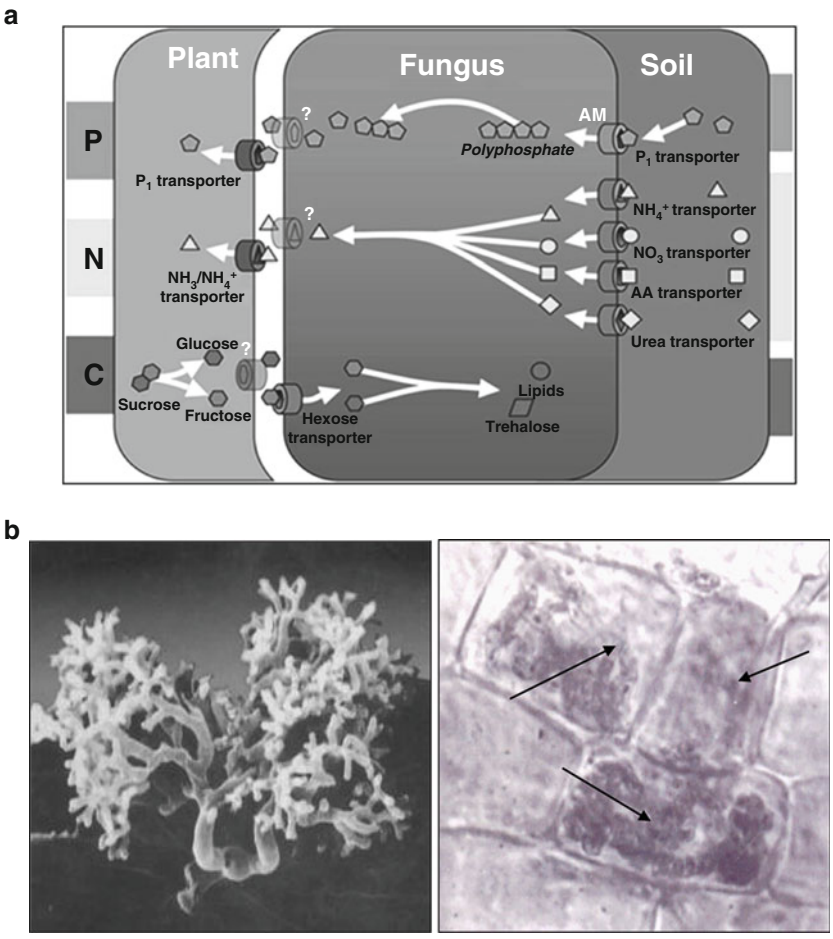

Fig. 1.1 (**a**) Scheme summarising the main nutrient exchange processes in AM symbiosis (c.f. Bonfante and Genre 2010). (**b**) *Left*: A typical arbuscule (adopted from Mycorrhiza, Varma and Hock 1999). *Right*: Intracellular root colonisation (*arrows*) by *P. indica* in maize plant as observed under light microscope (Varma et al. 2001)

the soil by means of hyphae, which are the "roots" of the fungus. These "roots" are extremely filamentous and explore every crack and cranny between and into soil particles to extract water, absorb and create nutrients and organic matter, and provide all of these to the plant through the roots (Fig. 1.1a). Mycorrhiza in soil aids in stability against erosion and most importantly aggregation of water-soluble components, and fungus receives carbohydrates from plants. This bidirectional flux is mediated by a very unique structure called arbuscules and/or highly branched intracellular structures (Fig. 1.1b). The AM fungi are obligate biotrophs and depend entirely on the plant for carbon source for reproduction. Fungi also protect the roots from pathogens by literally creating very specific solutions to ward off predators,

either physically or chemically. For this invaluable service, the plant "pays" the fungi 15–20 % of the foods it has converted from the energy of the sun (photosynthesis). This mutualistic relationship between plant roots and mycorrhizal fungi is an essential link between nearly all plants and their soil environment. Once established if the food flow from the roots to the fungi is interrupted in any way, the fungi respond accordingly by becoming more aggressive. This is why plants in stressed conditions benefit from mycorrhizas. Regardless of the cause, i.e. drought, high salt, parasites, negative organisms, etc., mycorrhizas have evolved to protect their food source—the plant. If the plant life cycle is finished, the mycorrhiza shifts its energy away from trying to keep the plant alive to perpetuating itself through the production of copious amounts of spores. Mycorrhizal fungi are a natural phenomenon—it is a natural part of a complete plant system.

1.1.2 Endophytes

Endophytic organisms are mostly fungi, bacteria and actinomycetes that live in the intercellular spaces of plant tissue and cause no apparent damage to their host (Raghukumar 2008). Carroll (1988) defined two different types of endophytic fungi: constitutive mutualists (type I endophyte) and inducible mutualists (type II endophyte). It is usually proposed that most type I clavicipitaceous endophytes (Epichloë/Neotyphodium) are systemic and vertically transmitted through seeds and exclusively infect grass. Instead, nonsystemic type II endophytes are taxonomically diverse and horizontally transmitted from plants to plants and colonise all plants in ecosystems (Yuan et al. 2010). Recently, nonsystemic endophytic fungi identified in a very wide range of host plant species have met with increasing attention due to their striking species diversity and multiple functions (Rodriguez and Redman 2008).

A number of fungi are known to colonise plant roots but do not cause disease. These include mycorrhizas, binucleate *Rhizoctonia* spp., *Trichoderma* and various plant growth-promoting rhizobacteria. Many of these organisms have been known for decades as agents that biocontrol plant diseases, but recent studies have demonstrated that they have many other useful attributes. *Trichoderma* strains have been described clearly as endophytic plant symbionts. The first step for any of these microorganisms is to colonise roots, and they demonstrated that plant-derived sucrose is an important resource which is critical for plant root colonisation by *Trichoderma virens* and a fungal invertase is key to initiation of the mechanisms of root colonisation (Harman 2011).

Plant use of endophytic fungi in defence from stressed conditions like drought and high salt concentrations in soil is a very common phenomenon, primarily involving the arbuscular mycorrhizal fungi and other members of Sebacinales.

1.2 Origin of Sebacinales

The order Sebacinales occupies a basal position among the Basidiomycetes and has a close phylogenetic affiliation with *Geastrum* spp. It has been hypothesised that the common ancestor of the Geastrum/Sebacinales clade or even of the whole Hymenomycetes was ECM (Taylor et al. 2003; Weiß et al. 2004; Smith and Read 1997). This basal order of Hymenomycetes (Basidiomycetes) encompasses fungi with longitudinally septate basidia and imperforate parenthesomes (i.e. the derivates of the endoplasmic reticulum covering septal pores and allowing communication between cells). They also lack cystidia and clamp connections.

Molecular ecology studies, based on rDNA sequences, uncover that Sebacinales are common mycorrhizal associates with many plant species all over the world. Sequences from members of the Sebacinales, either grown in cultures or mainly from fruit bodies and environmental samples, reveal that Sebacinales can be divided into two clades, A and B, that differ in their ecology (Weiß et al. 2004). Clade A sequences are obtained from fruit bodies (Weiß and Oberwinkler 2001), achlorophyllous (McKendrick et al. 2002; Selosse et al. 2002a; Taylor et al. 2003) and photosynthetic orchids (Selosse et al. 2004; Julou et al. 2005) as well as many trees (Selosse et al. 2002a; Urban et al. 2003; Moyersoen 2006). Fruit bodies are only formed by Sebacinales of the clade A. In contrast, clade B Sebacinales are found in autotrophic orchids (Warcup 1988; Bougoure et al. 2005), liverwort (Kottke et al. 2003) and Ericaceae (Berch et al. 2002; Allen et al. 2003; Bougoure and Cairney 2005; Setaro et al. 2006).

Weiß et al. (2011) reported that Sebacinales are extremely versatile in their mycorrhizal associations and universally present as symptomless endophytes. They were found in field specimens of bryophytes, pteridophytes and all families of herbaceous angiosperms which included liverworts, wheat, maize and the non-mycorrhizal model plant *Arabidopsis thaliana*. They have also detected these fungi in herbarium specimens originating from pioneering field trips to North Africa in the 1830s/1840s. No geographical or host patterns were detected. Their data suggested that the multitude of mycorrhizal interactions in Sebacinales may have arisen from an ancestral endophytic habitat by specialisation. Considering their proven beneficial influence on plant growth and their ubiquity, endophytic Sebacinales may be a previously unrecognised universal hidden force in plant ecosystems.

An amazing morphological and physiological diversity among numerous different types of these plant–fungal interactions has been observed. No other fungal group showed a diversity of mycorrhizal types comparable to that found in the Sebacinales, a basidiomycetous order (Weiß et al. 2004; Glen et al. 2002; Urban et al. 2003; Selosse et al. 2002a, b, 2007; Kottke et al. 2003; Setaro et al. 2006; McKendrick et al. 2002). DNA sequence analyses have demonstrated a high phylogenetic diversity in this group, which is divided into two distinct subgroups, informally designated group A and group B. Though it is known from molecular phylogenetic analyses that Sebacinales belong to the mushroom-forming

Basidiomycetes (Agaricomycotina) (Hibbett et al. 2007), only a few sebacinalean morphospecies producing basidiomes have been described, all of them belonging to group A. Morphological data on group B Sebacinales is very sparse.

1.2.1 Sebacina vermifera

In the year 1967, Warcup and Talcut identified *Sebacina vermifera* from the roots of Australian terrestrial orchids of the genera Microtis, Cyrtostylis and Eriochilus by their telemorphs, as forming mycorrhizas.

1.2.2 Piriformospora indica

Varma and his collaborators first described the filamentous fungus *Piriformospora indica* in 1998 as a cultivable, mycorrhiza-like fungus. The fungus was originally found in soil samples from the rhizosphere of the woody shrubs *Prosopis juliflora* and *Zizyphus nummularia* growing in the western part of Rajasthan, which is a typical desert region of the Indian subcontinent (Thar). The fungus was named *Piriformospora indica* based on its characteristic pear-shaped chlamydospores (Verma et al. 1998). Comparisons of 18S rDNA sequences indicated that a similar kind of fungus apparently occurs in the rhizosphere soil of *Leptochloa fusca* or *Oryza minuta* in Pakistan and in the Philippines, respectively. Later it was also found from foothills of Himalayas in India, Pakistan, Philippines, Australia, Portugal, Italy and several South American countries (Varma et al. 2001, 2012b, c).

1.2.3 Piriformospora williamsii

Basiewicz et al. (2012) have reported a new species of genus *Piriformospora* from the spore of *Glomus fasciculatum*. *Piriformospora williamsii* is a new member of the so far monotypic genus *Piriformospora* and shows that this genus contains still undescribed species that were recently discovered as endophytes of field-collected specimens of Anthyllis, Medicago and Lolium in Germany.

1.3 Taxonomy and Genome Sequencing

Molecular phylogenetic analyses (Weiß et al. 2004) have revealed that *P. indica* is a member of the basidiomycetous order Sebacinales (Basidiomycota: Agaricomycetes) (Hibbett et al. 2007; Qiang et al. 2012; Weiß et al. 2011). Molecular sequence data are useful for systematics where morphological characters

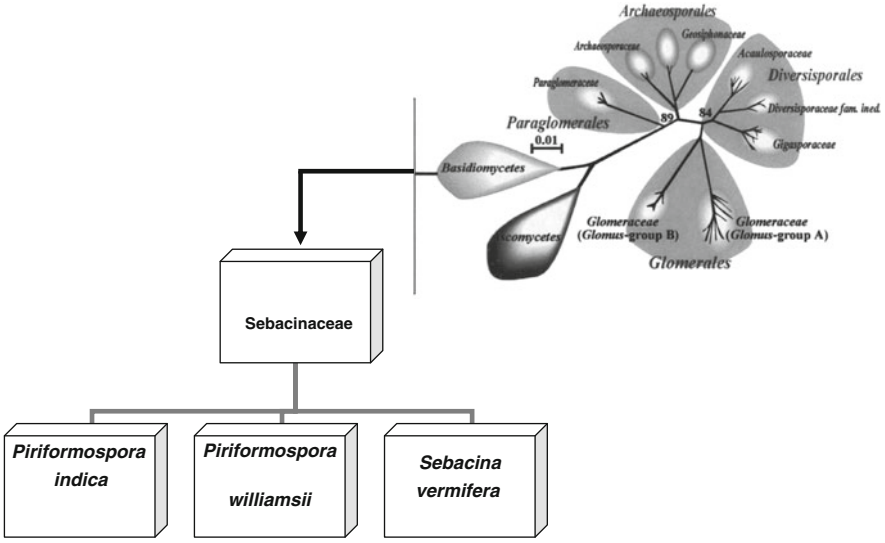

Fig. 1.2 Molecular taxonomic position of *P. indica* and in relation to symbiotic arbuscular mycorrhizal fungi

like the taxonomically decisive sexual states are missing. A neighbour-joining analysis of partial 18S rDNA sequences (525 nucleotide position) placed *P. indica* within the Basidiomycota close to the *Rhizoctonia solani* group (Ceratobasidiales). A maximum likelihood analysis on almost complete 18S rDNA sequences (1,550 nucleotide positions) confirmed this assignment. Similar results were obtained by distance and parsimony methods. A comprehensive phylogenetic analysis of Rhizoctonia using sequences from mitochondrial and nuclear rDNA on more representatives should provide an insight into the evolution of this important group and clearly the evolutionary relationship of *P. indica* and Rhizoctonia within the Hymenomycetes (Fig. 1.2). A 28S rDNA showed no change to the taxonomic status (Varma et al. 2001, 2002). Evaluations of 18S rDNA clone libraries suggested that the fungus was not abundant in any of these rhizospheres. Due to primitive similarities to Zygomycetes, this fungus is termed as AM-like fungus (Franken et al. 2000).

Nonetheless, *Sebacina vermifera* sensu, a fungus isolated from Bavaria, contains 28S rRNA (Oberwinkler 1964; Warcup and Talbot 1967; Weiß and Oberwinkler 2001). Therefore it is closely fitted to the family Sebacinaceae. The highly conserved 5.8s gene regions of the Neottia fungi are statistically similar to GenBank entries for many fungi. *S. vermifera* and *P. indica* are similar to the two Neottia fungi in the 5.8s gene region (McKendrick et al. 2002). Despite many similarities in macroscopical and also ultrastructural characters (Bandoni 1984), the Sebacinaceae are separated from the remaining taxa of Auriculariales sensu Bandoni in the present analysis, which is consistent with the previous molecular phylogenetic analysis of Weiß and Oberwinkler (2001).

Recent study reported 25 Mb genome of *P. indica* (Zuccaro et al. 2009, 2011). The main features of *P. indica* genome sequence include 50.68 % GC content, 4.68 % repeat rate, 11,769 protein-coding genes, 5.16 average exons per gene, gene density of 471(number of gene per Mb), 867 secreted proteins, 386 small secreted proteins (SSP), 3,134 unique gene models, 197 unique SSP and 58 tRNA genes. This breakthrough research is the first in-depth genomic study which describes a mutualistic symbiont with a biphasic lifestyle through extensive comparative analysis of the *P. indica* genome with other Basidiomycota and Ascomycota fungi that have diverse lifestyle strategies identified features typically associated with both, biotrophism and saprotrophism. The tightly controlled expression of the lifestyle-associated gene that sets during the onset of the symbiosis was revealed by microarray analysis which argues for a biphasic root colonisation (or biphasic lifestyle) strategy of *P. indica*. Cytological studies have shown an early biotrophic growth followed by a cell death-associated phase for its support. About 10 % of the fungal genes induced during the biotrophic colonisation have encoded putative small secreted proteins (SSP), including several lectin-like proteins and members of a *P. indica*-specific gene family (DELD) with a conserved novel seven-amino acid motif at the C terminus. Similar to effectors found in other filamentous organisms, the occurrence of the DELDs correlated with the presence of transposable elements in gene-poor repeat-rich regions of the genome.

Their study with *P. indica* has provided a global characterisation of fungal transcriptional responses associated with the colonisation of living and dead barley roots. The findings on the genome of *P. indica* have given an insight in understanding development of biotrophic plant symbionts and suggested a series of incremental shifts along the continuum from saprotrophy towards biotrophy in the evolution of mycorrhizal association from decomposer fungi. Basiewicz et al. (2012) have described a new species of *Piriformospora williamsii* and have established its phylogenetic relationship with other members of Sebacinales (Fig. 1.3).

1.4 Morphology of Sebacinales

In contrast to AM fungi, *P. indica* (a root endophyte) grows on a wide range of synthetic simple and complex media (Varma et al. 1999), for example, minimal media (MM1) normally used for in vitro germination of AM fungi with 2 % glucose as carbon source and on different media for *Aspergillus* medium and Moser b medium (Pham et al. 2004a, b). Mass cultivation of the fungus can be easily achieved on simplified broth culture. *P. indica* can be mass multiplied on a very cost-effective energy source "Jaggery" obtained from extract of *Saccharum officinarum* (patent 944/DEL/2012). Mycelium utilises a wide variety of inorganic and organic phosphate chemicals and produces acid phosphatases at the tip of hyphae (Singh et al. 2003). The morphology of the mycelium strongly varies depending on the composition of the nutrients of the culture medium and the conditions of cultivation (solid or liquid media). Most of the mycelium of *P. indica*

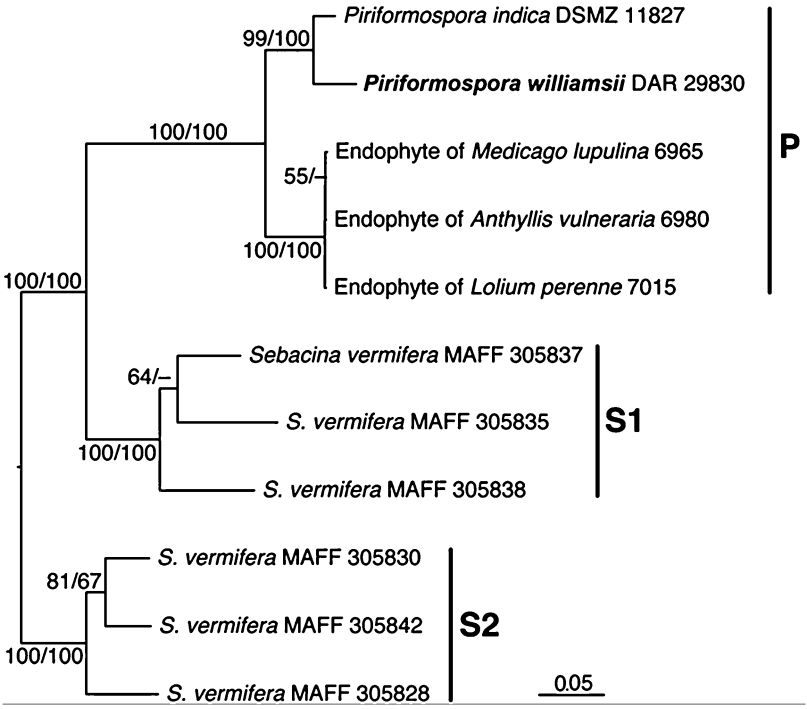

Fig. 1.3 Phylogenetic relationships of *P. williamsii*. The tree was derived by ML analysis (RAxML GAMMACAT) from a concatenated alignment of partial TEF, ITSD5.8S and partial 28S rDNA sequences. Branch labels are bootstrap values inferred from 1,000 replicates using ML/MP; values below 50 % are denoted with L. Rooting was inferred from an ML analysis of an ITSD5.8SD28S alignment, which additionally included an outgroup sequence of *Craterocolla cerasi* (Sebacinales group A). Branch lengths are given in terms of the expected number of substitutions per nucleotide (c.f. Basiewicz et al. 2012)

grows under the surface (subsurface) of the agar media. Using solid culture media, only few aerial hyphae are formed. The mycelium grows concentrically and covers the agar media homogenously. Sometimes the mycelium forms rhythmic rings in the petri dishes. Young mycelia cultures are white, but in age the colour turns to cream yellow. The structure of the mycelium is homogenous; no specialised hyphae could be observed, but some hyphae are arranged in coils. The patterns of the hyphal ramification are regular with the branching of acute and up to 90° angled hyphae. In submersed shaking cultures, the mycelia are conglomerated to small wadding-like globose balls (see Chap. 2 of this volume). The temperature range of the fungal growth is 20–35 °C, optimum temperature and pH being 30 °C and 5.8, respectively.

Morphologically, until now no fruit bodies and no basidia are formed by the mycelium within the cultures. The propagation of *P. indica* is only done by chlamydospores or by mycelium (see Chap. 2 of this volume).

P. indica hyphae are white and almost hyaline. Hyphae are thin walled and of different diameters ranging from 0.7 to 3.5 μm. Septate hyphae often show

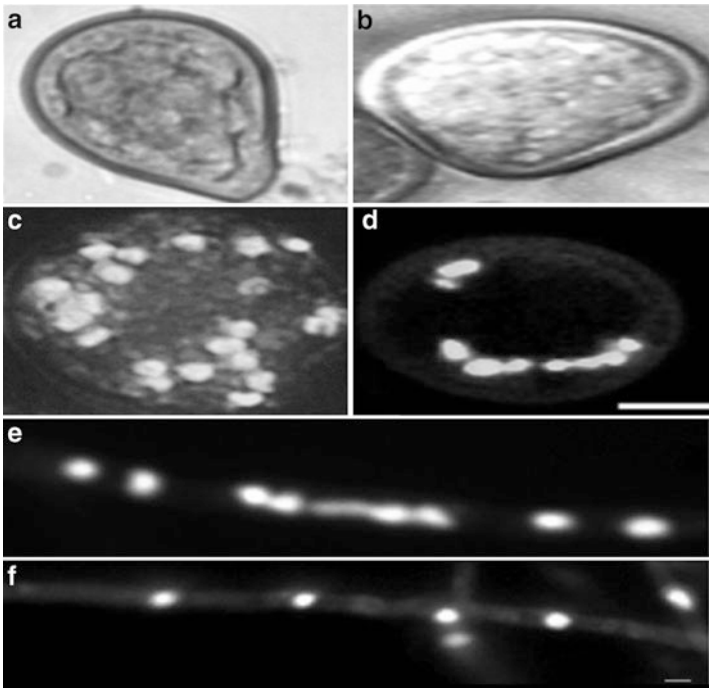

Fig. 1.4 DAPI-stained nuclei from *Piriformospora williamsii* (ex multinucleate Rhizoctonia DAR 29830) and from *P. indica*. (**a**) Bright field microscopy image of chlamydospores from *P. indica* (size ranging from 16 to 25 μm length and 10 to 17 μm in width). (**b**) Bright field microscopy image of chlamydospores from *P. williamsii* (size ranging from 8 to 12 mm), bar 7 mm. (**c**) DAPI-stained chlamydospores from *P. indica* (chlamydospore are multinucleate with up to 25 nuclei per cell). (**d**) DAPI-stained chlamydospores from *P. williamsii* (chlamydospore is multinucleate with up to ten nuclei per cell), bar 7 mm. (**e**) DAPI-stained hyphae of *P. williamsii* showing multinucleate cells, bar 2 mm. (**f**) DAPI-stained hyphae of *P. indica* showing a more regular distribution of nuclei in the hyphae. Measurements of the size of the hyphae and chlamydospores were performed using the Leica Confocal Software (LCS) in a confocal laser scanning microscope, Leica TCS SP2 (Leica, Bensheim, Germany)

anastomosis and irregularly septate. The hyphae are highly interwoven and often adhered together and give the appearance of simple intertwined cords. New branches emerge irregularly, and the hyphal walls show some external deposits (polysaccharides or some hydrophobic proteins) at regular intervals which stain deeply with toluidine blue. Since septation is irregular, the single compartments could contain more than one nucleus. The chlamydospores appear singly or in clusters with distinctive pear-shaped structure (Fig. 1.4). The chlamydospores were of 16–25 μm length and 10–17 μm in width. Each spore contains 8–25 nuclei. Very young spores have thin hyaline walls. At maturity, these spores have walls up to 1.5 μm thick. So far, neither clamp connections nor sexual structures could be observed (Varma et al. 2001).

Newly discovered species, *P. williamsii* is morphologically very similar to *P. indica*. The mycelium of *P. williamsii* is mostly flat and submerged into the medium. No aerial mycelium is observed. Similar to *P. indica*, this strain is also multinucleate and contains 2–6 nuclei. Chlamydospores are mostly spherical to rarely pear shaped. The spores are 8–12 μm in diameter and contain up to ten nuclei.

1.5 Functions of Sebacinales

P. indica is extremely versatile in its mycorrhizal associations capable of colonising members of bryophytes, pteridophytes, gymnosperms and angiosperms (Varma et al. 2012a, b). On root colonisation, endophyte increases nutrient uptake; allows plants to survive under water, temperature and salt stress and confers (systemic) resistance to toxins, heavy metal ions and pathogenic organisms along with other beneficial effects such as plant growth promotion, early flowering, higher seed yield and alteration in the secondary metabolites.

1.5.1 Plant Promotional Property

To author's best knowledge, no study has been conducted on the function of Sebacina and *P. williamsii*. *P. indica* displays an endophytic lifestyle and has the ability to colonise the roots of a wide range of mono- and dicotyledonous plants, including members of the Brassicaceae (e.g. *Arabidopsis thaliana*) which are known as non-host plants for ecto- and arbuscular mycorrhiza (Peskan-Berghofer et al. 2004). Plants colonised by *P. indica* display a wide range of beneficial effects including enhanced host growth (Table 1.1) and resistance to biotic and abiotic stresses, promotion of adventitious root formation in cuttings and enhanced nitrate and phosphate assimilation (Zuccaro et al. 2011).

Interaction of *P. indica* with barley has been extensively studied. It has shown growth-promoting activity by enhancing barley grain yield (Waller et al. 2005). Deshmukh et al. (2006) reported studies on mutualistic symbiosis of *P. indica* with barley and revealed that *P. indica* causes host cell death for proliferation in differentiated barley roots. Baltruschat et al. in 2008 studied biochemical mechanisms underlying *P. indica*-mediated salt tolerance in barley with special focus on antioxidants. *P. indica*-colonised barley roots in salt stress conditions had increased plant growth, elevated amount of ascorbic acid and increased activities of antioxidant. These findings have suggested that antioxidants might play role in both inherited and endophyte-mediated plant tolerance to salinity as reported.

Brassica napus L., a member of the Brassicaceae family, is an agriculturally and economically significant oilseed crop worldwide. Chen et al. (2012) reported the effects of *P. indica*-inoculated *B. napus* seedlings and analysed its developmental stages. The fungus-treated plants showed significant increase in the size and numbers of their leaves and the weights of their fresh roots, dry roots and shoots;

Table 1.1 Host of plants interacting with Sebacinales

Aneura pinguis Dumort	*Cicer arietinum*
Adhatoda vasica syn.	*Aristolochia elegans*
Daucus carota	*Arachis hypogaea*
Petroselinum crispum	*Medicago sativa*
Centella asiatica	*Glycyrrhiza glabra*
Cuminum cyminum	*Abrus precatorius*
Foeniculum vulgare	*Mimosa pudica*
Carum copticum	*Vigna unguiculata*
Coriandrum sativum	*Glycyrrhiza glabra*
Artemisia annua	*Acacia catechu* Willd.
Spilanthes calva DC	*Nilotica* Willd.
Stevia rebaudiana	*Prosopis chilensis* Stuntz sys.
Calendula officinalis	*P. juliflora* DC.
Arnica spp.	*Abrus precatorius*
Avena sativa	*Cicer arietinum*
Arabidopsis thaliana Heynh.	*Dalbergia sissoo* Roxburgh
Cassia angustifolia Senna Patti	*Curcuma longa*
Cajanus cajan	*Lathyrus odoratus*
Terminalia arjuna	*Pisum sativum*
Tephrosia purpurea Pers.	*Fragaria vesca*
Salvia officinalis	*Glycine max* Merr.
Mentha piperita	*Quercus robur* (clone DF 159)
Allium cepa	*Cymbopogon martinii* Staph Van motia
Allium sativum	*Colchicum luteum*
Chlorophytum borivilianum Baker	*Aloe vera* syn.
C. tuberosum Baker	*Zea mays* white
Azadirachta indica A. Juss	*Coffea arabica*
Helianthus annus	*Elettaria cardamomum*
Dactylorhiza fuchsii Druce	*Asparagus racemosus*
D. purpurella	*Populus tremula*
D. majalis Rchb. f.	*P. tremuloides* Michx. (clone Esch5)
D. maculata	*Bacopa monnieri*
D. incarnata	*Oryza sativa*
Setaria italica	*Secale cereale*
Sorghum vulgare Burm. fil.	*Saccharum officinarum*
Hordeum vulgare	*Panicum miliaceum*
Secale cereale	*Zingiber officinale*
Sorghum vulgare	*Coleus forskohlii*
Triticum aestivum	*Hyoscyamus niger*
Zizyphus nummularia Burm. fil.	*Datura stramonium*
Atropa belladonna	*Withania somnifera*
Solanum melongena	*Papaver somniferum*
Picrorhiza kurroa	*Ruta graveolens*
Tectona grandis	*Mentha piperita*
Physalis peruviana	*Arabidopsis thaliana*
Petunia hybrida	*Beta vulgaris*
Nicotiana tobaccum	*Brassica napus*

(continued)

Table 1.1 (continued)

N. attenuata	*Aristolochia elegans*
Solanum lycopersicum	*Tecoma radicans*
Tulsi	*Vinca rosea*
Brassica nigra	*Hemidesmus indicus*
Brassica oleracea var. botrytis	*Brassica oleracea* var. capitata
Spinach	Bamboo

early flowering and increased seed yield and oil content. Nutritional analysis revealed that fungus-treated plants had reduced erucic acid and glucosinolate contents and increased accumulation of N, P, K, S and Zn. Also, RT-PCR results showed that the expression of Bn-FAE1 and BnECR genes, encoding enzymes responsible for regulating erucic acid biosynthesis, was downregulated at mid and late life stages during seed development in colonised plants (Binggen Lou-personal communication). Thus the results confirmed that *P. indica* plays an important role in enhancing growth, seed yield and seed quality of *B. napus*.

1.5.2 Biotic Stress Cocultivation with Pathogenic Fungi

P. indica supports the host by protecting it from pathogenic fungi (Waller et al. 2005). The underlying mechanism of the fungus beneficial activity is not yet understood, but it is observed that the plant antioxidant system is activated and thus implicated in the improvement of abiotic and biotic stress tolerance (Waller et al. 2005; Deshmukh and Kogel 2007; Druge et al. 2007). Kumar et al. (2009) reported the bioprotection performance of *P. indica* against the root parasite *Fusarium verticillioides* in maize and observed stimulated increase in biomass, root length and root number compared to the plants grown with *F. verticillioides* alone. The increased activity of antioxidant enzymes minimised the chances of oxidative burst (excessive production of reactive oxygen species), and therefore, *F. verticillioides* might be protected from the oxidative defence system during colonisation. The decrease of antioxidant enzyme activities due to the presence of *P. indica* helped the plant to overcome the disease load of *F. verticillioides* (Kumar et al. 2009). They also suggested that *P. indica* may target a not-yet-identified signalling pathway to induce systemic resistance. Transmission electron microscopy studies in *Arabidopsis–P. indica* interaction have demonstrated that root colonisation is initiated by a biotrophic colonisation phase followed by a later cell death-dependent phase (Schäfer et al. 2007; Schäfer and Kogel 2009). The effect of *P. indica* as bioprotector on barley was assessed under seminatural conditions using Mitscherlich pots (Waller et al. 2005). In the reported work, the interaction between the plant and the fungus was established in growth chambers, followed by incubation outdoors. Under these conditions *P. indica* acted as both a biofertiliser and a biocontrol agent (Waller et al. 2005). The performance of

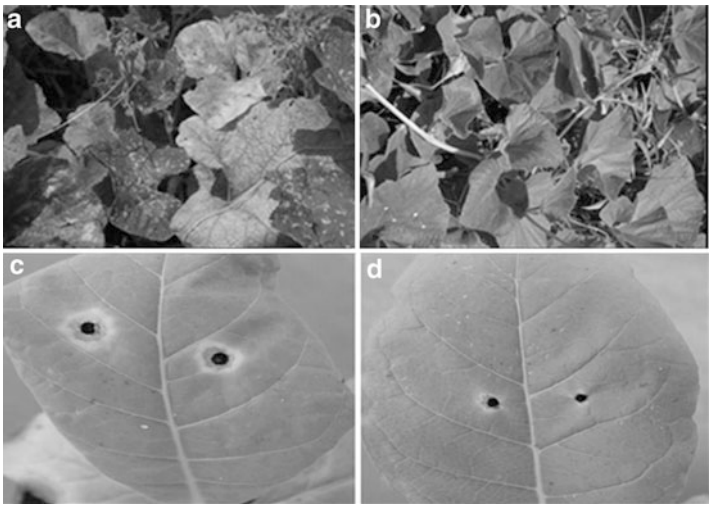

Fig. 1.5 *P. indica* protects against plant pathogens like fungi and viruses. (**a**) Bottle gourd infested with insects and virus in the field; (**b**) bottle gourd plants treated by *P. indica* are healthy; (**c**) Alternaria longipes infection status of untreated *Nicotiana tabacum*; and (**d**) *P. indica*-colonised plants (Courtesy Amit Kharkwal and Binggan Lau)

P. indica under field conditions against another pathogenic fungus *B. graminis* is reported by Serfling et al. (2007). The authors showed that the symptoms caused by the leaf pathogen in *P. indica*-colonised plants did not differ from those which were not colonised.

In another experiment in which wheat was treated with the pathogenic fungus Pseudocercosporella herpotrichoides, a significant decrease in the disease in the *P. indica*-colonised plants compared to non-colonised ones was observed. Similar results were obtained with the root-pathogenic fungus *Cochliobolus sativus*. In axenic culture, *P. indica* does not exhibit antifungal activity to *F. culmorum* or *C. sativus* indicating that the protective potential of the endophytic fungus does not rely on antibiosis. Besides the plant growth promotional effect, the disease symptoms in the control plants are visible in contrast to plants inoculated with *P. indica* in which its mutualism was established; those plants showed no or mild symptoms of the diseases. In ongoing studies, the control plants devoid of *P. indica* showed higher-percentage disease index against *Alternaria longipes* and *Colletotrichum falcatum* (Fig. 1.5). These results demonstrate that *P. indica* exerts beneficial activity against two major cereal pathogens that cause enormous worldwide economic losses (Waller et al. 2005). *P. indica* showed profound effect on disease control when challenged with a virulent root and seed pathogen *Gaeumannomyces graminis* (Varma et al. 2001; Serfling et al. 2007). The pathogen growth was completely blocked by *P. indica* indicating that the fungus acted as a potential agent for biological control of root diseases; however, the chemical nature of this inhibitory factor is not yet known. *P. indica* synthesises secondary metabolites like hydroxamic acids (DIBOA, DIMBOA) which act as natural pesticides (Varma et al. 2001).

Interaction of *P. indica* on eight dominant and virulent plant pathogenic fungi has been reported which includes *Alternaria* sp., *Fusarium oxysporum*, *F. udum*, *Colletotrichum falcatum*, *Verticillium* sp., *Rhizoctonia bataticola* and *R. solani* (Tripathi and Varma 2010). The effect of *P. indica* against pathogen based on inhibitory activity by dual bixenic culture technique, volatile metabolites against pathogen, *P. indica* culture filtrate and role of lytic enzymes in pathogen inhibition. They reported major inhibitory activity using potato dextrose agar media in *C. falcatum* and *Verticillium* sp., clearly indicating lytic effect on the mycelia and moderate inhibitory activity against *F. oxysporum*, *F. udum* and *R. bataticola* with clear area of inhibition, while *Alternaria* sp. and *S. rolfsii* showed least inhibitory response among all the pathogens. *P. indica* volatile metabolites showed inhibitory activity against all eight pathogenic fungi with maximum growth of inhibition in *R. bataticola* in comparison to others. Also, *P. indica* culture filtrate adversely affected the growth and general morphology of all the pathogenic fungi that revealed severe deformity, crinkling and hyphal coiling in broth cultures followed by Rose Bengal staining which showed hyphal lysis in case of all pathogenic fungi indicating involvement of fungal cell wall-degrading enzymes. Further, role of lytic enzymes produced by culture filtrate in pathogen inhibition was reported which indicated positive production of laminarinase (β-1, 3 exoglucanase) and chitinase enzymes (Tripathi and Varma 2010).

1.5.3 Abiotic Stress

Abiotic stresses have become an integral part of crop production. In general, plants suffer from dehydration or osmotic stress under drought, salinity and low temperature condition which causes reduced availability of water (dehydration) for cellular function and maintenance of cellular turgor pressure.

P. indica has been shown to increase resistance against tolerance to abiotic stress in plants. Fungal colonisation has shown to promote plant growth which is a characteristic effect and can also be observed under stress conditions. *P. indica* modulates the defence system and alters the metabolism to compensate the loss in photosynthesis and prevent oxidative damage caused by stress. During salt and drought stress, *P. indica* maintains a high antioxidative environment by primarily inducing the defence system, especially the ascorbate–glutathione (ASH–GSH) cycle. Also, it has shown to induce several antioxidative enzymes during salt and drought stress that are involved in detoxification of reactive oxygen species such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POD), mono-dehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). *P. indica* also increased the level of osmolytes (polyamines, proline, etc.), in response to salinity and drought stress. Interplay of antioxidative environment mediated by ASH and osmolytes (polyamine, proline, etc.) along with strong activity of antioxidative enzyme system leads to maintenance of plastid integrity and therefore enhanced photosynthetic

efficiency in colonised plant during abiotic stress. In addition, *P. indica* has also shown to induce salt and drought stress-responsive genes of the plant, which may play an important role in enhanced abiotic stress tolerance of crop plants (Kumar et al. 2012).

1.5.3.1 Cold

Influence of *P. indica* on seed germination at extreme low temperatures was studied in 12 leafy vegetable plants in an experiment which was carried out in Leh–Ladakh (DIHAR, DRDO laboratory), India at an altitude of 3,500 m. In a period of 25 days up to 100 %, seed germination was observed in case of cabbage, endive, Swisschord (Palak), Swisschord (Red), radish and onion, whereas not a single seed germinated in untreated control. In about 3 months' growth in microplots, significant increase in cabbage, cauliflower's heads and beetroot bulbs was recorded (Varma et al. 2012a; Murugan 2011). As *P. indica* was screened from extreme hot sand dunes of Western Rajasthan, India (+40–50 °C), this fungus has also interacted with leafy hosts at extreme low temperature (−30 to +4 °C) thereby demonstrating unique features which are not yet described in the literature (see Patent vide no. 709/DEL/2011 dated 15th March 2011).

1.5.3.2 Salt Stress

Interaction between barley and *P. indica* has been studied with respect to salt tolerance, and findings have suggested that barley was able to tolerate moderate salt stress (100 mM) in hydroponic culture (Waller et al. 2005). They investigated salt stress tolerance in barley leaves which were exposed to moderate (100 mM NaCl) and high (300 mM NaCl) salt concentrations in hydroponic culture. They found that detrimental effect of moderate salt stress was completely abolished by *P. indica*, with a higher biomass gain. However, under high salt stress conditions, both noninfested and infested plants exhibited a severe biomass reduction. Also, *P. indica* has shown to induce elevated amount of ascorbic acid and increase activities of antioxidant enzymes in barley roots during its symbiotic association under salt stress conditions suggesting that antioxidants might play a role in both inherited and endophyte-mediated plant tolerance to salinity (Baltruschat et al. 2008).

Salt stress on axenic growth of *P. indica* has been recently reported which suggests that *P. indica* can tolerate salt concentrations up to 219.14 mM, i.e., about 0.2 M (Chordia 2011) Interaction of *P. indica* with extremely sensitive crop species (glycophytes) may help them in salt tolerance and provide a helping hand in sustainable agriculture.

Zarea et al. (2011) have reported an experimental study on the salt stress tolerance abilities of the endophytic fungi, *P. indica*, and *Azospirillum* strains, isolated from nonsaline and saline soil, at five NaCl levels (0, 0.1, 0.2, 0.3, 0.4,

0.5 mol L^{-1}) followed by a greenhouse experiment for analysing the effects of these selected microorganisms under increasing salinity levels on seedling growth, solute accumulation (proline and sugars) and photosynthetic pigments (Chl a, b, ab) of seedling wheat. They found that the upper threshold of *P. indica* salinity tolerance was 0.4 mol L^{-1} NaCl in both liquid and solid broth medium and that of salt-adapted and non-salt-adapted *Azospirillum* strains were 0.2 and 0.4 mol L^{-1} NaCl, respectively. *P. indica* had a greater threshold for salinity tolerance than both salt-adapted and non-salt-adapted *Azospirillum*. Their results indicated a positive influence of the organisms on salinity tolerance, more with the saline-adapted diazotrophs than the nonsaline-adapted strains. They concluded that the results could be related to a better water status, higher photosynthetic pigment contents and proline accumulation in wheat seedlings inoculated with *P. indica*.

Green house experiments revealed that inoculation with the two isolates has increased salinity tolerance of wheat plants; the saline-adapted *Azospirillum* strains showed better performance with respect to improved fresh and dry weights at 80 and 100 days after sowing under both nonsaline and saline conditions. When compared to plants inoculated with nonsaline-adapted *Azospirillum* strains, those inoculated with adapted *Azospirillum* strains had much better performance with respect to the presence of photosynthetic pigment (Chl a, b and ab) and proline accumulation. The levels of proline in the leaves were found to increase in inoculated plants in response to inoculation with two isolates as compared to uninoculated plants among which *P. indica* showed greater proline accumulation in leaves, while the difference in sugar concentrations was not found to be significant under saline conditions or between inoculated and uninoculated plants. Overall, results indicated that *P. indica* was more tolerant to salt stress than *Azospirillum* strains and the symbiotic association between *P. indica* fungus and wheat plants improved wheat growth, regardless of the salinity (Kumar et al. 2012).

1.6 Physiology and Biochemistry of *P. indica*

1.6.1 Phytohormones

PYK10 is required for the beneficial interaction between *Arabidopsis* and *P. indica*. PYK10 codes for a root- and hypocotyl-specific b-glucosidase/myrosinase, which is implicated to be involved in plant defences against herbivores and pathogens and located in the endoplasmic reticulum (Varma et al. 2012a).

1.6.1.1 Mineral Transport

The members of Sebacinales are established to transport a variety of essential nutrients responsible for plant productivity and to accomplish the metabolic

Fig. 1.6 The Ratoon crop of sugar cane receiving the fungus had dark green leaves where the controls showed light green to yellow leaves, a typical sign of sulphur deficiency photographed after one year of field trial

Table 1.2 Soil analysis

Treatments	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	K (%)	P (%)	S (%)
Control	202.2	25.0	4.9	1.87	0.24	0.086	0.095
P. indica	281.4	30.2	10.8	1.31	0.40	0.088	0.092
CD (5%)	47.07	NS	0.88	0.33	NS	NS	NS

A field trial experiments were performed at Punjab Agricultural University, Ludhiana (courtesy—Dr. Gosal)

activities. In one of the experiments in sugarcane under field trial, *P. indica* obviated the iron deficiency in sugarcane (Fig. 1.6, Table 1.2). This fungus is also known to reduce the toxicity caused by heavy metals (Bhardwaj 2011).

1.6.2 Phosphate Transport

Phosphorus is an essential mineral nutrient for the plant growth. Plants acquire this mineral from the environment either directly by their roots or indirectly from mycorrhizal fungi which form inter- or intracellular symbiotic association with the roots. AM colonisation may increase the rate of phosphorus accumulation beyond the limits which can be currently utilised, thus reducing the rate of phosphorus utilisation efficiency. Such momentary "luxury consumption" of phosphorus may, however, serve a storage function and be utilised subsequently, allowing mycorrhized plants ultimately to outperform non-mycorrhized plants (Koide 1991). The 32P experiments suggested that *P. indica* is important for P-acquisition by the root especially in the arid and semiarid regions.

Recently cloning and functional analysis of a gene encoding a phosphate transporter (PiPT) from this root endophyte has been reported (Yadav et al. 2010). The

PiPT polypeptide belongs to the major facilitator superfamily (MFS) and exhibits 12 transmembrane helices divided into two halves that are connected by a large hydrophilic loop in the middle. The function of the protein encoded by PiPT was confirmed by complementation of a yeast phosphate transporter mutant. PiPT belongs to high affinity phosphate transporter family (Pht1). Electroporation and RNA interference were used to prepare knockdown (KD) transformants of the gene for understanding the physiological role of PiPT. KD transformants transported a significantly lower amount of phosphate to the host plant than wild-type *P. indica*. Higher amounts of phosphate were found in plants colonised with wild-type *P. indica* than that of non-colonised and plants colonised with KD-PiPT *P. indica*. These observations suggested that PiPT is actively involved in the phosphate transportation, and in turn *P. indica* helps to improve the nutritional status of the host plant (Yadav et al. 2010; Kumar et al. 2011).

1.7 Conclusion and Future Directions

The purpose of this chapter is to review the origin, structure, taxonomy status and various important functions of the members of Sebacinales. Information known till date is summarised as follows: plant promoter, induces early flowering, value addition to medicinal plants, active phosphate transporter, nitrate/nitrite reductase activity, novel phytohormone, potent biocontrol agent and helps plant to overcome biotic and abiotic stresses. The future research may be focused on what is the exact mechanism for plant promotion; bio-protection against virus and phytopathogens; resistance against high temperature, low temperature, salinity and nutrient stress tolerance; enhancement by nanomaterials; what is the active ingredient in culture filtrate promoting plant growth and what triggers the early flowering, and *P. indica* is looking for a partner to activate sexual stages.

References

Allen TR, Millar T, Berch SM, Berbee ML (2003) Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. New Phytol 160:255–272

Arora M, Kharkwal AC, Bains KS, Varma A (2012) Novel and new nutrient for cultivation of Piriformospora indica. (Application no. 944/DEL/2012)

Baltruschat H, Fodor J, Borbala H, Niemczyk E, Barna B, Gullner G, Janeczko A, Kogel K, Schafer P, Schwarczinger I, Zuccaro A, Skoczowski A (2008) Salt tolerance of barley induced by the root endophyte Piriformospora indica is associated with a strong increase in antioxidants. New Phytlogist 180:501–510

Bandoni RJ (1984) The Tremellales and Auriculariales and alternative classification. Trans Mycol Soc Jpn 25:489–520

Basiewicz M, Weiß M, Kogel K-H, Langen G, Zorn H, Zuccaro A (2012) Molecular and phenotypic characterization of Sebacina vermifera strains associated with orchids, and the description of Piriformospora williamsii sp. nov. Fungal Biol 116:204–213

Berch SM, Allen TR, Berbee ML (2002) Molecular detection, community structure and phylogeny of ericoid mycorrhizal fungi. Plant Soil 244:55–66

Bhardwaj S (2011) Micronutrient enhancement in rice. Ph. D. Thesis. Amity University, Uttar Pradesh

Bonfante P, Genre A (2010) Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. Nat Commun 1:1–11

Bougoure DS, Cairney JWG (2005) Assemblages of ericoid mycorrhizal and other root-associated fungi from Epacris pulchella. Environ Microbiol 7:819–827

Bougoure JJ, Bougoure DS, Cairney JW, Dearnaley JD (2005) ITS-RFLP and sequence analysis of endophytes from Acianthus, Caladenia and Pterostylis (Orchidaceae) in southeastern Queensland. Mycol Res 109:452–460

Carroll G (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69:2–9

Chen Y-Y, Yang J, Gao Qi-K, Lin Fu-C, Lou B-G (2012) Piriformospora indica promotes growth, seed yield and quality in Brassica napus L. (Personal Communication)

Chordia PS (2011) Salt stress on axenic growth of Piriformospora indica. M.Phil Thesis, Amity University Uttar Pradesh

Deshmukh S, Kogel KH (2007) Piriformospora indica protects barley from root rot disease caused by Fusarium. J Plant Dis Protect 114:263–268

Deshmukh S, Huckelhoven R, Schafer P, Imani J, Sharma M, Weiss M, Waller F, Kogel K (2006) The root endophytic fungus requires hosts cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Druge U, Baltruschat H, Franken P (2007) Piriformospora indica promotes adventitious root formation in cutting. Sci Hortic 112:422–426

Franken P, Requena N, Butehorn B, Krajinski F, Kuhn G, Lapopin L, Mann P, Rhody D, Stommel M (2000) Molecular analysis of the arbuscular mycorrhiza symbiosis. Arch Acker Pft Boden 45:271–286

Glen M, Tommerup IC, Bougher NL, O'Brien PA (2002) Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? Mycorrhiza 12:243–247

Harman GE (2011) Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. New Phytol 189:647–649

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Thorsten Lumbsch H, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson KH, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo JM, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüssler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N (2007) A higher-level phylogenetic classification of the Fungi. Mycol Res 111:509–547

Julou T, Burghardt B, Gebauer G, Verveiller D, Damesin C, Selosse MA (2005) Evolution of mixotrophy in orchids: insight from a comparative study of green and achlorophyllous Cephalanthera damasonium. New Phytol 166:639–653

Koide RT (1991) Tansley Review No. 29. Nutrient supply, nutrient demand and plant response to mycorrhizal infection. New Phytol 117:365–386

Kottke I, Beiter A, Weiß M, Haug I, Oberwinkler F, Nebel M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while Aneura pinguis (Metzgeriales) is associated with a Tulasnella sp. Mycol Res 107:957–968

Kumar M, Yadav V, Tuteja N, Johri AK (2009) Antioxidant enzyme activities in maize plants colonized with Piriformospora indica. Microbiology 155:780–790

Kumar M, Sharma R, Jogawat A, Singh P, Dua M, Gill SS, Trivedi D, Tuteja N, Verma A, Oelmuller R, Johri A (2011) Piriformospora indica, a root endophytic fungus, enhances abiotic stress tolerance of the host plant. In: Tuteja N, Gill SS, Tiburcio AF, Tuteja R (eds) Improving crop resistance to abiotic stress. Weinheim, Wiley-Blackwell, pp 543–548

Kumar M, Sharma R, Jogawat A, Singh P, Dua M, Gill SS, Trivedi DK, Tuteja N, Varma AK, Oelmueller R, Johri AK (2012) Piriformospora indica, a root endophytic fungus, enhances abiotic stress tolerance of the host plant. In: Tuteja N, Gill SS, Tiburcio AF, Tuteja R (eds) Improving crop resistance to abiotic stress. Wiley-Blackwell, Weinheim, pp 543–548

McKendrick SL, Leake JR, Taylor DL, Reed DJ (2002) Symbiotic germination and development of the myco-heterotrophic orchid Neottia nidus-avis in nature and its requirement for locally distributed Sebacina sp. New Phytol 154:222–247

Moyersoen B (2006) Pakaraimaea dipterocarpacea is ectomycorrhizal, indicating an ancient Gondwanaland origin for the ectomycorrhizal habit in Dipterocarpaceae. New Phytol 172:753–762

Murugan P (2011) Enhancing winter vegetable production in greenhouse of cold arid desert of Ladakh by inoculation of Piriformospora indica. DRDO Technology Spectrum, 164–170

Oberwinkler F (1964) Intrahymeniale heterobasisiomyceten. Fruchtkorperlose Sebacina- Sippen und ihre systematische Stellung. Nova Hedwigia 7:489–498

Peskan-Berghofer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmuller R (2004) Association of Piriformospora indica with Arabidopsis thaliana roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plant 122:465–477

Pham GH, Kumari R, Singh A, Sachdev M, Prasad R, Kaldorf M et al (2004a) Axenic cultures of Piriformospora indica. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Germany, pp 593–616

Pham HG, Kumari R, Singh A, Malla R, Prasad R, Sachdev M, Kaldorf M, Buscot F, Oelmueller R, Hampp R, Saxena AK, Rexer K-H, Kost G, Varma A (2004b) Axenic cultures of Piriformospora indica. In: Varma A, Abbott LK, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Germany, pp 593–613

Qiang X, Weiss M, Kogel KH, Schäfer P (2012) Piriformospora indica—mutualistic basidiomycetes with an exceptionally large plant host range. Mol Plant Pathol. doi:10.1111/J.1364-3703.2011.00764.X

Raghukumar C (2008) Marine fungal biotechnology: an ecological perspective. Fungal Divers 31:19–35

Rodriguez R, Redman R (2008) More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J Exp Bot 59:1109–1114

Schäfer P, Kogel KH (2009) The sebacinoid fungus Piriformospora indica: an orchid mycorrhiza which may increase host plant reproduction and fitness. In: Deising HB, Esser K (eds) The mycota, vol 5, Plant relationships. Springer, Germany, pp 99–112

Schäfer P, Khatabi B, Kogel KH (2007) Root cell death and systemic effects of Piriformospora indica: a study on mutualism. FEMS Microbial Lett 275:1–7

Selosse M-A, Weiß M, Jany J-L, Tillier A (2002a) Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid Neottia nidus-avis (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. Mol Ecol 11:1831–1844

Selosse MA, Bauer R, Moyersoen B (2002b) Basal hymenomycetes belonging to Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol 155:183–195

Selosse MA, Faccio A, Scappaticci G, Bonfante P (2004) Chlorophyllous and achlorophyllous specimens of Epipactis microphylla (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb Ecol 47:416–426

Selosse M-A, Setaro S, Glatard F, Richard F, Urcelay C et al (2007) Sebacinales are common mycorrhizal associates of Ericaceae. New Phytol 174:864–878

Serfling A, Wirsel SGR, Lind V, Deising HB (2007) Performance of the biocontrol fungus Piriformospora indica on wheat under greenhouse and field conditions. Phytopathology 97:523–531

Setaro S, Weiß M, Oberwinkler F, Kottke I (2006) Sebacinales form ectendomycorrhizas with Cavendishia nobilis, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. New Phytol 169:355–365

Singh A, Singh A, Kumari M, Rai M, Varma A (2003) Biotechnological importance of Piriformospora indica—A novel symbiotic mycorrhiza like fungus: an overview. Indian J Biotechnol 2:65–75

Smith S, Read D (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London

Taylor DL, Bruns TD, Szaro TS, Hodges SA (2003) Divergence in mycorrhizal specialization within Hexalectris spicata (Orchidaceae), a non-photosynthetic desert orchid. Am J Bot 90:1168–1179

Tripathi S, Varma A (2010) Development of Piriformospora indica based bioinoculant for some economicaly important plants. Ph.D thesis

Urban A, Weiß M, Bauer R (2003) Ectomycorrhizas involving sebacinoid mycobionts. Mycol Res 102:3–14

Varma A, Verma S, Sudha, Sahay N, Britta B, Franken P (1999) *Piriformospora indica* – a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol, USA 65:2741–2744

Varma A, Singh A, Sudha, Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) Piriformospora indica: an axenically culturable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) The mycota, vol 9, Fungal associations. Springer, Heidelberg, Germany, pp 123–150

Varma A, Singh AR, Sudha Sahay NS, Kumari M, Bharati K, Sarbhoy AK, Maier W, Walter MH, Strack D, Franken P, Singh AN, Malla R, Hurek T (2002) Piriformospora indica: a plant stimulator and pathogen inhibitor arbuscular mycorrhizalike fungus. In: Markandey DK, Markandey NR (eds) Microorganisms in bioremediation. Capital Book Company, New-Delhi, pp 71–89

Varma AK, Singh S, Srivastava RB, Bakshi M, Murugan P (2011) Piriformospora indica obivates extreme temperatures stress. (Application no. 709/DEL/2011)

Varma A, Bakshi M, Lou B, Hartmann A, Oelmueller R (2012) Functions of a novel plant growth-promoting mycorrhizal fungus: Piriformospora indica. Agricultural Research, NAAS (accepted)

Varma A, Tripathi S, Prasad R, Das A, Sharma M, Bakshi M, Arora M, Rastogi K, Agrawal A, Kharkwal AC, Tsimilli-Michael M, Strasser RJ, Bagde US, Bisaria VS, Upadhyaya CP, Malla R, Kost G, Joy SI, Chen Y, Ma J, Lou B, Oelmuller R (2012b) The symbiotic fungus Piriformospora indica: update. In: Hock B (ed) The Mycota XXL. Springer, Berlin, Heidelberg, New York

Varma A, Fekete A, Srivastava A, Saxena AK, Frommberger M, Li D, Gschwendter S, Sheramati I, Oelmueller R, Kopplin PS, Tripathi S, Hartmann A (2012c) Inhibitory interactions of Rhizobacteria with the symbiotic fungus Piriformospora indica. In: Varma A, Oelmueller R (eds) Sebacinales. Springer, Berlin, Heidelberg, New York

Verma S, Varma A, Rexer KH, Kost G, Sarbhoy A, Bisen P, Butehorn B, Franken P (1998) Piriformospora indica, gen. et sp. nov., a new root-colonizing fungus. Mycologia 95:896–903

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, Von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus reprograms barley to salt –stress tolerance, disease resistance and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Warcup JH (1988) Mycorrhizal associations of isolates of Sebacina vermifera. New Phytol 110:227–231

Warcup JH, Talbot PHB (1967) Perfect states of Rhizoctonia associated with orchids. New Phytol 66:621–641

Weiß M, Oberwinkler F (2001) Phylogenetic relationships in Auriculariales and related groups-hypotheses derived from nuclear ribosomal DNA sequences. Mycol Res 105:402–415

Weiß M, Selosse M-A, Rexer K-H, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Weiß M, Sýkorová Z, Garnica S, Riess K, Martos F (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS One 6:e16793. doi:10.1371/journal.pone.0016793

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathy T, Tuteja N, Saxena AK, Johri AK (2010) A Phosphate transporter from a root endophytic fungus Piriformospora indica plays a role in the phosphate transfer to the plants. J Biol Chem 285:26522–44

Yuan Z, Zhang C, Lin F (2010) Role of diverse non-systemic fungal endophytes in plant performance and response to stress: progress and approaches. J Plant Growth Regul 29:116–126

Zarea MJ, Hajinia S, Karimi N, Mohammadi Goltapeh E, Rejali F, Varma A (2011) Effect of Piriformospora indica and Azospirillum strains from saline or non-saline soil on mitigation of the effects of NaCl. Soil Biol Biochem 45:139–146, http://www.elsevier.com/locate/soilbio

Zuccaro A, Basiewicz M, Zurawska M, Biedenkopf D, Kogel K-H (2009) Karyotype analysis, genome organization, and stable genetic transformation of the root colonizing fungus Piriformospora indica. Fungal Genet Biol 46:542–550

Zuccaro A, Lahramann U, Idener U, Langen G, Pfiffi S, Biedenkop D, Wong P, Samans B, Grimm C, Basiewicz M, Murat C, Martin F, Kogel K-H (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont Piriformospora indica. PLoS Pathog 7:e1002290. doi:10.1371/journal.ppat.1002290

Chapter 2
Morphology and Ultrastructure of *Piriformospora indica*

Gerhard Kost and Karl-Heinz Rexer

2.1 History

The first finding of the fungus *Piriformospora indica* is like it happened sometimes in science; when you want to investigate and solve an unclear problem, you reveal another new phenomenon. During a routine isolation of AM fungi in the desert Thar (Rajasthan, India), *P. indica* was isolated for the first time from the rhizospheres of *Prosopis juliflora* (Schwartz) DC. and *Ziziphus nummularia* (Burm. fil.) Wt. & Arn. as a contaminant (Verma et al. 1998). Some AM spores of *Glomus mosseae* were picked from these roots. At the first superficial view, this spore looked very similar to some vesicular spore-types of Glomerales. It seems that the isolated spore is a vesicle of an AM fungus and belongs to a fungus of the Glomeromycota. But this spore was well growing on artificial complex media. Therefore this species could not be a member of the Glomeromycota because Glomeromycota are growing in biotrophic interaction with plants only. A detailed ultrastructural and molecular genetic analysis (Verma et al. 1998) elucidated, that the systematic position of this species is in the lower Agaricomycotina (Basidiomycota). It was necessary to describe the isolated fungus as a new species of the order Sebacinales, Basidiomycota. Because of the pear-like form of the spores and the geographic origin of the first collection of this fungus, we selected the name *P. indica* (Verma et al. 1998; Varma et al. 1999, 2001).

First experiments with *P. indica* revealed that the cocultivation of the fungus together with young maize seedlings causes a strong support for the growth of their shoots. Further experiments using a broad spectrum of interacting plants documented a broad growth-supporting effect of *P. indica*. Several studies revealed the different ways of the interaction between the mycelium and the host plant.

G. Kost (✉) • K.-H. Rexer
Philipps University of Marburg, FB Biology, Systematic Botany and Mycology, Karl-von-Frisch Str. 8, 35032 Marburg, Germany
e-mail: kost@biologie.uni-marburg.de; rexer@biologie.uni-marburg.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_2,

A deeper knowledge about the morphological structures of *P. indica* is necessary and important for a better understanding of the biology of this fungus and its interactions. This species is very important for basic science and technology and it has a broad spectrum of applications in agriculture and biotechnology. After the description of *P. indica*, many scientific projects have been started. Recently a new species, *Piriformospora williamsii*, of the genus *Piriformospora* was described (Basiewicz et al. 2012).

After 15 years of intensive investigations in several labors around the world, much information is revealed about this fungus. Now it is obvious that *Piriformospora* is a model species in science, but additionally it is also a very important and useful one for application in biotechnology and agriculture.

In this chapter, the morphological and ultrastructural data about this member of the Sebacinales—*P. indica*—is collected from own studies, and others are compiled from literature to give a complete overview about this quite interesting fungus.

2.2 Morphological Structures

Until now no fruit bodies and no basidia are found formed by the mycelium within the cultures. The propagation of *P. indica* is only done by chlamydospores or by mycelium.

2.2.1 Hyphal Cells

The hyphal cells of *Piriformospora* are thin walled, and they are always hyaline and not pigmented. They have an obvious small diameter in comparison to other hyphal types of the Basidiomycota. The dimensions of the hyphae strongly depend on the culture conditions. In water agar or under low nutrient conditions, the hyphal cells are very long and extremely small. Cultivating *Piriformospora* in complex media, the hyphal diameter ranges from 0.6 µm up to 3.5 µm. The hyphal length in MOM-agar is (10) 12–18 µm (20) but in water agar the hyphal cells can obtain a length of 50 µm and more. The form of the hyphae is tubular but very often the hyphae are strongly moniliform. The hyphae are regularly septated but the number of nuclei per cell is not fixed. The hyphal cells are multinucleate. There is no formation of clamp connection at the septa. Sometimes the hyphae are coiled in several layers. It is not rare that the hyphae are interconnected by several anastomoses.

Within the hyphal cells, the highest number of observed nuclei was up to 8, but in the most case, 2–6 nuclei per cell only. The single nucleus is regularly distributed within the hyphal cell. In the apical, hyphal-tip, cell, only one nucleus was observed mostly. Because no conjugated nuclei (dicaryon) could be examined within the hyphae, the nuclear stages of the hyphal cells were considered to be haploid.

But actual molecular studies revealed that *P. indica* is heterokaryotic (Zuccaro et al. 2009, 2011).

2.2.2 Mycelium

Piriformospora indica can be cultured on several synthetic and complex media; it grows under solid and liquid culture conditions. The morphology of the mycelium strongly varies depending on the composition of the nutrients of the culture medium and the conditions of cultivation (solid or liquid media). The mostly used media for *P. indica* is MYP and MA. Most of the mycelium of *P. indica* grows under the surface of the agar media. Using solid culture media, only few aerial hyphae are formed. The mycelium grows concentric and covers the agar media homogenously. Sometimes the mycelium forms rhythmic rings in the petri dishes. Young mycelium cultures are white but in age the color turns to cream yellow. The structure of the mycelium is homogenous, no specialized hyphae could be observed, but some hyphae are arranged in coils. The patterns of the hyphal ramification are regular with the branching of acute and up to 90° angled hyphae. In submerse shaking cultures the mycelia are conglomerated to small wadding like globose balls (see Fig. 2.3a).

During our infection experiments with *Populus* saplings of the *Populus* hybrid (Esch5) and *P. indica* the mycelium shows a more aggressive behavior against host plant (Kaldorf et al. 2005). This was actuated by the media conditions during the experiments. This violent effect to the poplar shoots seems to depend on nitrogen concentration of media. The mycelium overgrows the shoots of the plant, invades into the plant cell like a phytopathogenic fungus, and destroys them by infection of their plant cells of aerial parts (Kaldorf et al. 2005).

The hyphae of the mycelium never formed basidia. Warcup (1981, 1988) used several species of lower Agaricomycotina also members of the Sebacinales for his mycorrhizal experiments with orchids. He described advanced techniques for the induction of basidia of these orchid inhabiting fungal mycelia (Warcup & Talbot 1967). But all tests to induce the forming of basidia of *P. indica* failed until today. Therefore only the anamorphic stage of *P. indica* and no teleomorph are known.

2.2.3 Chlamydospores (Fig. 2.1)

The hyphae form colorless chlamydospores in culture. The chlamydospores emerge from terminal hyphal tips. Their development starts with a swollen thin-walled vesicle separated by a cell wall. The mature spores are almost globular, but one side is a little bit smaller and they look similar to the shape of a pear. The size of the mature chlamydospores varies between (14) 16 and 25 (33) µm in length and (9) 10

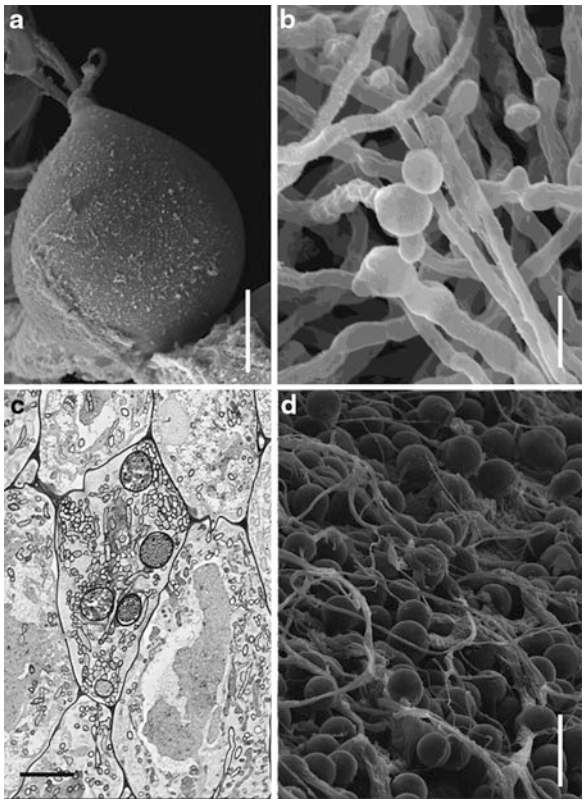

Fig. 2.1 (**a**) Chlamydospore bar = 5 μm. (**b**) Ontogenies of chlamydospores bar = 10 μm. (**c**) Endocellular formed chlamydospores bar = 30 μm. (**d**) Chlamydospore formation on petri dish culture bar = 30 μm

and 17(20) μm in width. Because of this shape of the spores, the genus name of the fungus was selected. The chlamydospore-forming hyphae are irregularly swollen near the point of the first spore formation and a little bit inflated. Young developing spores are clavate. Successively some more chlamydospores are developed at the end of short branching hyphae. Therefore, the chlamydospores are very often clustered in groups.

Their surface is smooth and at the beginning uncolored. The spore wall is thickening during the maturing process up to max 1.5 μm, and the color of the wall changes to light yellowish. The surface structure of the chlamydospores was revealed by scanning electron microscopy. The cytoplasm of the spores is refracting in the LM because it is completely filled with granular material. Many strongly refracting lipoid bodies can be observed inside the spores. The chlamydospores contain many nuclei (8–25), which can be seen after staining with DAPI by fluorescence microscopy.

On suitable media but also on water agar, the chlamydospores form a germination tube and grow with very small hyphae. The growth rate of these primary hyphae is depending on the composition of the media. In media with water or in low concentrated nutrient, the germination hyphae are unbranched over a longer distance, but in normal concentrated media, many branching hyphae can be seen.

2.3 Interaction Structures

One of the specific features of *P. indica* is the ability to interact with the roots of many plant species of different taxa. The morphology of the interacting structures depends on the involved partners. These interacting partners could be mosses, ferns, gymnosperms, and angiosperms. Depending on the taxa of the angiosperms, *P. indica* interacts with quite different modes of interaction. The behavior of *Piriformospora* during the interaction with the roots is depending on the host plant. All plant species, which are able to form one of the different mycorrhizal types, can be used by *Piriformospora* as a host. The morphological structures during the interaction with *P. indica* are quite similar to that what it is known from their common interaction structures. These different types will be described below.

Together with ectomycorrhiza-forming plants, *P. indica* promotes the plant growth also, but the fungus is not able to build up a well-developed ectomycorrhiza with mantle and Hartig net. Only a loose net of hyphae covers the surface of the roots and some intercellular growing hyphae penetrate into the roots of such plants. The root cells react to the endophytic growing mycelium with thickened walls of the adjacent cells.

In these cases, where *P. indica* penetrates the root cells, two different modes of reactions caused by the invading hyphae are known.

2.3.1 Endophytic and Necrotrophic Mode of Life

P. indica is able to destroy the cells at the beginning of the interaction and behave itself like a necrotrophic parasite in some cases (Deshmukh et al. 2006; Kogel et al. 2006; Schäfer et al. 2007). If hyphae of *Piriformospora* invade into a plant cell, the hyphal tip forms a small inconspicuous appressorium on the surface of the outer wall of the rhizoids or root cells. During the contact phase of the hyphae of *Piriformospora*, the living plant cells rarely respond by accumulating additional cell wall material near the infection site. After the plant cell wall is locally dissoluted by fungal enzymes, the hyphal tip intrudes into plant cell. But only in few cases the plant cells are able to stop the infection by the fungus. Regularly theses root cells die very quickly. Mostly the invaded root cells of plant species are killed before infection, or they die off during the contact phase of the hyphae. Therefore no additional cell wall material could be seen at the infection point. The fungal hyphae cross the cell wall with thin penetration peg. The cell content will be saprophytically decomposed by the fungus. Using FDA staining, which distinguishes living and dead cell, it can be observed that many cortex cells of the interacting root have died after an infection of the root by *P. indica*, while uninfected root cells are still alive. Very often *P. indica* develops its chlamydospores inside these dead root cells.

Table 2.1 List of some selected and tested host plants of *Piriformospora indica* compiled by literature and own results

Acacia nilotica	*Abrus precatorius*	*Adhatoda vasica*
Aneura pinguis	*Arabidopsis thaliana*	*Artemisia annua*
Azadirachta indica	*Bacopa monnieri*	*Brassica rapa*
Cassia angustifolia	*Chlorophytum borivilianum*	*Chlorophytum tuberosum*
Cicer arietinum	*Coffea arabica*	*Coleus forskohlii*
Cymbopogon martinii	*Dactylorhiza fuchsii*	*Dactylorhiza incarnata*
Dactylorhiza maculata	*Dactylorhiza majalis*	*Dactylorhiza purpurella*
Daucus carota	*Dalbergia sissoo*	*Foeniculum vulgare*
Glycine max	*Helianthus annuus*	*Hordeum sativum*
Linum album	*Medicago truncatula*	*Mentha piperita*
Nicotiana tabacum	*Nicotiana attenuata*	*Oryza sativa*
Oryza granulata	*Panicum virgatum*	*Petroselinum crispum*
Picea abies	*Pinus sylvestris*	*Piper nigrum*
Pisum sativum	*Populus tremula*	*Populus tremuloides*
Prosopis chilensis	*Prosopis juliflora*	*Quercus robur*
Saccharum officinarum	*Setaria italica*	*Solanum lycopersicum*
Solanum melongena	*Sorghum vulgare*	*Spilanthes calva*
Tectona grandis	*Tephrosia purpurea*	*Terminalia arjuna*
Thymus vulgaris	*Vigna mungo*	*Withania somnifera*
Zea mays	*Ziziphus nummularia*	

2.3.2 *Biotrophic Interaction with Living Plant Cells*

In contrast to the widely distributed necrotrophic type of interaction between the hyphae of *Piriformospora* and the root cells of plants, a small group of plants have developed an equilibrated biotrophic interaction between the hyphae of *Piriformospora* and the living cells of the plant. In case of this interaction, the invading hyphae of *P. indica* do not destroy the invaded cells of orchids and liverworts. *P. indica* establishes a typical orchid mycorrhiza or a mycothallus together with some bryophytes. This interaction could be interpreted as biotrophic and mutualistic.

Piriformospora has a wide spectrum of hosts. During the last years many plant species are tested under defined conditions. A list of tested plant species is given below (Table 2.1):

2.4 Mycorrhiza Types of *P. indica*

The members of the order Sebacinales are able to form different mycorrhizal types depending on the interacting plant species. In this book, some of these different interaction types are described in detail. During the last decade, we have studied the

wide spectrum of host plants forming different types of mycorrhiza together with *P. indica* (Glen et al. 2002).

2.4.1 Interactions with VA-Mycorrhiza-Forming Plants

The first interaction studies of *P. indica* were done with *Zea mays*, where the plant-supporting effect was observed for the first time. At the first glance the chlamydospores of *P. indica* within the root cells and outside of the roots of the plants remind to the interacting structures of an arbuscular mycorrhiza. The chlamydospores appear inside the root cortex cells and outside of the root. The hyphae penetrate the cell walls and most of the infected cells are dead. But during this infection process, the central cylinder of the root will not be invaded by hyphal cells. Many of the tested plants belongs to this interaction type.

2.4.2 Orchid Mycorrhiza (Fig. 2.2)

Under defined conditions it is tested that *P. indica* promotes the germination of orchid seeds and promotes the formation of the protocorm (Blechert et al. 1999). During the experiments it could be shown that the developing protocorm has the same morphology and anatomy during the interaction together with *P. indica* as with autochthonic fungal species. The orchids' cell is completely filled up with hyphae; the nucleus of these cells expands during the higher cell activity (Fig. 2.2a).

In some cases, especially in orchids, the root hairs of the orchids show a defense reaction against the intruding hyphae. Around the point of invasion, a small lignotuber is visible. These lignotubers are only formed if the infected plant cells are living and active during the first phase of the invasion processes (Fig. 2.2b, c). During the interaction between *P. indica* and the root cells of *Dactylorhiza majalis*, the protoplast of the rhizoid seems to be influenced by the penetration of the hyphae (Fig. 2.2a, c).

2.4.3 Ectomycorrhiza-Forming Plants (ECM Plants)

During cocultivation experiments with the ECM-forming species *Quercus robur*, an undeveloped network of hyphae covers the surface of the roots, and few intercellular hyphae that live inside the roots were observed here. Very thin hyphae intercellularly grow, but the typical hyphal arrangements such as a Hartig net and palmetti structures were not formed during our experiments.

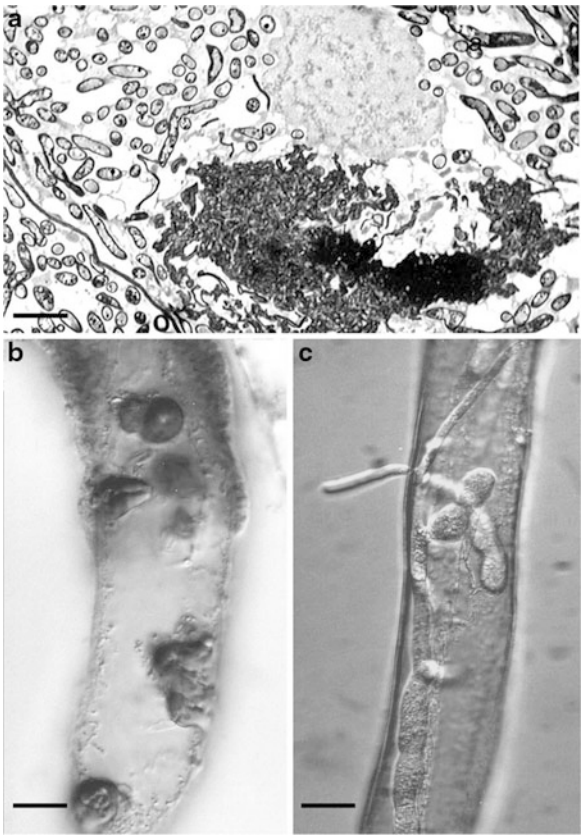

Fig. 2.2 (**a**) Infected root cells of *Dactylorhiza majalis* by *Piriformospora indica*, swollen nucleus of the orchid cell, dark material remains of a former fungal infection of *Piriformospora indica;* bar = 30 μm. (**b**) Lignotuber of a rhizoid of *Dactylorhiza majalis* after infection of *Piriformospora indica;* bar = 20 μm. (**c**) Infected rhizoid of *Dactylorhiza* with moniliform hyphae of *Piriformospora indica;* bar = 20 μm

2.4.4 Piriformospora *and* Arabidopsis thaliana *(Brassicaceae)*

The morphological structures which appear during the interaction between the hyphae *P. indica* and the root cells *Arabidopsis thaliana* are very similar to those described from other AM-forming plants (Peškan-Berghöfer et al. 2004). Although many species of the Brassicaceae are not always living together with Glomerales, *P. indica* interacts with these members of the Brassicaceae. The growth rate of the specimens, which interact with *Piriformospora*, is obviously higher. During the interaction, the hyphae of *P. indica* destroy some cortex cells of the root. Cocultivation studies of both partners revealed that *Arabidopsis thaliana* forms interacting structures similar to those which are described from other AM plants. The roots are loosely covered by the mycelium of *Piriformospora*. Some infected outer root cells are dead, but the hyphae of *P. indica* do not cross the pericycle and do not invade into the internal parts of the roots.

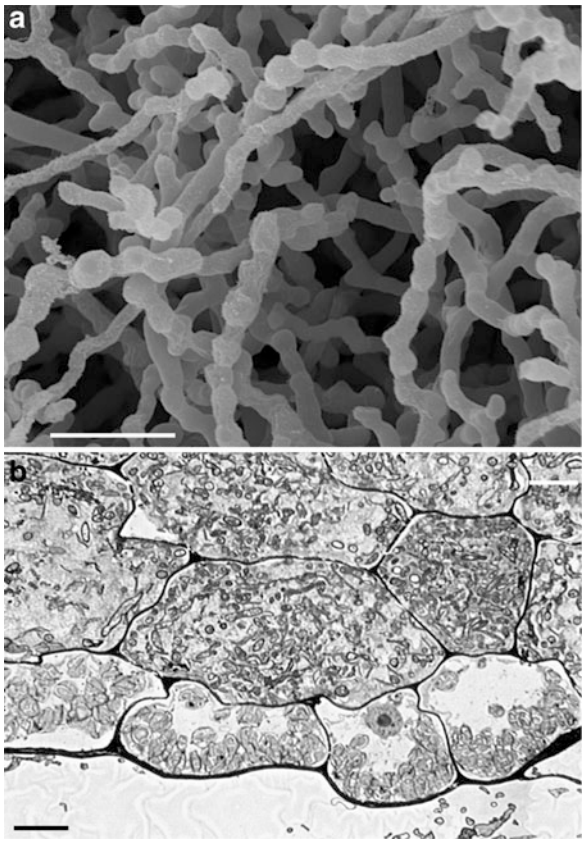

Fig. 2.3 (**a**) Moniliform hyphae of *Piriformospora indica* on solid culture medium; bar = 10 μm. (**b**) Infected thallus cells of *Aneura pinguis* by *Piriformospora indica;* bar = 20 μm

2.4.5 Interactions with Aneuraceae (Fig. 2.3)

By testing the host spectrum of *P. indica*, we cocultivated liverwort *Aneura pinguis* together with *P. indica* under sterile conditions. After a short time, both partners intensively interacted. The hyphae of *P. indica* invaded the moss thalli. The hyphae seem to be accumulated in a layer under the epidermic cells. The living moss cells are completely filled with hyphae (Kottke et al. 2003, Preussing et al. 2010).

2.4.6 Interactions with Ericaceae

The different groups of the family of Ericaceae are able to establish quite diverse interaction types depending on the taxonomic position within the Ericaceae. Field experiments also demonstrated the growth-supporting effect of *P. indica* in

cocultivation experiments. A special chapter in this book is attended to this type of interaction (Selosse et al. 2007).

2.5 Ultrastructure

Whereas the light microscopy gave no indication to the systematic relationship, the studies of the ultrastructural characters of the hyphae of *Piriformospora* clearly revealed the relationship to Basidiomycota by transmission electron microscopy. Later molecular genetic studies affirmed this interpretation of the taxonomic position. Ultrathin sections of the hyphal wall showed that the hyphal wall is multifaceted by different opacity of the layers of the cell wall. At the septa, the cell wall is swollen around the septal pore forming a distinct dolipore. These both characters are typical for Basidiomycota. To recognize the correct classification within the Basidiomycota the analyses of the specific structures of the cell wall and the septa were very useful (Verma et al. 1998). The parenthesomes cover both sides of the dolipore like a flat membranaceous disc without any detectable perforations. The membrane of the parenthesomes is arranged by different electron opaque layers. Both outer layers are electron dense and appear as a dark line. The inner layer is less dense and appears much lighter. Additionally to these structures, a small darker line can be observed in the middle of the inner layer. All these characters can be found in the order of the Sebacinales.

Although the morphology of *P. indica* does not show so quite different structures and specializations like fruit body forming Asco- and Basidiomycota, it is very interesting to observe the different adaptations and specializations of the mycelium of *P. indica* during the interaction processes with the different hosts. With this fungus it can be proved that interacting structures formed by one strain of *P. indica* together with different host plants belong to quite diverse mycorrhizal types. These data impressively prove again that the host plants induce the type of mycorrhization. Several publications of the last years documented that species of Sebacinales are overlooked (Selosse et al. 2009; Weiß et al. 2004) and they are underestimated in their importance of the influence of the composition of the vegetation on the earth.

References

Basiewicz M, Weiß M, Kogel K-H, Langen G, Zorn H, Zuccarod A (2012) Molecular and phenotypic characterization of Sebacina vermifera strains associated with orchids, and the description of *Piriformospora williamsii* sp. nov. Fungal Biol 116:204–213

Blechert O, Kost G, Hassel A, Rexer RH, Varma A (1999) First remarks on the symbiotic interactions between *Piriformospora indica* and terrestrial orchid. In: Varma A, Hook B (eds) Mycorrhizae, 2nd edn. Springer, Germany, pp 683–688

Deshmukh S, Hückelhoven R, Schäfer P, Imani J, Sharma M, Weiss M, Waller F, Kogel KH (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Glen M, Tommerup IC, Bougher NL, O'Brien PA (2002) Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? Mycorrhiza 12:243–247

Kaldorf M, Koch B, Rexer KH, Kost G, Varma A (2005) Patterns of interaction between Populus Esch5 and *Piriformospora indica*: a transition from mutualism to antagonism. Plant Biology 7:210–218

Kogel KH, Franken P, Hückelhoven R (2006) Endophyte or parasite-what decides? Curr Opin Plant Biol 9:358–363

Kottke I, Beiter A, Weiss M, Haug I, Oberwinkler F, Nebel M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while Aneura pinguis (Metzgeriales) is associated with a Tulasnella species. Mycol Res 107:957–968

Peškan-Berghöfer T, Shahollari B, Giang PH, Hehl S, Markert C, Blanke V, Varma AK, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiologia Plantarum 122:465–477

Preussing M, Nebel M, Oberwinkler F, Weiss M (2010) Diverging diversity patterns in the Tulasnella (Basidiomycota, Tulasnellales) mycobionts of Aneura pinguis (Marchantiophyta, Metzgeriales) from Europe and Ecuador. Mycorrhiza 20:147–159

Schäfer P, Khatabi B, Kogel KH (2007) Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism. FEMS Microbiol Lett 275:1–7

Selosse MA, Setaro S, Glatard F, Richard F, Urcelay C, Weiss M (2007) Sebacinales are common mycorrhizal associates of Ericaceae. New Phytol 174:864–878

Selosse MA, Dubois MP, Alvarez N (2009) Do Sebacinales commonly associate with plant roots as endophytes? Mycol Res 113:1062–1069

Varma A, Singh A, Sudha, Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) Mycota IX. Springer, Germany, pp 123–150

Varma A, Verma S, Sahay NS, Bütehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Verma SA, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:898–905

Warcup JH (1981) The mycorrhizal relationships of Australian orchids. New Phytologist 87:371–381

Warcup JH (1988) Mycorrhizal associations of isolates of *Sebacina vermifera*. New Phytologist 110:227–231

Warcup JH, Talbot PHB (1967) Perfect states of rhizoctonias associated with orchids. New Phytologist 66:631–641

Weiß M, Sykorova Z, Garnica S, Riess K, Martos F, Krause C, Oberwinkler F, Bauer R, Redecker D (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS One 6:16793

Weiß M, Selosse M-A, Rexer K-H, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Zuccaro A, Lahrmann U, Guldener U, Langen G, Pfiffi S, Biedenkopf D, Wong P, Samans B, Grimm C, Basiewicz M, Murat C, Martin F, Kogel K-H (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. PLoS Pathog 7:1–26

Zuccaro A, Basiewicz M, Zurawska M, Biedenkopf M, Kogel K-H (2009) Karyotype analysis, genome organization, and stable genetic transformation of the root colonizing fungus *Piriformospora indica*. Fungal Genet Biol 46:543–550

Chapter 3
Similarity Between *Piriformospora indica* and *Sebacina vermifera* Sensu Members of the Order *Sebacinales* Based on Immunological Techniques

Rajani Malla, Utprekshya Pokharel, and Ajit Varma

3.1 Introduction

Phosphatases are the enzymes of wide specificity which cleave phosphate ester bonds, and this plays an important role in the hydrolysis of polyphosphates and organic phosphates. Acid and alkaline phosphatases are the two forms of intracellular phosphatase active at acidic and alkaline conditions, respectively. ACPase was found to be mainly involved in uptake of P by the fungal mycelia, and alkaline phosphatase (ALPase) is linked with its assimilation (Fries et al. 1998).

Acid phosphatase in soil originates from both plants and fungi, while ALPase is believed to be of purely microbial origin (Gianinazzi-Pearson and Gianinazzi 1978; Tarafdar and Rao 1996). Indications are found that ACPase of fungal origin has a higher hydrolyzing efficiency than enzymes of plant origin (Tarafdar et al. 2001). Studying ACPase is difficult due to their multiform occurrence in organisms, their relative nonspecificity, their small quantity, and their instability in dilute solution. Their study is also complicated by wide variations in the activity and property of isozyme between species and between different stages in each plant's development (Alves et al. 1994) (Fig. 3.1).

Alkaline phosphatase has been proposed as a marker for analyzing the symbiotic efficiency of colonization (Tisserant et al. 1993). The argument for this was that ALPase is an important enzyme in metabolic processes leading to P transfer to the

R. Malla (✉)
Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
e-mail: rajanimalla2000@yahoo.com

U. Pokharel
Department of microbiology, Punjab University, Chandigar, India
e-mail: utp_pok@yahoo.co.in

A. Varma
Amity Institute of Microbial Technology, Amity University Uttar Pradesh, E-3 Block, IV Floor, Amity University Campus, Sector -125, Noida 201303, U.P, India
e-mail: ajitvarma@amity.edu

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_3,

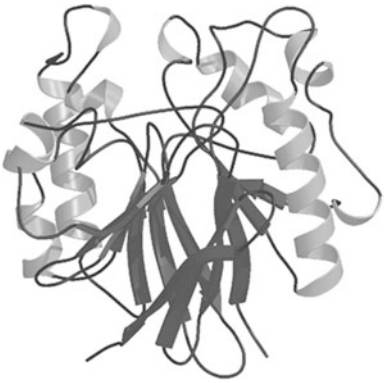

Fig. 3.1 3D structure of purple acid phosphatase from animal (Lindqvist et al. 1999)

host plant. The alkaline phosphatase activity is shown to be increased sharply prior to mycorrhizal stimulation of plant growth and then declined as the mycorrhizal colonization gets aged and P accumulated within the host. Arbuscule is speculated to be a site of nutrient exchange between the host plant and AM fungi (Cox et al. 1980). Phosphate efflux from the fungi to the host plant at arbuscules is supported by the recent discovery of novel plant Pi transporters that are localized around arbuscules and acquire Pi from the fungi (Rausch et al. 2001; Harrison et al. 2002; Paszkowski et al. 2002).

3.1.1 Sebacinales

Mycorrhizal taxa of *Sebacinaceae*, including mycobionts of ectomycorrhizas, orchid mycorrhizas (McCormick et al. 2004), ericoid mycorrhizas, and jungermannioid mycorrhizas, are distributed over two subgroups. One group contains species with microscopically visible basidiomes, whereas members of the other group probably lack basidiomes. *Sebacina* appears to be phylogenetic; current species concepts in *Sebacinaceae* are questionable. *S. vermifera* sensu consists of a broad complex of species possibly including mycobionts of jungermannioid and ericoid mycorrhizas. Extrapolating from the known rDNA sequences in *Sebacinaceae*, it is evident that there is a cosm of mycorrhizal biodiversity yet to be discovered in this group. Taxonomically, the *Sebacinaceae* recognized a new order, the *Sebacinales* (Weiß et al. 2004; Malla et al. 2005). The order primarily contains the genera *Sebacina*, *Tremelloscypha*, *Efibulobasidium*, *Craterocolla*, and *Piriformospora*. Proteomics and genomics data about *P. indica* fungus have recently been described (Peškan-Berghöfer et al. 2004; Shahollari et al. 2005; Kaldorf et al. 2005; Malla et al. 2007a).

Piriformospora indica and Sebacina vermifera from *Sebacinales* are documented to function as bio-fertilizer, bioregulator, bioprotector, and supplement to the health of the plant and the soil (Verma et al. 1998; Varma et al. 1999; Malla

et al. 2002). More recently, they are documented to act as agent for biological hardening of tissue culture-raised plants. Despite their enormous potential, their biotechnological applications could not be exploited to the level they deserve. The axenic cultivability of these fungi provided ample opportunity to study the comparative immunological relationship and molecular marker like random amplified polymorphic DNA (RAPD) to establish variability in between these two fungi (Weiß et al. 2004; Malla and Varma 2004; Malla et al. 2004).

3.2 Materials and Methods

Actively growing colonies of *P. indica* and *S. vermifera* sensu were transferred in modified Aspergillus medium (Hill and Kaefer 2001; Pham et al. 2004b), incubated for 10 days at 28 ± 2 °C in the dark with constant shaking at 120 rpm. The morphological features of the fungi were studied with the aid of Leica microscope (Type 020–518.500, Germany).

3.2.1 Gel Filtration Chromatography

The crude protein extracted was fractionated by 80 % saturation of ammonium sulfate, dialyzed overnight against phosphate buffer (80 mM), and passed through column chromatography using Sephadex G-100 (Cutler 2001). The flow rate was 0.5 ml/min and fractions of 4 ml each were collected. The relative ACPase activity was measured in 25 µl aliquots of selected fractions at OD 420 nm using 2 mM *p*-NPP as substrate at standard condition of 37 °C. Fraction with higher activity was pooled and passed through ion exchange chromatography using DEAE Sephadex (Bollag et al. 1996). The column was neutralized with phosphate buffer (80 mM) and eluted with 0.1 M NaCl.

3.2.2 Molecular Mass

The active fractions pooled from ion exchanger were separated in 10 % non-denaturing PAGE (native PAGE). The gel after separation at 4 °C for 6 h was reacted with 2 mM *p*-NPP with sodium acetate buffer at room temperature. Yellow visible phosphatase bands thus formed were eluted from the native gel using elution buffer (ammonium carbonate, 100 mM) and separated in 12 % SDS-PAGE to determine molecular mass.

3.2.3 Developing Antiserum

New Zealand white rabbit were used for the production of antiserum. Polyclonal antibodies were raised against *P. indica* ACPase detected in native gel as yellow band. The band was manually cut by blade and prepared as immunogen, as described by Amero et al. 1996.

3.2.4 Preparation of Antiserum

The antiserum was prepared according to Hahn et al. (1998). The antiserum was precipitated up to 50 % by adding an equal amount of saturated ammonium sulfate solution. Further purification of antiserum was done by ion exchanger, DEAE—Sepharose CL-6B (Johnstone and Thorpe 1996).

3.2.5 Immunodetection

The quick method of detection of antiserum was done by single radial and double immunodiffusion (Vaerman 1981) and ELISA (Harlow and Lane 1999; Voller et al. 1980) method. The optimal concentrations of the conjugate as well as of primary antibody were determined by crisscross serial dilution analysis.

3.2.6 Immunoblotting

Western blotting of the antiserum was done according to Towbin et al. (1979) and Horst (2000). Nitrocellulose sheet (Schleicher and Schuell, Germany) 0.45 μm pore size was used for transblot of protein. The blots after transfer were detected with Ponceau S stain (Sigma), destained with deionized water. The purified antiserum using DEAE—Sephadex CL-6B served as primary antibody. Enzyme-conjugated secondary antibody (HRPO, anti-rabbit IgG, Sigma, A-9169) was used for binding of primary antiserum. 3, 3′-Diaminobenzidine tetrahydrochloride (DAB) in combination with urea peroxide (Sigma Co.) as substrate provided for visualization of immunological complex.

3.2.7 Immunofluorescence

The hyphae and spores were fixed for 15 min at 4 °C with 3.7 % paraformaldehyde, mixed with an equal amount of double-strength phosphate buffer, and passed through a 0.45 μm Millipore filter. Washed the treated sample with PBS (3 × 5 min) containing 100 mM glycine. Biomass was permeabilized with 0.1 % Triton X-100 in PBS for 4 m. Unspecific binding of the antibodies was blocked by 1 % BSA (Meyberg 1988). After several washings with PBST (containing 0.02 % Tween 20), the biomass was incubated with antiserum fractionated with ammonium sulfate diluted to 1:100. Fluorescein isothiocyanate (FITC) (Sigma Aldrich, F-0382) conjugated 2nd antibody diluted 1:100 was used for visualization of antigen–antibody complex and screened with confocal microscope (Olympus).

3.2.8 Immunogold

Four day old samples were fixed in 1 % glutaraldehyde and 2 % paraformaldehyde, filtered through a 0.45 μm pore-sized paper kept for 18 h at 4 °C, and postfixed for 2 h in 1 % osmium tetroxide. Samples were dehydrated with graded alcohol/acetone solutions, embedded in LR white resin (Botton and Chalot 1991). Ultrathin-sectioned (60–90 nm thick) labeled grid with primary antibody IgG (1:100 diluted) was kept overnight at 4 ° C and washed with 0.1 M PB four times. The grid was labeled with secondary antibody anti-rabbit-goat IgG conjugated with 15 nm gold particle (1:100) and stained in 0.5 % aqueous uranyl acetate for 10 min and lead citrate for 5 min. The stained sections were observed with Philips CM-10 electron microscope operated at 60–80 KV.

3.2.9 Two-Dimensional PAGE

The first dimension was performed with the tube cell model 175 (Bio-Rad, Hercules, CA) and with glass capillary tube (1.0–1.4 mm internal diameter and 210 mm long) as described by Gravel and Golaz 1996. Ampholyte pH 5.0–7.0 and 3.0–10.0 were from Bio-Rad. The samples were loaded on the top of the capillary (cathodic side). 80 μg of the sample was loaded using Hamilton syringe. IEF was carried out at 200 V constant voltage for 2 h, followed by 500 V constant voltage for 2 h, and finally 800 V constant voltage for 16 h (overnight). Tube gels were equilibrated in equilibration buffer for ½ h at RT. The protean II chamber (Bio-Rad) is employed for second dimension. The gel (160 × 200 × 1.5 mm) was casted in the casting chamber (Bio-Rad). Tube gels were transferred on the top of SDS gels and separated at 90 V constant voltage in stacking gel and 120 V constant voltage in separating gel. Proteins in the 2D gel were stained with silver-staining method.

3.2.10 Staining of Acid Phosphatase with Fast Garnet GBC

The crude extract of biomass was separated in 10 % non-denaturing PAGE. The gel after separation was stained with Fast Garnet GBC in sodium acetate buffer containing α-naphthyl phosphate as substrate at room temperature (Ho and Zak 1979).

3.2.11 Detection of Phosphatase by ELF-97 Substrate

The fungal cultures grown for 48 h in broth were fixed in 3.7 % formaldehyde in phosphate buffer saline for 1 h in 4 °C. The samples were permeabilized in 0.02 % Tween 20 in PBS buffer for 10 min at room temperature. The samples were rinsed four times with PBS. The diluted ELF-97 substrate was filtered through 0.2 μm pore-sized spin filters E-6606 (Ingrid and van Aarle, 2001). Since the reaction occurs very fast, the reaction was performed on the microscope using excitation filter and emission filter from the fluorescein set, which provided the appropriate UV excitation and transmits wavelengths greater than 400 nm. 50 μl of substrate solution was added. Immediately, the sample was placed on the microscope and the development of the signal observed was monitored under the fluorescence microscope (Olympus, model, FV-300).

3.2.12 RAPD Technique

Isolation and purification of fungal DNA was done following modified CTAB protocol of Moller et al. (1992). DNA amplification was performed in a total volume of 25 μl containing (in μl) 2.5, buffer (10× without $MgCl_2$); 2.5, $MgCl_2$; 0.8, dNTPs (10 mM); 1.0, primer (30 ng/μl); 0.5 *Taq* polymerase (3U/μl); and DNA concentration ranging from 5 to 25. Random 10 bp oligonucleotide primers (Operon Technologies Alameda, California) were used to produce amplification. The DNA was amplified in PTC-200 Thermal Cycler (Techne, UK) electrophoresed on 1.5 % agarose gel in 1 % TAE at 3.5 V/cm for 2 h, stained with ethidium bromide.

Statistical analysis was performed using the NTSYS-pc program (Rohlf 1992). The degree of genetic relatedness or similarity was estimated using the Jaccard coefficient. Clustering of similarity matrices was done by UPGMA (unweighted pair group method with arithmetic mean) and projection by TREE program of NTSYS-pc.

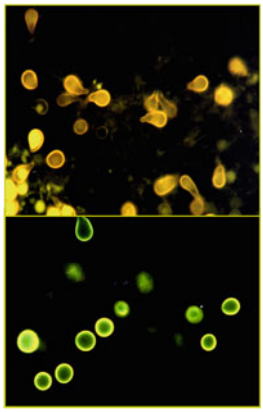

Fig. 3.2 Autofluorescence shown by *P. indica* (*left*) and *S. vermifera* (*right*) chlamydospores (*arrow*) seen under Leica microscope (model, 020–518.500) using I3 filter with excitations range 450–490 λ at ×400. The characteristic pear-shaped chlamydospores are more frequently common in *P. indica* and *S. vermifera* sensu

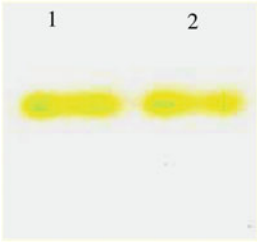

Fig. 3.3 50 μg crude protein extracted from fresh biomass of fungus, loaded in native PAGE, was separated in 10 % gel for 6 h at 4 °C, was washed with 50 mM sodium acetate buffer to neutralize, and was shaken in water bath with solution of 2 mg/ml *P*-NPP. Yellow color is *p*-nitrophenol, product of enzyme reaction upon substrate. *Lane 1 P. indic*a, *Lane 2 S. vermifera*. The banding pattern of the fungus shows precise similarity in between two fungi in their molecular mass and ionic strength

3.3 Results

P. indica and *S. vermifera* sensu belonging to the same taxonomic group show similar morphology (Fig. 3.2). The molecular mass of ACPase in *P. indica* and *S. vermifera* sensu was identical. The enzyme was eluted from 8 % native gel and was separated in SDS gel. The identical molecular mass of acid phosphatase in both of the fungi suggests the close relationship of those fungi (Figs. 3.3 and 3.4).

The antibody raised against acid phosphatase of *P. indica* showed maximum ELISA reading with *S. vermifera* sensu. The immunoblot showed the strong reactivity of antiserum with own protein of cytoplasmic (CF) and wall membrane (W/MF) fraction and with protein of CF and W/MF of *S. vermifera* sensu.

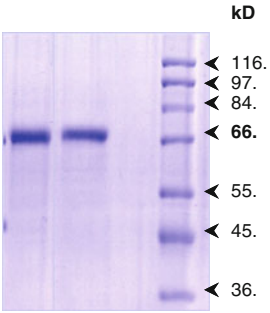

Fig. 3.4 Molecular weight profile of ACPase eluted from native gel after several purification procedures. *Lane 1 P. indica*, *Lane 2 S. vermifera* sensu, *Lane 3* molecular marker (Sigma wide range). The crude enzyme after selective precipitation of ammonium sulfate followed by gel filtration (Sephadex G-100) and ion exchange chromatography (DEAE-Sephadex) was separated in 8 % native PAGE and detected by assay using *p*-NPP. The eluted protein from native PAGE was separated by 12 % SDS-PAGE along with wide-range marker. The pure acid phosphatase showed 66kD molecular mass

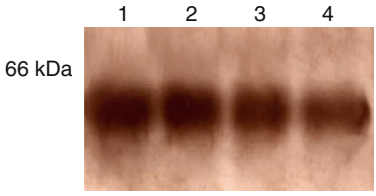

Fig. 3.5 Western blot analysis of *P. indica* and *Sebacina vermifera* sensu separated by 10 % SDS-PAGE. *Lane 1* CF. *Lane 2* W/MF of *P. indica* reacted with homologous antiserum. *Lane 3* CF. *Lane 4* W/MF of *S. vermifera* sensu cross-reacted with *P. indica* antiserum. The result shows precisely defined bands in all samples. All blotted bands represent similarity in their molecular mass supporting immunologically highly related species. CF cytoplasmic fraction, W/MF wall membrane fraction

The *P. indica* antiserum blotted the bands of *S. vermifera* sensu at precise location of *P. indica* ACPase in 12 % SDS-PAGE (Fig. 3.5). Using fluorescein isothiocyanate (FITC)-labeled antibodies, localization of the enzyme was found on spores and hyphal walls. The antiserum localized the enzyme in *S. vermifera* sensu showing strong relationship of this fungus with *P. indica*. The precise localization was not distinct in hyphae. The antibody densely bound the spore wall in comparison to the inner spore may be due to poor penetration of antibody to wall. The antiserum also precisely localized the enzyme in cytoplasm and vacuoles of *S. vermifera* sensu supporting the immunological link between these two fungi (Fig. 3.6).

Silver-stained 2-dimensional maps of mycelial protein of *P. indica* and *S. vermifera* sensu showed similarity in most of the major protein bands. The gel indicated differences in some of the minor proteins (Fig. 3.7).

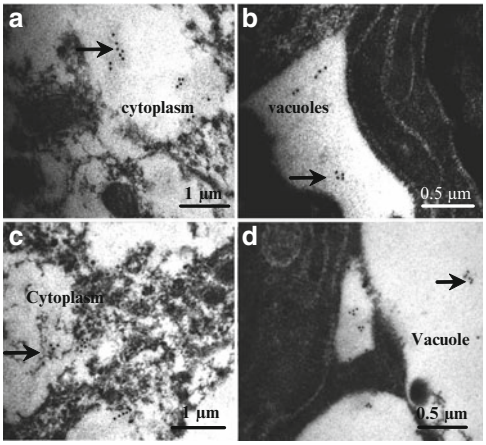

Fig. 3.6 Immunolocalization of acid phosphatase in *P. indica* (*top*) and *S. vermifera* sensu (*bottom*). Electron micrograph of an ultrathin section of *P. indica* and S. *vermifera* sensu treated with secondary antibody (goat anti-rabbit) coupled to colloidal gold 15 nm size. Dark dots are gold particles indicating localization of the enzyme acid phosphatase. Localization is prominent in cytoplasm and vacuoles. The cells were fixed with 1 % glutaraldehyde and postfixed with 1 % osmium tetroxide. The primary antibody was raised against acid phosphatase of *P. indica*

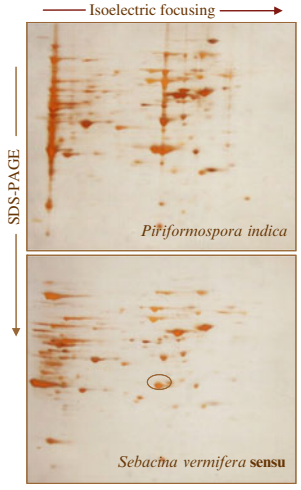

Fig. 3.7 Silver-stained two-dimensional maps of mycelial protein of *P. indica* and *S. vermifera* sensu loaded with 80 µg of protein onto IEF gels. Separation in the horizontal dimension was achieved by isoelectric focusing using carrier ampholyte in the pH range of 3–10 in the presence of 9.2 M urea and separation in the vertical dimension by 12 % SDS-PAGE

Using α-naphthyl phosphate as substrate, different isoforms of ACPase were obtained by Fast Garnet GBC staining. *P. indica* and *S. vermifera* sensu showed three distinct isoforms of ACPase each, one with higher molecular mass and two

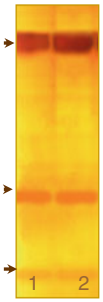

Fig. 3.8 Fast Garnet GBC staining of ACPase. The ACPase isoforms of *P. indica* and *S. vermifera* sensu show similar banding pattern. *Lane 1 P. indica*. *Lane 2 S. vermifera* sensu. The native PAGE separated for 6 h at 4 °C was neutralized with 50 mM sodium acetate buffer, stained with Fast Garnet GBC using α-naphthyl phosphate as substrate. The precisely localized band shows similar molecular mass and ionic strength of isoforms between *P. indica* and *S. vermifera* sensu

other with lower molecular mass. The pattern of isoforms was similar in both the fungi (Fig. 3.8). The higher molecular mass isoform is suspected to be dimeric form of the enzyme and lower is suspected to be monomeric form of ACPase on the basis of the position they formed in native gel.

The 48 h old hyphae of *P. indica* grown in *Aspergillus* medium when reacted with enzyme-labeled fluorescence (ELF-97) substrate developed thick fluorescent precipitation in *P. indica* hyphae with dense production of ELF crystal. The reaction was comparable with control (Fig. 3.9). The activities were prominent but exact position or location of phosphatase was not confirmed. No any extracellular phosphatases and alkaline phosphatase activity were detected by this method.

The RAPD result showed about 60 % similarity between these two fungi according to the dendrogram (data not given). The RAPD data confirmed that even among these two species of *Sebacinales* belonging to the same morpho-zymographical groups, the level of variation was substantially high according to RAPD.

3.4 Discussion

The present experiment was undertaken to have an insight to the immunological relationship of *P. indica and S. vermifera* sensu grown in vitro. Soil fungi release the extracellular phosphatases in their environment (Nahas et al. 1982). These phosphatases are introduced into the soil by active exudation, leakage, or cell lysis (Dick et al. 1983; Tadano et al. 1993; Malla et al. 2007a). The phosphatase from *P. indica* and *S. vermifera* sensu separated in native PAGE was assayed with 2 mM of *p*-NPP at pH 5.3. The finding suggests that the mobility of the ACPase enzymes was identical in those two fungi exhibiting the same overall banding pattern. The bands separated were appearing to be homologous for both the isolates.

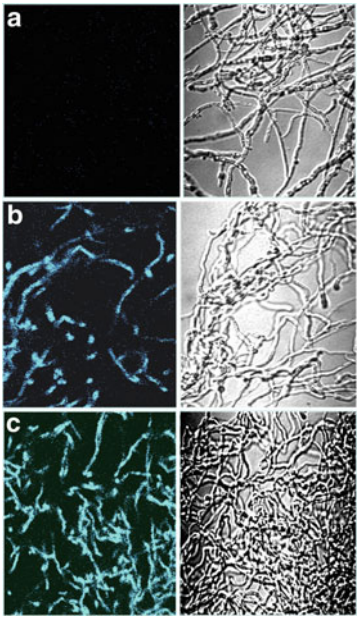

Fig. 3.9 Detection of ACPase activity by enzyme-labeled fluorescent substrate (ELF 97, molecular probe). (**a**) Control without substrate. (**b**) *P. indica*. (c). *S. vermifera* sensu. The result observed under Olympus standard microscope using excitation filter and dichroic mirror from the DAPI filter set and emission filter from the fluorescein set provides the appropriate UV excitation and transmits wavelengths greater than 400 nm. The microscope is equipped for epi-fluorescence with a high pressure Hg lamp. The results show uniform activity of phosphatase throughout the mycelium

Certain enzymes and isozymes are more suitable for taxonomic studies of the organisms (Malla 2008). These enzymes were especially important since they allowed closely related species to be readily separated. Usually, however, the species within each genus had very similar (probably homologous) banding patterns for various enzymes in terms of band number, relative mobility, and definition (Loxdale et al. 1983). The enzyme of ACPase had identical mobilities (which are based on molecular size and charge) between these two species. When *p*-NPP was used to visualize the bands in native gel, *P. indica* and *S. vermifera* sensu gave single precisely identical banding pattern of 66 kDa molecular mass.

Phosphatase activities, both ALPase and ACPase, have been visualized and shown to be associated with the hyphal wall and also in vacuoles of the extraradical mycelium using the enzyme-labeled fluorescent (ELF) substrate. In the intraradical mycelium, ACPase activity has been localized in arbuscules (Ezawa et al. 1995) and ACPase activity seemed to decrease with aging of the colonization. In the intraradical hyphae this activity is mainly located in the vacuoles (Saito 1995). The polyclonal antibodies raised against cytosolic ACPase of *P. indica* immunolocalized the enzyme on the vacuoles and cytoplasm of the mycelium.

The antiserum precisely localized the enzyme in *S. vermifera* sensu, displaying strong relationship between these two fungi.

Separation of protein in two-gel system is a useful technique for distinguishing different fungi. The protein patterns of mycelium in two-dimensional PAGE of *P. indica* and *S. vermifera* sensu gave the massage that those two fungi are different in some minor proteins. So far one-dimensional PAGE showed no distinct bands of dissimilarity. Because of its resolution, the 2D PAGE has been applied to a great number of biological problems ranging from the analysis of proteins in different tissues under various hormonal states. The result obtained from 2D PAGE was comparable for the placement of those two fungi in the same genus not in species.

Staining of the native gel from both the fungi with Fast Garnet GBC using α-naphthyl phosphate as reaction substrate showed three isoforms of ACPases. It is noticeable that all those three bands of *P. indica* were identical to *S. vermifera* sensu. Stricto *P. indica* and *S. vermifera* sensu stricto, CF and W/MF showed three distinct isoforms of ACPase each, one with higher molecular mass and two with lower molecular mass. The mobility of the isozymes and patterns were similar in both the cases, supporting the closeness of these fungi to each other.

Several attempts were made to purify and characterize the enzyme from AM fungi; most of the result were based on activities of the enzyme related to extra- and intraradical hyphae, cytochemical localization, use of enzyme-labeled fluorescent substrate (ELF), and through expression of transporter genes responsible for ACPase (Aono et al. 2004). Due to unculturability of the arbuscular mycorrhizal fungus, very little is known about its purification. The enzyme-labeled fluorescent (ELF) substrate method could be used for visualizing phosphatase activity associated with colonized as well as noncolonized hyphae of fungus. Its high sensitivity provides the opportunity to study more in details the exudation or release of phosphatases by fungal mycelium in mono-axenic root organ cultures. Furthermore, the use of in situ hybridization may enable localization of the expression of phosphatase gene(s) at the cellular level. The ELF substrate method has proven to be a suitable and sensitive method in the determination of ACPase activity associated with axenic culture of *P. indica*. An advantage of ELF substrate compared to fast blue RR salt is its high sensitivity. When *P. indica* and *S. vermifera* sensu hyphae were incubated with ELF acid buffer solution, ELF precipitated all over the internal hyphal structure within 30 s except spores. The use of the ELF substrate in combination with laser scanning confocal microscopy could probably provide better insight into the location of phosphatase activity of fungi inside the roots and external hyphae. Comparing ELF with FB staining the ELF crystals was more abundant than those of FB. Almost similar observation was noticed in *S. vermifera* sensu showing closeness of these fungi to each other.

PCR-based techniques have already been applied to endo- and ectomycorrhizal fungi where morphological characters are in conflict, ambiguous, and missing (Podila and Lanfranco 2004). This approach has allowed the development of molecular tools for their identification (Hartmann et al. 2004; Malla et al. 2005; Malla and Varma 2007). The RAPD data confirmed that even among these two species of *Sebacinales* belonging to the same morpho-zymographical group, the

level of variation was substantially high. The genetic variation of the isolates was grouped in the dendrogram (data not given). *P. indica* and *S. vermifera* sensu showed low similarity. The polymorphism showed by RAPD of these two fungi *P. indica* and *S. vermifera* sensu suggests placing these fungi in far-related group or separated genus although morphologically and immunologically they are closely related (Malla et al. 2006; Malla et al. 2007b).

3.5 Conclusion

The application of serological technique for localization and characterization of ACPase in these two fungi has provided new imminent into important aspects of this field. Result presented in this study provides, antibodies raised against protein in native gel band was an important technique. The benefits derived from this technology supply specific antibody which is difficult by ordinary purification procedure. *Piriformospora indica* and *Sebacina vermifera* sensu belonging to the same taxonomic group show similar morphology, functions, protein profiles, and isozymes characterization along with close immunological relationships. However, they show distinct genetic variation based on the random amplified polymorphic DNA analysis. Thus, it is suggested that such isolates should be considered into separate species. Molecular characterization offers an alternative approach for more reliable and reproductive identification at species level. We therefore suggest that along with morphology and immunological characterization, RAPD analysis could be used to distinguish indigenous species in natural ecosystem.

Acknowledgements Rajani Malla is thankful to Dr. Ashok K. Chauhan, founder president, Amity—RBEF New Delhi—for his support. The author is also thankful to Dr. Upendra and Sweta from All India Institute of Medical Sciences and Dr. Ram Prasad from Amity University.

References

Alves JM, Siachakr CD, Allot M, Tizroutine S, Mussio I, Servaes A (1994) Isozyme modifications and plant regeneration through somatic embryogenesis in sweet potato. Plant Cell Rep 13:437–441

Amero SA, James TC, Elgin SCR (1996) Production of antibody using proteins in gel bands. In: Walker JM (ed) The protein protocols handbook. Humana, NJ, pp 717–720

Aono T, Maldonado-Mendoza IE, Dewbre GR, Harrison MJ, Saito M (2004) Expression of alkaline phosphatase genes in arbuscular mycorrhizas. New Phytol 162:525–534

Bollag DM, Rozycki MD, Edelstein SJ (1996) Protein methods, 2nd edn. Wiley-Liss, New York, pp 231–269

Botton B, Chalot M (1991) Techniques for the study of nitrogen metabolism in ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology 23. Academic, New York, pp 203–252

Cox G, Kg M, Sanders F, Nokolds C, Tinker PB (1980) Translocation and transfer of nutrients in vesicular arbuscular mycorrhizas.III. Polyphosphate granules and phosphorus translocation. New Phytol 84:649–659

Cutler P (2001) Chromatography on the basis of size. In: Simon R (Eds) Protein purification techniques. a practical approach, 2nd Edn. pp 187–211

Dick WA, Juma NG, Tabatabai MA (1983) Effects of soil and acid phosphatase and inorganic pyrophosphatase of corn roots. Soil Sci 136:19–25

Ezawa T, Saito M, Yoshida T (1995) Comparison of phosphatase localization in the intraradical hyphae of arbuscular mycorrhizal fungi Glomus spp and *Gigaspora* spp. Plant Soil 176:57–63

Fries LLM, Pacovsky RS, Safir GR, Kaminski J (1998) Phosphorus effect on phosphatase activity in endomycorrhizal maize. Physiol Plantarum 103:162–171

Gianinazzi-Pearson V, Gianinazzi S (1978) Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. II. Soluble alkaline phosphatase specific to mycorrhizal infection in onion roots. Physiol Plant Pathol 12:45–53

Gravel P, Golaz G (1996) Two-Dimensional PAGE using carrier ampholyte pH gradients in the first dimension. In: Walker JM (ed) The protein protocols handbook. Humana, NJ, pp 127–32

Hahn A, Goebel C, Hock B (1998) Polyclonal antibodies for the detection of arbuscular mycorrhizal fungi. In: Varma A (ed) Mycorrhiza manual. Springer, Berlin, pp 255–270

Harlow ED, Lane D (1999) Using antibodies. Cold Spring Harbour Laboratory Press, NY, pp 386–388

Harrison MJ, Dewbre GR, Liu JY (2002) A phosphate transporter from Medicago truncatula involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. Plant Cell 14:2413–2429

Hartmann A, Pukall R, Rothballer M, Gantner S, Metz S, Schloter MB (2004) Microbial community analysis in the rhizosphere by in situ and ex situ application of molecular probing, biomarker and cultivation technique. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin, Heidelberg, Germany, pp 449–469

Hill TW, Kaefer E (2001) Improved protocols for aspergillus medium: trace elements and minimum medium salt stock solutions. Fungal Genet News Lett 48:20–21

Ho I, Zak B (1979) Acid phosphatase activity of six ectomycorrhizal fungi. Can J Bot 57:1203–1205

Horst MN (2000) Molecular approaches to glycobiology. pp 2–30

Ingrid M, van Aarle IM (2001) Microscopic detection of phosphatase activity of saprophytic and arbuscular mycorrhizal fungi using a fluorogenic substratr. Mycologia 93:17–24

Johnstone A, Thorpe R (1996) Immunochemistry in practice, 3rd edn. Blackwell Scientific, Oxford

Kaldorf M, Koch B, Rexer K-H, Kost G, Varma A (2005) Patterns of interaction between Populus Esch5 and *Piriformospora indica*: a transition from mutualism to antagonism. Plant Biol 7:210–218

Lindqvist Y, Johansson E, Kaija H, Vihko P, Schneider G (1999) Three-dimensional structure of a mammalian purple acid phosphatase at 2.2 Å resolution with a mu-(Hydr)oxo bridged di-iron center. J Mol Biol 291:135–147

Loxdale HD, Castañera P, Brookes CP (1983) Electrophoretic study of enzyme from cereal aphid population. Bull Ent Res 73:645–657

Malla R (2008) Isozymes involved in phosphate metabolism in *Piriformospora indica*. J Inst Sci Technol 15:1–13

Malla R, Varma A (2004) Phosphatase (s) in microorganisms. Biotechnological applications of microbes. IK International/Kluwer academic Press, India, New York/Holland pp. 125–50

Malla R, Varma A (2007) Use of short oligonucleotide primers in random amplified polymorphic DNA techniques for species identification. In: Oelmüller R, Varma A (eds) Advanced techniques in soil microbiology, vol 11. Springer, Germany, pp 235–244

Malla R, Singh A, Md Z, Yadav V, Suniti, Verma A, Rai M, Varma A (2002) *Piriformospora indica* and plant growth promoting Rhizobacteria: An Appraisal. In: Rao GP, Bhat DJ, Lakhanpal TN, Manoharichari C (Eds) Frontiers of fungal diversity in India. 401–19

Malla R, Prasad R, Giang PH, Pokharel U, Oelmueller R, Varma A (2004) Characteristic features of symbiotic fungus *Piriformospora indica*. Endocytobiosis Cell Res 15:579–600

Malla R, Pokharel U, Varma A (2005) Random amplified polymorphic DNA of the two fungi from Sebacinales. J Institute Sci Technol 14:34–43

Malla R, Zeaullah MD, Pokharel U, Varma A (2006) Immunological characterization of acid phosphatase in an endophytic fungus. Nepal J Sci Technol 7:77–84

Malla R, Pokharel U, Prasad R, Varma A (2007a) Proteomics and genomics approach to study plant–microbe cross communication. In: Chauhan AK, Harsha K, Varma A (eds) Microbes for human life, vol 4. IK International, India, pp 609–636

Malla R, Pokharel U, Prasad R, Oelmüller R, Varma A (2007b) Immuno-technology for the localization of acid phosphatase using native gel bands in *Piriformospora indica* and other Soil Microorganism. In: Modern tools and techniques. Springer, Germany, pp 211–234

McCormick MK, Whigham DF, O'Neill J (2004) Mycorrhizal diversity in photosynthetic terrestrial orchid. New Phytol 163:425–438

Meyberg M (1988) Selective staining of fungal hyphae in parasitic and symbiotic plant fungus associations. Histochemistry 88:197–199

Moller EM, Bahnweg G, Sandermann H, Geiger HH (1992) A simple and efficient protocol for isolation of high molecular weight DNA from filament fungi, fruit bodies and infected plant tissue. Nucleic Acids Res 20:6115–6116

Nahas E, Terenzi HF, Rossi A (1982) Effect of carbon source and pH on the production and secretion of acid phosphatase and alkaline phosphatase in neurospora crassa. J Gen Microbiol 128:2017–2021

Paszkowski U, Kroken S, Roux C, Briggs SP (2002) Rice phosphate transporters includes an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci 99:13324–13329

Peškan-Berghöfer T, Shahollari B, Pham HG, Hehl S, Markent C, Blank V, Kost G, Varma A, Oelmueller R (2004) Association of Piriformospora indica with Arabidopsis thaliana roots represent a novel system to study beneficial plant-microbe interactions and involve in early plant protein modifications in the endocytoplasmic reticulum and in the plasma membrane. Physiol Plant 122:465–71

Pham GH, Kumari R, Singh A, Malla R, Prasad R, Sachdev M, Kaldorf M, Buscot F, Oelmüller R, Hampp R, Saxena AK, Rexer K-H, Kost G, Varma A (2004b) Axenic cultures of *Piriformospora indica*. In: Plant Surface Microbiology. Springer, Germany. pp 593–613

Podila GK, Lanfranco L (2004) Functional genomic approaches for studies of mycorrhizal symbiosis. Plant surface microbiology. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin, Heidelberg, Germany, pp 567–592

Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, Amrhein N, Bucher M (2001) A phosphate transporter expressed in arbuscule-containing cells in potato. Nature 414:462–466

Rohlf F.J, NTSYS-pc. (1992) Numerical taxonomy and multivariate analysis system. Vers. 1.70. Exeter Software, Setauket, New York

Saito M (1995) Enzyme activities of the internal hyphae and germinated spores of an arbuscular mycorrhizal fungus. Gigaspora margarita Becker and Hall. New Phytologist 129:425–431

Shahollari B, Varma A, Oelmüller R (2005) Expression of a receptor kinase in Arabidopsis roots is stimulated by the basidiomycete Piriformospora indica and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol Holland 162:945–958

Tadano T, Ozawa K, Sakai H, Osaki M, Matsui H (1993) Secretion of acid phosphatase by the roots of crop plants under phosphorus-deficient conditions and some properties of the enzyme secreted by lupin roots. Plant Soil 156:95–98

Tarafdar JC, Rao AV (1996) Contribution of Aspergillus strains to acquisition of phosphorus by wheat (Triticum aestivum L.) and chick pea (Cicer arietinum L.) grown in a loamy soil. Appl Soil Ecol 3:190–214

Tarafdar JC, Yadav RS, Meena SC (2001) Comparative efficiency of acid phosphatase originated from plant and fungal sources. J Plant Nutr Soil Sci 164:279–282

Tisserant B, Gianinazzi-Pearson V, Gianinazzi S, Gollotte A (1993) In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol Res 97:245–250

Towbin H, Staehlin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci 76:4350–4354

Vaerman JP (1981) Single radial immunodiffusion. Meth Enzymol 73:291–305

Varma A, Verma S, Sudha, Sahay N, Britta B, Franken P (1999) Piriformospora indica—a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:2741–2744

Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarabhoy A, Bisen P, Bütenhorn B, Franken P (1998) Piriformospora indica, gen. et sp. nov., a new root colonizing fungus. Mycologia 90:896–903

Voller A, Bidwell DE, Bartlett A (1980) Enzyme linked immunosorbent assay. In: Rose NR, Friedmans H (eds) Manual of clinical immunology. American Society for Microbiology, Washington, DC, pp 359–371

Weiß M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with abroad mycorrhizal potential. Mycol Res 108:1–8

Chapter 4
Piriformospora indica: Perspectives and Retrospectives

Satwant Kaur Gosal, Anu Kalia, and Ajit Varma

4.1 Introduction

Soil is a dynamic system consisting of soil minerals/clay, soil organic matter, and soil microorganisms which play an important role in improving soil quality, enhancing soil fertility, and maintenance of soil structure stability. This soil microbial diversity is thus a precious resource that indicates the agricultural prosperity of a nation with a single gram of soil that may consist several thousand types of microbes in terms of both species richness and species number (Wardle et al. 2004). Soil microbial diversity encompasses the prokaryotic forms like bacteria, cyanobacteria, actinomycetes, and myxomycetes and eukaryotic forms like fungi (both soil yeasts and molds) and protozoa (amoeba and ciliates) as well as bacteriophages/viruses. Among these diverse life forms, the soil fungi are of great ecological importance pertaining to organic matter decomposition and nutrient recycling. Fungi are multicellular, heterotrophic, uni- to multinucleated microbes having four major classes and several orders. Certain oomycetes and basidiomycetous fungi exhibit symbiosis with higher plants; the "symbiotic root fungi" possess higher potential for application in agriculture as these are known to improve soil quality in terms of soil stabilization by secretion of particle-binding proteins (like glomalin) and function as plant growth promotive as well as plant probiotic

S.K. Gosal (✉)
Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab 141004, India
e-mail: skgosal@rediffmail.com

A. Kalia
Electron Microscopy and Nanoscience Laboratory, Punjab Agricultural University, Ludhiana, Punjab 141004, India
e-mail: kaliaanu@pau.edu; kaliaanu@gmail.com

A. Varma
Amity Institute of Microbial Technology, Amity University Uttar Pradesh, E-3 Block, IV Floor, Amity University Campus, Sector -125, Noida 201303, U.P, India
e-mail: ajitvarma@amity.edu

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_4,

microbes. The major genera of symbiotic fungi, commonly termed as "mycorrhizal fungi," include the structurally and molecularly diverse ectomycorrhiza, endomycorrhiza, ectendomycorrhiza, and arbuscular mycorrhizal (AM) fungi (*Glomus, Scutellospora, Acaulospora, Gigaspora* genera). The mycorrhizal associations are plant mutualistic interactions that have appeared due to coevolution of certain specific plant genera with these fungi over several eras which resulted in transformation followed by initial colonization of aquatic or semiaquatic plant forms (Ligrone et al. 2007) to true terrestrial vascular land plants (Brundrett 2002). Due to their enormous ecological and agricultural importance, these are one among the elaborately studied symbiotic associations at the molecular genetics, proteome, and interactome levels. These interactions result in formation of certain specific or in general morphologically distinct structures of fungal origin (extramatricular hyphal extensions, mantle, arbuscules, and vesicles) which result in capturing of nutrient elements from the soil solution beyond the depletion zone considering the plant benefit perspectives and in increasing the host–fungal interaction surface volume for better nutrient exchange between both partners. The AM fungal associations are unique and could be exploited for attaining higher crop yields with suboptimal chemical fertilizer and pesticide inputs (Kuo and Huang 1982; Jeffries et al. 2003; Arpana and Bagyaraj 2007; Hu et al. 2009) though the mass production of authentic culture for commercial success suffers a basic hitch due to the obligate symbiont characters of the fungi. Several advancements in the culturing of these fungal forms have paved towards achieving potential culture inoculants but not to match the commercial scale production levels. Any mycorrhizologist would prefer for studies on a cultivable AM fungi-like organism, and the discovery of *Piriformospora indica* puts forth the possible solution for it by offering an AM fungi-like benefits supermounted by the premiere characteristics of easy cultivation on known semisynthetic to synthetic media like potato dextrose agar and Kaefer's agar (Verma et al. 1998).

P. indica belongs to phylum Hymenomycetes, class Basidiomycota, and order Sebacinales having enormous potential as a plant growth-promoting fungal endophytic agent to a variety of plant hosts belonging to diverse families or groups, even the members of families like Cruciferae which are not infected by the AM fungi. It has been named due to formation of typical pyriform chlamydospores which act as perennation bodies similar to the AM spores. Another similarity among the two is the formation of extended hyphal structures that ramify the soil in vicinity of the host roots hunting and accumulating nutrients like phosphorus, iron, manganese, zinc, and many more from a diameter of several square meters of the soil. Within the host plant root tissues, structures similar to arbuscules and vesicles occur in the intercellular spaces as well as may be packed intracellularly which enhances the interaction interface among the microsymbiont and the host plant tissues or may act as storage organ of the microsymbiont. A perusal of current literature has shown that *P. indica* has enormous potential for growth promotion of plants by colonization of roots. So a lot of focus is given on studies related to *P. indica* to fully exploit the enormous potential encapsulated in this fungus. Though it mimics the morphology, it shares physiological and application potentials of arbuscular mycorrhizal

fungi, but it has its unique features, which command its elaborative commercial success over the AM fungal counterparts.

4.2 Discovery and Taxonomic Position

Though the fungal endophytes are known for asymptomatic infection of the root tissues of several aquatic, semiaquatic, and terrestrial plants since ages (Rodriguez and Redman 2008), the first report of plant beneficial root fungal association was given by Kamienski (1881) followed by reports on the mycorrhizal associations in plants. The discovery of plant root-interacting, endophytic, and arbuscular mycorrhiza-like fungus took place in 1998 by isolation of *P. indica* from the sandy rhizospheric soil of woody shrubs *Prosopis juliflora* and *Zizyphus nummularia* in Thar Desert in northwestern Rajasthan, India (Verma et al. 1998). This fungus, though related to *Rhizoctonia,* is known for its ability to infect a variety of host plants and acts as a potent plant growth-promoting microbe through an array of morphological and physiological attributes which are now patented from the European Patent Office, Muenchen, Germany, with patent number 97121440.8-2105 (Nov. 1998) (Varma and Franken 1997). This fungal culture is available from culture repositories in India (National Bureau of Agriculturally Important Microorganisms, Mau Nath, UP, India) and in Europe (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunsweich, Germany).

On the basis of latest molecular fungal classification including information from molecular analysis of 18S rRNA taxonomically, *P. indica* belongs to order Agaricini of class Hymenomycetes among division Basidiomycota which includes basidiospore-producing, hymenium-bearing fungal forms involved in intimate association with the plant roots particularly related to the ectomycorrhizal genera *Epulorhiza* to *Tulasnella* and *Sebacina*, *Ceratorhiza* to *Ceratobasidium*, or *Moniliopsis* to *Thanatephorus* (Selosse et al. 2002; Hibbett et al. 2007).

Ultrastructurally, the hyphae of *P. indica* bear characteristic dolipore septa with continuous parenthosomes under transmission electron microscope, while serologically this endophyte shares many antigenic properties with that of the mycorrhizal fungi (Varma et al. 2001; Weiss et al. 2004). The 5′ terminal large subunit rRNA sequence analysis and the ultrastructural analysis have led to reclassification of *P. indica* in a separate newly defined order monotypic Sebacinales housing globally distributed great variety of ericoid, orchid, cavendishioid, jungermannioid, and ectomycorrhizae (Setaro et al. 2006; Kirk et al. 2008). On basis of the phylogenetic analysis, order Sebacinales contains two distinct groups or clades, viz*.,* clade/group A comprising of basidiomes and sebacinoid mycobionts harboring ectomycorrhizas and orchid mycorrhizas and clade/group B forming a heterogeneous group including ericoid, cavendishioid, and jungermannioid mycorrhizas (Weiß et al. 2004; Setaro et al. 2006). Order Sebacinales has a single family Sebacinaceae comprised of eight genera and 29 species (Cannon and Kirk 2007; Kirk et al. 2008). *P. indica* has been classified as a member of genus *Sebacina* spp. in the group B of order

Sebacinales. Similar to the other members of its order, *P. indica* bears tremendous plant growth-promoting potentials which result in enormous improvement in the overall plant biomass and thus yield (Singh et al. 2003). Moreover, due to its wider host range, it can effectively infect the orchids and may act as a specific orchidaceous mycorrhizal fungus particularly for genus *Dactylorhiza* (Deshmukh et al. 2006).

4.3 Structure, Life Cycle, and Host Range

P. indica shows morphological, physiological, functional, and serological similarities with AM fungal genera *Glomus* and *Gigaspora* though due to the difference in the large subunit rRNA sequence and presence of dolipore septa, it is not classified among zygomycetous AM fungi. The fungus can easily be axenically cultivated on a number of defined, complex, synthetic media (Hill and Kaefer 2001) which facilitate mass cultivation of this fungus without the requirement of the living organs/tissues of the host plant as are required for cultivation of obligatory symbionts AM fungi.

Morphologically, *P. indica* produces thin-walled, irregularly septate, white, and almost hyaline hyphae which exhibit multinucleate character though there exists variability in number of nuclei in each hyphal segment. These hyphae typically range from 0.7 to 3.5 μm in diameter and show frequent anastomosis, i.e., fusion between branches of the same or different hyphae. The ramifying hyphae bear multinucleate (8–15 nuclei), large pyriform chlamydospores (16–25 μm × 10–17 μm) at their tips and/or at the intercalary positions, individually or in clusters that are formed in the root cortical cells and later are present in soil; however, the sexual structures, hyphal knots, and clamp connections have yet to be reported in this fungus (Varma et al. 2001; Varma 2008). It infects the host roots due to germination of the chlamydospores in soil lying in the vicinity of the host roots and forms inter- and intracellular hyphae in the root cortex, often differentiating into dense hyphal coils and chlamydospores in higher plants (Singh and Varma 2000).

P. indica being a versatile root endophyte possesses a wide spectrum of host plants encompassing angiospermous dicots and monocotyledonous plants including herbaceous, shrubs, as well as tree species like poplar (Pham et al. 2004; Prasad et al. 2005). The host plants being benefited by *P. indica* may involve economically important cash crops like sugarcane, maize, and wheat (Rai et al. 2001; Waller et al. 2005; Baltruschat et al. 2008) to medicinal plants like *Aloe vera, Adhatoda, Artemisia, Abrus, Bacopa, Chlorophytum, Coleus, Spilanthes, Tridax, and Withania* (Kumari et al. 2004; Oelmuller et al. 2009); legume crops like *Glycine max, Pisum sativum,* and *Nicotiana tobaccum* (Barazani et al. 2005) to members of family Chenopodiaceae and Brassicaceae like spinach, mustard, cabbage, and *Arabidopsis thaliana* which are non-mycorrhizal owing to secretion of large amounts of glucosinolates (Kumari et al. 2003; Peskan-Berghofer et al. 2004); and terrestrial orchids (Bougoure et al. 2005; Kaldorf et al. 2005) to bryophytes

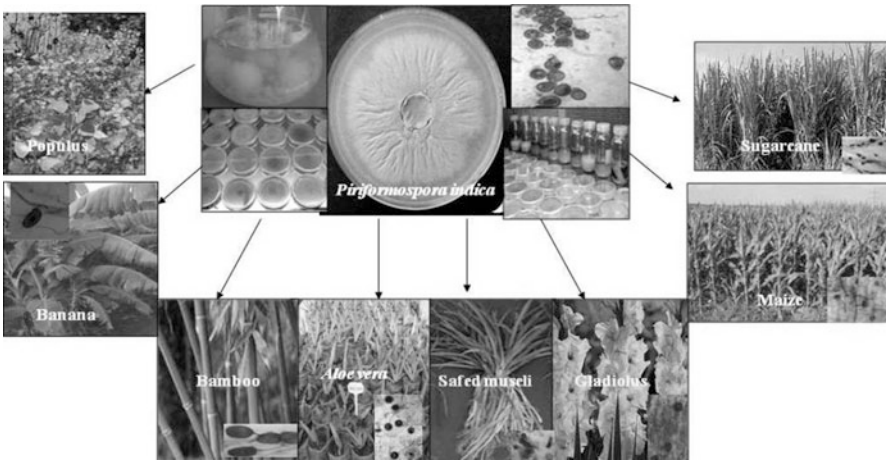

Plate 4.1 Different host plants studied for *P. indica* infection and colonization under green house and field conditions

like liverwort (*Aneura pinguis*) as well as pteridophytes (*Pteris ensiformis*) and gymnosperms (*Pinus halepensis*) (Kottke et al. 2003; Urban et al. 2003; Bidartondo and Duckett 2010). A variety of plant hosts have been studied to exhibit infection and colonization by *P. indica* inoculation in greenhouse as well as field experiments at Punjab Agricultural University, Ludhiana, Punjab, India (Plate 4.1).

4.4 Plant–Fungal Interactions and Mycorrhizosphere Associations

Rhizosphere is the most dynamic zone in soil exhibiting interactions which could be positive, negative, or neutral among a variety of microbial forms with plant roots. Mycorrhizosphere is an extended version of the soil region encompassing plant roots, soil, and extramatricular hyphal extensions of the symbiotic mycorrhizal/endophytic fungi along with the interacting bacteria, fungi, protozoa, and viruses. The dynamicity of the rhizo- or mycorrhizospheric interactions lies in the intricate microbe–microbe or plant–microbe cross talks using secretion of (may be minute quantities of) signaling compounds which may be phenolics or the modified glucosides (Harrison 2005; Paszkowski 2006; Bonfante and Anca 2009). Mycorrhizosphere is characterized by involvement of the bacterial component among the subsets of beneficial rhizomicroflora existing in the vicinity of the growing plant roots due to presence of abundant nutrients. These mycorrhiza-associated

rhizobacteria or helper bacteria may either exist in the vicinity (loosely) or cling tightly on the surface of the fungal extramatricular hyphae to provide the additional benefits to plants for being mycorrhizal host by supporting establishment of the mycorrhiza.

The extent of interaction state and interface between the plant–microbial symbionts is speculated to exist in equilibrium with both partners being equally benefitted in a mutualistic interaction. Over the evolutionary scale, microsymbionts (bacteria, fungi, actinomycetes, cyanobacteria) have modified their external structures and signals to cope up with the plant defense mechanisms as well as both have maneuvered each other using finely orchestrated signal molecules to develop an inter/intracellular coexistence. The microbes particularly alter (initiate or subdue) the defense-related molecules or phytohormones, i.e., salicylic acid, jasmonic acid, and ethylene along with minor players like abscisic acid, gibberellins, and auxins, which are intricately dependent on each other to form a network (Wang et al. 2007; Asselbergh et al. 2008; Koornneef and Pieterse 2008; Navarro et al. 2008). The most popular bacterial symbionts rhizobia secrete chitooligosaccharides, i.e., nod factors which result in changes that lead to suppression of salicylic acid accumulation and reactive oxygen species production in the host plant, and these suppressions help in intrusion of rhizobia through root-hair cells (Shaw and Long 2003).

Arbuscular mycorrhizal associations have been most elaborately studied in symbiotic plant–fungal associations that involve production of diffusible signaling molecules (Myc factors) followed by their release by fungal hyphae and perception by the plant without even actual physical contact between the two members (Kosuta et al. 2003). Similarly, the plant roots excrete few chemicals that result in induction of hyphal branching of the germinating mycorrhizal spores, e.g., presence of strigolactone 5-deoxystrigol compound in root exudates enhances hyphal branching (Akiyama et al. 2005). At the tissue level, mycorrhizal infection of the epidermis followed by the outer and inner cortex of the plant roots occurs largely intercellular and then intracellular by formation of branched arbuscules to increase the fungal–plant interaction interface.

Being an endophytic mycorrhiza, *P. indica* exhibits many common signal and receptor molecules to that of AM fungi though this symbiosis differs from plant–AMF associations in terms of extent of intrusion of the host root tissue which is extensive for the former with both cellular and extracellular fungal growth in epidermal and cortical tissue. The infection process starts with the germination of the pyriform chlamydospores occurring in the vicinity or on the surface of the root followed by invasion of the host roots within one to 2 days by the fungal hyphae that penetrate the epidermal cells *via* anticlinal cell walls to reach intercellular space between cells, while the intracellular invasion is particularly through the plasmodesmata of the plant cell (Deshmukh et al. 2006). The sites of *P. indica* infection include the root tip, root hairs, and the root differentiation zone from where the fungus reaches the cortical tissue and starts producing spores in epidermis and cortex within a week. The intracellular existence of this fungus indicates its

potential to exist inside root cells as hyphae and spores, without provoking tissue necrotization (Deshmukh et al. 2006).

The spectrum of benefits imparted by interaction of this fungus with its host plant includes the growth promotion, production of higher yields, as well as stress resistance to several biotic and abiotic factors. The former two benefits may be attributed to the production of phytohormone by the fungus itself as well as modulation of the host phytohormone profile (amount and type of phytohormone). This endophytic fungus is also known to produce phytohormones like auxins and cytokinins, though amounts of fungal auxins produced are minuscule, but it is known to modulate the plant auxin production and also enhances auxin production by the plant *in vivo* and produces substantial amount of cytokinins itself as well as increases plant cytokinin levels (Vadassery et al. 2008). However, the stress resistance is *via* the suppression/elevation of the molecules/products involved in the host defense-related circuits. A study by Camehl et al. (2010) exhibits the role of the ethylene signaling components and ethylene-targeted transcription factors in balancing or maintaining the beneficial interaction between *P. indica* and the infected host.

4.5 Molecular Dissection of Interaction at Genome and Proteome Level

As most of the information on the molecular sequences of majority of mycorrhizal plants and mycorrhiza are still fragmentary, there is plenty of room for the development of innovative tools and techniques for deciphering the mycorrhizal–host plant interactions at the molecular scales (Schafer et al. 2007). With the popularization of the novel tools and techniques of the -omic sciences, it is now possible to dissect the level of interactions and explore the diversity of interaction signals during development of symbiosis and signal pathways at the molecular levels, be it genetic/phylogenomic, proteomic, to metabolomic to reveal the share of contribution of each partner in the association as the genomic sequences of both mycorrhiza and host plant are now readily available (Hata et al. 2010). Both rhizobia and mycorrhizal fungi share a common signaling pathway with many signaling proteins of same origin or type (Table 4.1). This suggests a common ancestry of the mode of infection by the two endosymbionts which later segregated or rather became specialized for both the counterparts in their respective hosts. The rhizobia specialized to interact with the signaling compounds of the legume hosts, while the mycorrhiza retained the broad host spectrum range and only curtailed few plants from getting benefits from the association. Molecularly, the plant–mycorrhizal associations result in elicitation of responses among which the rapid and transient shifts in the intracellular calcium (most common intracellular messenger in signal transduction by plants) happen most commonly as the mycorrhizal signals are usually perceived by the receptor-like kinases which elicit phosphorylation

Table 4.1 The genes involved in the common symbiosis pathway (CSP) shared by rhizobia and mycorrhiza

Plant genes	Role or function	References
Nodulation receptor kinase (NORK)	Contains three leucine-rich repeats (LRRs) involved in ligand binding and cell signaling Plays an intermediary part and connects the activity of Myc factor receptors to subsequent steps	De Mita et al. (2007), Gianinazzi-Pearson et al. (2007), Markmann et al. (2008)
Symbiosis leucine-rich repeat receptor kinase (SYMRK)	Epistatic to other common symbiosis pathway genes	Stracke et al. (2002), Parniske (2004), Reinhardt (2007)
CASTOR and POLLUX (cation channels)	Predicted to be membrane-integrated channel proteins showing structural homology with Ca-gated potassium channels Involved in upstream of intracellular calcium spiking Localized in the plastids of root cells, indicating plastid role in controlling intracellular symbioses	Imaizumi-Anraku et al. (2005), Banba et al. (2008)
NUP85 and NUP133 (nucleoporins)	Components of the nucleopore complex involved in trafficking and/or localization of factors essential for induction of Ca signals	Kanamori et al. (2006), Saito et al. (2007), Banba et al. (2008)
Ca/calmodulin (CaM)-dependent protein kinase (CCaMK)	Structural similarity to CaMKII in mammals Act as a key regulator protein for the downstream genes leading to activation of the downstream signaling pathways	Tirichine et al. (2006), Banba et al. (2008)
CYCLOPS/IPD3	Known to interact with CCaMK Activates the downstream gene cascade(s) leading to successful infection of mycorrhiza	Messinese et al. (2007), Yano et al. (2008)
Does not Make Infection genes (DMI1, DMI2, and DMI3)	Involved in formation of the transient prepenetration apparatus as revealed by the *dmi* mutants that exhibit total or partial block of epidermal penetration by *Gigaspora* hyphae	Genre et al. (2005)
MtMSBP1 gene	Encodes a membrane-bound steroid-binding protein Involved in mycorrhizal development as evidenced by occurrence of aberrant mycorrhizal phenotype with thick and septated appressoria, decreased number of arbuscules, and distorted arbuscule morphology by RNAi downregulation of gene Speculated to play role in sterol homeostasis in root	Kuhn et al. (2009)

followed by cation channel-based induction of calcium changes in nucleus and finally activation of calcium calmodulin-dependent kinase (Navazio et al. 2007). Vadassery et al. (2009a) have reported induction of intracellular calcium in roots of *Arabidopsis* in response to cell wall extract of *P. indica*. The inevitability of the presence of certain genes responsible for production of signaling components could be ascertained by pinpointing the absence of gene homologs of several signaling components in certain non-mycorrhizal plants which suggests the exact genetic basis of the non-mycorrhizal behavior of members of Chenopodiaceae and Brassicaceae.

P. indica possesses 6 chromosomes comprising a total genome size of 15–20 Mb, and this would be helpful in identification of the loci involved in production of signaling proteins/receptor or enzymes for initiation and establishment of infection in host root cells. Moreover, the fungus is transformable which provides an added advantage of scrutinizing the events of infection during interaction using conventional and advanced microscopy techniques like confocal laser scanning microscopy.

P. indica exhibits similarities as well as variations in the expression or repression of certain common genes involved in initiation and establishment of mycorrhizal–plant interaction (Peskan-Berghofer et al. 2004). The majority of reports on molecular basis of *P. indica* host interactions including the elucidation of the molecular mechanisms responsible for host recognition, root colonization, and subsequent beneficial activities have been obtained by studies on plants whose genome is known, particularly the model plant *Arabidopsis thaliana* and *Nicotiana tobaccum*. The structural proteomic tools, i.e., 2D gel electrophoresis and mass spectrometry, are most useful techniques to identify the amount and type of the expressed proteins (Kalia and Gupta 2005). The physiological changes brought about on infection of this microsymbiont in the plant can be traced down even at the molecular level particularly using the structural proteomic tools. The physiological alterations involve the changes in the protein profile of the membrane (MATH, i.e., meprin and tumor necrosis factor receptor-associated factor (TRAF) homology domain containing protein) and endoplasmic reticulum proteins (a leucine-rich repeat protein LRR2 and PYK10 beta-glucosidase) (Shahollari et al. 2007; Sherameti et al. 2008). Similarly, other genes like nitrate reductase and glucan water dikinase are upregulated by *P. indica* infection (Sherameti et al. 2005). Shahollari et al. (2005) have also documented transient upregulation of a plasma membrane receptor kinase during the recognition period of both organisms. Vadassery et al. (2009b) have reported the crucial role of monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 for development of a mutualistic interaction between *P. indica* and *Arabidopsis*.

There are certain genes which are prerequisite for mycorrhizal colonization but are not required by *P. indica* to cause infection like deactivation of *dmi-1* gene encoding an ion carrier which affects the mycorrhizal infection but not infection by *P. indica*. Gutjahr et al. (2009) have reported induction of starch accumulation in *Lotus japonicus* roots due to release of certain presymbiotic factors by *Gigaspora margarita*.

For establishment of successful infection by *P. indica* in host, certain specific genes need to be up/downregulated, e.g., transient upregulation of the membrane receptor leucine-rich repeat protein while partial deactivation of the sphingosine kinase gene. Schafer et al. (2009) have reported the role of gibberellin (GA) synthesis and perception for mutualisitic interaction with *P. indica*. The various benefits imparted by *P. indica* to its inoculated host can also be traced at the molecular level to ascertain the role of particular gene or set of genes in a specific function and would be more elaborately discussed in the applications section.

4.6 Applications

There are several benefits of *P. indica* inoculation in a variety of plants which may range from phenotypically identifiable enhanced growth and development to better biomass and grain yields. Moreover, the benefits may also be traced down at the biochemical or molecular level with increased production of certain phytochemicals (biochemical), endurance to sustain/withstand abiotic stresses like cold, drought, and salt-stress tolerance (physiological), as well as development of resistance to several potential phytopathogens. The fungus possesses positive phytopromotional effects due to production/modulation of phytohormone levels (plant bioregulation ability), apart from its role in mobilization and transportation of the plant unavailable phosphorous reserves in soil beyond the depletion zone (P-mobilizer ability). The role of this endophytic fungus in relieving the abiotic and biotic stresses could be elaborately utilized for biological hardening of micropropagated plantlets and as biological control agent. It is also well established that *P. indica* exhibits synergistic interactions with other plant growth-promoting rhizobacteria (PGPRs) similar to mycorrhiza (Bonfante and Genre 2008; Sharma et al. 2008).

4.6.1 Nutrient Uptake

Similar to AM fungi, *P. indica* is well known to possess ability to extract, mobilize, and transport two major macronutrients (phosphorus and nitrogen) as well as several micronutrients from soil and is actively involved in transferring these nutrients to the infected host plant *via* plant–fungal interfaces.

4.6.1.1 Macronutrient Uptake

P. indica acts as a potential phosphate mobilizer as it produces high amounts of phosphatase enzymes (cleave phosphate ester bonds to hydrolyze insoluble polyphosphates and organic phosphates) (Yadav et al. 2004) as well as can indirectly mobilize soil P-reserves by interacting/communicating with diverse

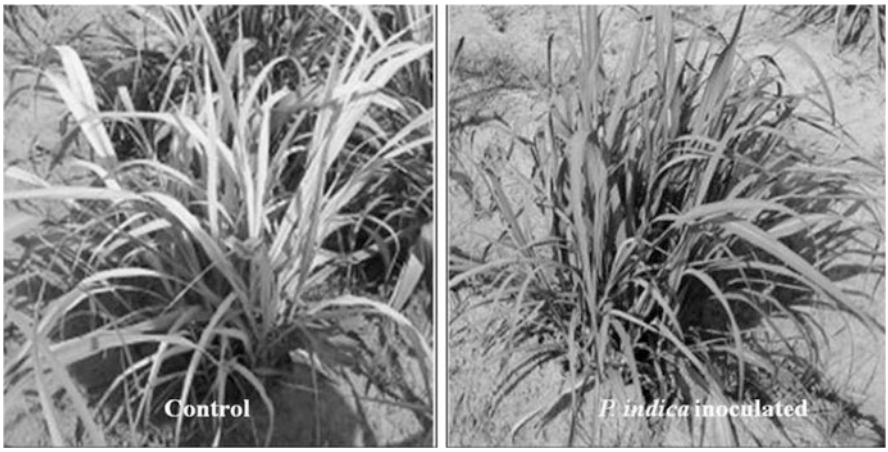

Plate 4.2 Effect of *P. indica* inoculation on iron acquisition in ratoon sugarcane var CoJ 88

rhizobacteria having inorganic P-solubilizing capabilities by virtue of production of a variety of organic acids (Singh et al. 2009). The recent studies using molecular tools have revealed that coinoculation of *P. indica* with P-solubilizing rhizobacteria (PSRB) results in enhanced P-uptake and P-content in host plant due to better establishment of PSRB in the mycorrhizosphere (Meena et al. 2010). Moreover, the *in vitro* laboratory studies have also revealed the ability of *P. indica* to grow on a variety of P-sources, i.e., inorganic, organic, and polyphosphates, which emanates its role as an active P-solubilizer apart from being P-mobilizer. *P. indica* also mediates nitrate uptake from the soil, which is in contrast to AMF, where nitrogen is preferentially absorbed as ammonium (Sherameti et al. 2005).

4.6.1.2 Micronutrient Uptake

Micronutrients are the elements essentially required in very small quantities for maintaining plant growth and health, among which include iron, boron, copper, zinc, molybdenum, manganese, and chlorine. The majority of micronutrients act as cofactors for several enzyme/enzyme complexes and have a greater role as essential members of the electron transport system proteins. Similar to mycorrhiza, *P. indica* is known to extract, mobilize, and translocate micronutrients particularly zinc, iron (Plate 4.2), manganese, and copper from soil and make it available to the plant (Gosal et al. 2010b; Achatz et al. 2010; Gosal et al. 2008a; Gosal et al. 2007).

4.6.2 Mechanism of Nutrient Uptake

Phosphorus solubilization from soil is performed by action of two types of phosphatase enzymes, viz., acid and alkaline phosphatases with the former shared by both the symbionts and involved in uptake of phosphorus, while the latter is mainly of fungal origin and involved in assimilation of phosphorus (Tisserant et al. 1993; Tarafdar and Rao 1996; Fries et al. 1998). Cytochemically, acid phosphatases could be localized on the membrane surfaces or plasmalemma in mycorrhizal roots, while alkaline phosphatases are localized on plant membranes surrounding the arbuscular or periarbuscular membrane of intercellular fungal hyphae. The *P. indica*-specific phosphate transporter has recently been deciphered to be a high-affinity P-transporter belonging to major facilitator superfamily and structurally contains 12 transmembrane helices divided into two halves connected by a large hydrophilic loop in the middle. The expression profiling of this 1,815 bp P-transporter has showed activity to be localized to the external hyphae of *P. indica* colonized with host plant root and shares significant sequence similarity with the already known GvPT (*Glomus versiforme*) and PHO84 (*S. cerevisiae*) phosphate transporters (Yadav et al. 2010). Similar to arbuscular mycorrhiza, *P. indica* also exhibits uptake and assimilation of the nitrogen species from the soil. *P. indica* stimulates NADH-dependent nitrate reductase activity in roots of *Arabidopsis* and tobacco plants with the enhanced enzymatic activity that could be correlated with an increased transcription of the corresponding plant gene (Sherameti et al. 2005). *P. indica* mediates nitrate uptake from the soil, which is in contrast to AMF, where nitrogen is preferentially absorbed as ammonium.

4.6.3 Biotization or Biological Hardening

Biotization is a common term used to signify inoculation of a plant beneficial microbe on surface of seed, roots, leaves, or other aerial parts of the plant that results in enhancement of growth, vigor, and finally yield (in biomass or grains) of inoculated plant. The germinated seed or even vegetatively propagated plants exhibit elaborate cross talks with the soil rhizomicrobes and thus have attained knowledge regarding the inevitability of the microbial counterparts as well as show dependence on beneficial microbes for better growth and development. However, the micropropagated plants are reared in sterile or aseptic conditions creating a microbiological vacuum, and thus these plants have to be primed with beneficial microbes for redemption of the microbiological void which equips these plants to acclimatize well to counteract the problems concerning survival and development of plantlets (Gosal et al. 2010a, b, 2008b). The micropropagated plants exhibit higher mortality rate due to the "transient transplant shock," on transfer of plantlets from lab to land. Above all, several survived plants may exhibit stunted growth or are often attacked by pathogenic soil microbes which further decrease the economic benefits

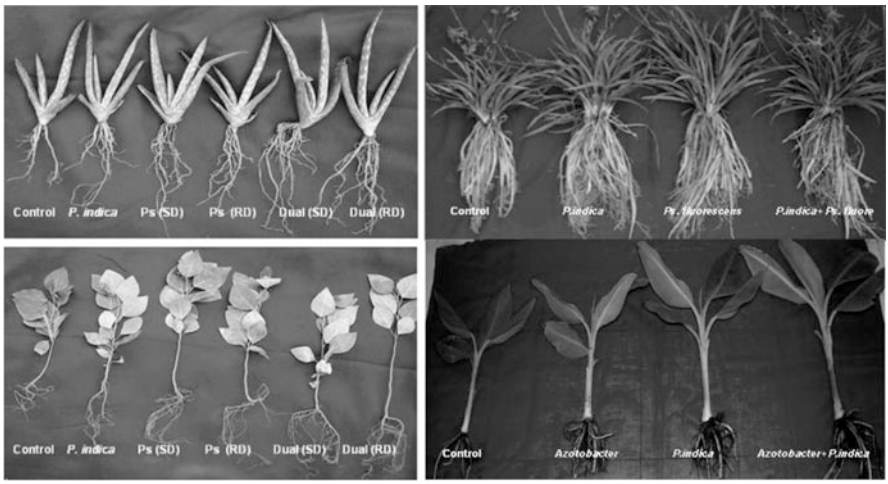

Plate 4.3 Effect of inoculation of *P. indica* and plant growth promoting bacteria on growth and development of different micropropagated plants under green house conditions

particularly in case of tissue-cultured floricultural or horticultural crop plants. However, at the weaning stage, about 10–40 % of plantlets either die or do not attain market standard, thereby causing significant losses at the commercial level.

The application of arbuscular mycorrhizal fungi (AMF) as a tool for biological hardening has solved a part of the problem. The lack of an authentic AMF axenic culture is an inherent problem for commercial application. Because of its ease of culture and plant growth promotional effect, Sahay and Varma (1999) evaluated the potential of *P. indica* to improve the survival and establishment of tissue-culture-raised plants. They recorded 88–94 % survival rate among the *P. indica*-inoculated regenerated plantlets of tobacco over uninoculated control plantlets and also observed maximum revival capacity of the inoculated plantlets to overcome the stress as compared to the control. Thus, the fungus has the potential to render protection to the micropropagated plantlets and help them escape the "transient transplant shock" (Shende et al. 2006). *P. indica* is known to exhibit similar brilliant positive effects on several plants belonging to diverse families like micropropagated *Chlorophytum borivilianum*, *Aloe vera*, *Populus*, *Dendrocalamus strictus*, sugarcane, and maize plants (Gosal et al. 2010a, b, 2009, 2008a, b, 2007) (Plate 4.3). Above all the inoculation of *P. indica* in medicinal plants also increases the production of amount of the medicinally active compound in the inoculated plants (Gosal et al. 2010b; Baldi et al. 2008). The saponin content of safed musli has been reported to increase on inoculation (Gosal et al. 2010b). Similarly, the amount of podophyllotoxin and 6-methoxypodophyllotoxin production (possess pharmacological anticancer properties) (Farkya et al. 2004) was increased by about four- and eightfold by co-cultivation of cell suspensions of *Linum album* with *P. indica* and *S. vermifera* (Baldi et al. 2008).

Plate 4.4 Effect of *P. indica* inoculation on growth and development of ratoon sugarcane var CoJ 88 under field conditions

4.6.4 Plant Growth-Promoting Activity

As this endophytic fungus exhibits AM fungi-like mechanisms regarding colonization of the roots of a wide variety of plant species and positive benefits imparted to even non-mycorrhizal hosts, it could be well speculated that on inoculation it will act by several known/unknown mechanisms to impart benefits. In general, as discussed above, *P. indica* exhibits root growth promotion even before noticeable root colonization which may be attributed to the enhanced nutrient (P particularly) availability and massive transfer to the aerial parts of the inoculated plant/seedlings (Shahollari et al. 2005). However, experimental input by Barazani et al. (2005) elaborates the plant growth promotional effects of inoculation of *P. indica* and also comprehends that the improvement of the nutritional status (P and N status) of the plant is not always responsible for visible enhanced vegetative or reproductive performance. They recorded that seed inoculation of axenic cultures of *P. indica* and *Sebacina vermifera* in *Nicotiana attenuata* resulted in stimulated seed germination and increased growth/stalk elongation. Plant growth promotion was observed in all the crop plants inoculated with *P. indica* under greenhouse as well as field conditions. Gosal et al. (2008a) have reported the increase in the shoot and root length of the maize plants under field conditions. Similar increase in the plant height was recorded by *P. indica* inoculation in *Dendrocalamus strictus* grown under greenhouse conditions (Gosal et al. 2007). A significant increase in tiller number, cane number, cane height, and cane yield were reported in *P. indica*-inoculated ratoon sugarcane plants as compared to uninoculated conventionally propagated sugarcane var CoJ 88 (Gosal et al. 2010c) (Plate 4.4). The plant growth promotional (PGP) activities of *P. indica* encompass both production of the phytohormones by itself and ordered alteration in the secretion pattern or amount of phytohormone production by the inoculated plant. Fakhro et al. (2010) reports

increase in *P. indica*-inoculated tomato fruit biomass in hydroponic culture with an approximately 100 % increase in fresh weight and approximately 20 % increase in dry matter content. Even with the application of fungicides like Bavistin, *P. indica* exhibited infection and colonization and finally increased yield in maize crop under field conditions (Gosal et al. 2009). Prajapati et al. (2008) have demonstrated the enhanced plant growth promotional effect on rice plant by coinoculation of *P. indica* with a known PGPR *Azotobacter chroococcum* and application of vermicompost. However, a report by Ray and Valsalakumar (2010) exhibits the nongrowth promotional behavior of *P. indica* in green gram, and the report concludes that *P. indica* is not a good synergist on green gram.

4.6.5 Tolerance to Abiotic and Biotic Stresses

Apart from these effects on vegetative and generative plant development, *P. indica* mediates stress tolerance to infested plants. The stress tolerance could be imparted in the inoculated host towards a variety of factors which could be largely classified or categorized into abiotic factors (including temperature extremes, saline/alkaline soil conditions, low moisture/soil–water conditions) and biotic factors (plant disease-causing microbial agents particularly including phytopathogenic bacteria and fungi) (Yuan et al. 2010).

4.6.5.1 Abiotic Stress Tolerance

Plant exhibits complex responses to abiotic stresses like salinity, heat, and drought stresses and involves elicitation of complex signal transduction pathways that are manifested as elaborate responses at the genetic as well as physiological scales. Plants being living organisms do respond, in positive or negative manner, towards various types of abiotic stresses; however, relatively very few plant species are known to withstand elevated abiotic stress levels (Alpert 2000). During their initial years of coevolution with the beneficial soil microbes, plants have marked out few mechanisms to overcome the abiotic stresses which either may be controlled by the plant genome or may involve the active role of the colonizing microbe, i.e., endophyte-mediated plant tolerance (Baltruschat et al. 2008). The abiotic stress tolerance attribute of the mycorrhizal fungi has been well deciphered. The mechanisms of plant growth promotion by *P. indica* are quite similar to the mycorrhizal fungi as a lengthened endophytic period in the inoculated host suggests its coevolution of common signaling and response pathways similar to the elaborately explored mycorrhizal fungi (Kogel et al. 2006); however, the pathways involved in imparting the abiotic as well as biotic stress tolerance are quite dissimilar to the mycorrhizal infections.

P. indica is known to impart tolerance towards many abiotic stress factors like extreme cold temperature, drought, and saline soil conditions. The saline and

drought-stress tolerance are most deciphered responses though meager research regarding the cold temperature stress tolerance due to *P. indica* inoculation has been reported. Many researchers have designed experimental setups to study the various mechanisms of *P. indica*-induced salt-stress tolerance. A report by Baltruschat et al. (2008) provides insight on several biochemical mechanisms responsible for induction of salt-stress tolerance in *P. indica*-inoculated barley plants by monitoring the key physiological markers affected by salt stress. They observed that on colonization of salt-stressed plant roots, *P. indica* attenuated the salt-induced lipid peroxidation, metabolic heat efflux, and fatty acid desaturation in plant leaves while elevating the amount of antioxidants such as ascorbic acid and antioxidant enzymes. These findings suggest that antioxidants might play a role in both inherited and endophyte-mediated plant tolerance to salinity.

Similar to induction of salt-stress tolerance, *P. indica* also confers drought-stress tolerance to inoculated plants which could be traced down at the molecular scale by figuring out alteration in expression of quite diverse set of stress-related genes (Seki et al. 2002). A report by Sheramati et al. (2008) suggests the induction of drought tolerance in *Arabidopsis* by co-cultivating or mock treating the plants with *P. indica* before exposing to mild drought stress. The further transfer of plants to soil resulted in better survival in comparison to the uninoculated controls. At the molecular level, the *P. indica*-inoculated plants exhibited faster and stronger upregulation of the message levels for several key drought-stress inducible enzymes, viz., phospholipase Dδ, CBL1, and HAT. The drought-stress inducible genes may either produce proteins that have a direct role in conferring tolerance to low water availability stress (like chaperones, late embryogenesis-abundant proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases) or indirectly regulate the stress response genes (like transcription factors, protein kinases/phosphatases, phospholipid metabolism enzymes, signaling molecules such as calmodulin-binding proteins) (Shinozaki and Yamaguchi-Shiozaki 2007) by playing a vital role in the signal transduction cascades involved in induction/suppression of direct drought-related genes (Shinozaki et al. 2003).

4.6.5.2 Biotic Stress

The plant has to bear several biotic stresses like the infection by a variety of phytopathogens belonging to diverse groups of viruses, bacteria, fungi, nematodes, insects, and higher animals particular concern for the herbivores. The beneficial rhizomicrobes exhibit a tendency to prime the plant from the attack by the phytopathogens, thereby imparting inducible systemic stress resistance to the pathogen attack. Different mechanisms have been suggested as being responsible for their biocontrol activity, which include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism, and production of inhibitory compounds.

P. indica colonizes the cortex of roots of a wide variety of plant species and promotes their growth and induces resistance against soilborne fungal pathogens in a manner similar to arbuscular mycorrhizal fungi (Ghahfarokhi and Goltapeh 2010). The inoculation of *P. indica* followed by colonization of the internal tissues of the plant may impart systemic resistance to various fungal and bacterial pathogens though the molecular mechanisms for these benefits have yet to be deciphered. Waller et al. (2008) have reported induction of systemic resistance against the leaf pathogenic fungus *Blumeria graminis* f. sp. *hordei* by colonization of *P. indica* and *Sebacina vermifera* strains in barley roots. Similar report of induction of resistance by *P. indica* in *Arabidopsis* for powdery mildew pathogen *Golovinomyces orontii* has been provided by Stein et al. (2008) which not only shows the reduction in conidia formation by the pathogen but also provides insight on the mechanism of *P. indica*-conferred ISR.

The inoculation of *P. indica* may also result in increase in the content of plants' own antimicrobial component(s). A report by Raj et al. (2004) advocates the enhancement of the antifungal activity of the *Spilanthes calva* plant extract of *P. indica*-inoculated plant against *Fusarium oxysporum* and *Trichophyton mentagrophytes*. On the other hand, Knecht et al. (2010) have reported the expression of *Beta vulgaris* plant germin-like proteins BvGLP-1 gene in transgenic *Arabidopsis* that elevated the H_2O_2 content and conferred significant resistance to two fungal pathogens *Verticillium longisporum* and *Rhizoctonia solani*. They have also reported that BvGLP-1 expression in *Arabidopsis* constitutively activates the expression of a subset of plant defense-related proteins such as PR-1 to PR-4 and PDF1.2 but not PDF2.1 and PDF2.3. An elaborate report of Felle et al. (2009) provides the molecular alteration exhibited by the *P. indica*-inoculated barley (*Hordeum vulgare* L.) plant. They suggested alteration in the surface pH characteristics by a constant flow of *P. indica* chlamydospores along primary roots. The root zones exhibit enhanced H^+ extrusion resulting in occurrence of transiently alkalized root-hair zone, while the elongation zone remains acidified within short period (8–10 min) of *P. indica* inoculation. The initial response is also observed in the aerial portion like leaves where the leaf apoplast began to acidify, thus providing potentiated systemic response to *Blumeria graminis* f. sp. *hordei* induced by *P. indica* in barley. Contrasting reports of involvement of the phytohormones in imparting systemic resistance in *P. indica*-induced resistance have also been proposed.

P. indica is also known to possess the ability to recruit the plant hormone signaling in order to manipulate plant defense. Schafer et al.'s (2009) experimental data would help identify gibberellin signaling as potential target for successful fungi and also reveals the complexity of compatibility mechanisms in host–microbe interactions. The traditional stress-related molecule like salicylic acid is largely not much affected by the *P. indica* inoculation. However a report by Stein et al. (2008) correlates the role of jasmonic acid and priming of jasmonic acid-responsive vegetative storage protein expression and its subsequent elevation to powdery mildew fungus (*Golovinomyces orontii*) infection. This report further suggests the

involvement of the reminiscent of induced systemic resistance for the resistance conferred by *P. indica*.

On inoculation, *P. indica* vigorously infects host root tissue and blocks the receptors for the attachment and adsorption of the phytopathogens which could be considered as the nonspecific physical way of this beneficial fungus to safeguard the inoculated plant. The fungus exhibits a scorable bioprotectant activity against a known fungal phytopathogen *Fusarium verticillioides*. The *P. indica*-primed or *P. indica*-inoculated maize plants showed a decrease in the antioxidant enzyme activity which curtails the *F. verticillioides* infection of new tissues as well as further controls the colonization of already infected tissues. This phytopathogen proliferates in the host tissue by playing and befooling the antioxidant enzyme machinery of the host. It causes increased antioxidant enzyme activity which minimizes the chances of oxidative burst (excessive production of reactive oxygen species), and therefore *F. verticillioides* might be protected from the oxidative defense system during colonization. Thus, this report explains the bioprotection ability of *P. indica* against the phytopathogens dissected at the molecular scale (Kumar et al. 2009). Similar report of biocontrol action of *P. indica* in tomato against *Verticillium dahliae* has been provided by Fakhro et al. (2010) which states a more than 30 % decrease in the disease severity caused by *V. dahliae* on tomato plants colonized by the endophyte.

4.7 Conclusions

P. indica possesses the phytopromotional to biocontrol properties, imparts stress tolerance to abiotic as well as biotic factors, and also exhibits enhanced nutritional availability to the inoculated plant. It has enormous application potentials for the biotization of the micropropagated plants and thus has a pivotal role to play in the new age phenomena of sustainable agriculture for enhanced productivity. The multifunctional abilities of this novel endophyte are well known though the underlying molecular mechanisms are yet to be deciphered in several hosts. The dissection of the molecular mechanisms to these activities would not only be useful to understand the basics of its action spectrum but would also provide the required knowledge for manipulation of the genomics of endophyte which could be harnessed for formation of multiple spectrum biofertilizer for a varied host plants and for the traditional grain and forage crops.

References

Achatz B, von Ruden S, Andrade D, Neumann E, Pons-Kühnemann J, Kogel KH, Franken P, Waller F (2010) Root colonization by *Piriformospora indica* enhances grain yield in barley under diverse nutrient regimes by accelerating plant development. Plant Soil 33:59–70

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824–827

Alpert P (2000) The discovery, scope, and puzzle of desiccation tolerance in plants. Plant Ecol 151:5–17

Arpana J, Bagyaraj DJ (2007) Response of Kalmegh to an arbuscular mycorrhizal fungus and a plant growth promoting rhizomicroorganism at two levels of phosphorus fertilizer. Am-Euras J Agric Environ Sci 2:33–38

Asselbergh B, De Vleesschauwer D, Hofte M (2008) Global switches and fine-tuning-ABA modulates plant pathogen defence. Mol Plant Microbe Interact 21:709–719

Baldi A, Jain A, Gupta N, Srivastava AK, Bisaria VS (2008) Co-culture of arbuscular mycorrhiza-like fungi (*Piriformospora indica* and *Sebacina vermifera*) with plant cells of *Linum album* for enhanced production of podophyllotoxins: a first report. Biotechnol Lett 30:1671–1677

Baltruschat H, Fodor J, Hauuach BD, Niemczyk E, Barma B, Gullner G, Janeczko A, Kogel KH, Schafer P, Schwarczinger I, Zuccaro A, Skoczowski A (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytol 180:501–510

Banba M, Gutjahr C, Miyao A, Hirochika H, Paszkowski U, Kouchi H, Imaizumi-Anraku H (2008) Divergence of evolutionary ways among common sym genes: CASTOR and CCaMK show functional conservation between two symbiosis systems and constitute the root of a common signaling pathway. Plant Cell Physiol 49:1659–1671

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. Oecologia 146:234–43

Bidartondo MI, Duckett JG (2010) Conservative ecological and evolutionary patterns in liverwort–fungal symbioses. Proc R Soc B 277:485–492

Bonfante P, Anca IA (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annu Rev Microbiol 63:363–383

Bonfante P, Genre A (2008) Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective. Trends Plant Sci 13:492–498

Bougoure JJ, Bougoure DS, Cairney JW, Dearnaley JD (2005) ITS-RFLP and sequence analysis of endophytes from Acianthus, Caladenia and Pterostylis (Orchidaceae) in southeastern Queensland. Mycol Res 109:452–460

Brundrett MC (2002) Coevolution of roots and mycorrhizas of land plants. New Phytol 154:275–304

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Oelmuller R (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol 185:1062–1073

Cannon PF, Kirk PM (2007) Fungal families of the World. CABI, Wallingford, p 328

De Mita S, Santoni S, Ronfort J, Bataillon T (2007) Adaptive evolution of the symbiotic gene *NORK* is not correlated with shifts of rhizobial specificity in the genus *Medicago*. BMC Evol Biol 7:210. doi:10.1186/1471-2148-7-210, cited online at URL http://www.biomedcentral.com/1471-2148/7/210

Deshmukh S, Huckelhoven R, Schafer P, Imani J, Sharma M, Waller F, Kogel KH (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Fakhro A, Andrade-Linares DR, von Bargen S, Bandte M, Buttner C, Grosch R, Schwarz D, Fanken P (2010) Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens. Mycorrhiza 20:191–200

Farkya S, Bisaria VS, Srivastava AK (2004) Biotechnological aspects of the production of the anticancer drug podophyllotoxin. Appl Microbiol Biotechnol 65:504–519

Felle HH, Waller F, Molitor A, Kogel KH (2009) The mycorrhiza fungus *Piriformospora indica* induces fast root-surface pH signaling and primes systemic alkalinization of the leaf apoplast upon powdery mildew infection. Mol Plant Microbe Interact 22:1179–1185

Fries LLM, Pacovsky RS, Safir GR, Kaminski J (1998) Phosphorus effect on phosphatase activity in endomycorrhizal maize. Physiol Plantarum 103:162–171

Genre A, Chabaud M, Timmers T, Bonfante P, Barker DG (2005) Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before Infection. Plant Cell 17:3489–3499

Ghahfarokhi RM, Goltapeh ME (2010) Potential of the root endophytic fungus *Piriformospora indica*; *Sebacina vermifera* and Trichoderma species in biocontrol of take-all disease of wheat *Gaeumannomyces graminis* var. *tritici* in vitro, in Iran. J Agric Technol 6:11–18

Gianinazzi-Pearson V, Sejalon-Delmas N, Genre A, Jeandroz S, Bonfante P (2007) Plants and arbuscular mycorrhizal fungi: cues and communication in the early steps of symbiotic interactions. Adv Bot Res 46:181–219

Gosal SK, Baddesha HS, Kalia A, Varma A (2008a) Role of endophytic fungus *Piriformospora indica* in nutrient acquisition and grain yield of maize in Phosphorus deficient soil under field conditions. J Environ Ecol 26:2172–2175

Gosal SK, Chandi GK, Gosal SS, Varma A (2008b) Role of *Piriformospora indica* and *Pseudomonas fluorescens* as growth promoting agents in biotized micropropagated plants of Populus deltoids. Indian J Agroforestry 10:84–90

Gosal SK, Bhatia P, Chawla JS, Chhibba IM, Varma A (2009) Biofortification of maize using symbiotic Fungi. In: Oral paper presented at 12th Punjab Science Congress held at Punjab Agricultural University Ludhiana, Punjab from February 7–9, 2009

Gosal SK, Chandi GK, Gosal SS, Varma A (2010a) Biotization of micropropagated *Aloe vera* with *Pseudomonas fluorescens* and *Piriformospora indica*. J Med Arom Plant Sci 32:91–96

Gosal SK, Kalrupia A, Gosal SS, Chhibba IM, Varma A (2010b) Biotization with *P. indica* and *Pseudomonas fluorescens* improves survival rate, nutrient acquisition, field performance and saponin content of micropropagated *Chlorophytum* sp. Indian J Biotechnol 9:289–297

Gosal SK, Sharma M, Gosal SS, Chhibba IM, Bhatnagar K, Varma A (2011) Biohardening with *Piriformospora indica* improves survival rate, growth, iron uptake and cane yield of micropropagated sugarcane. Int Sugar J 113:382–88

Gosal SK, Kumar L, Kalia A, Chauhan R, Varma A (2007) Comparative role of *Piriformospora indica* as biofertilizer for plant growth promotion and micronutrient acquisition activity on *Dendrocalamus strictus* seedlings. J Bamboo Rattan 6:223–228

Gutjahr C, Novero M, Guether M, Montanari O, Udvardi M, Bonfante P (2009) Presymbiotic factors released by the arbuscular mycorrhizal fungus *Gigaspora margarita* induce starch accumulation in *Lotus japonicus* roots. New Phytol 183:53–61

Harrison MJ (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 59:19–42

Hata S, Kobae Y, Banba M (2010) Interactions between plants and arbuscular mycorrhizal fungi. Int Rev Cell Mol Biol 281:1–48

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Thorsten-Lumbsch H, Lutzoni F, Matheny PB, Mclaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson KH, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo JM, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüßler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N (2007) A higher-level phylogenetic classification of the Fungi. Mycol Res 111:509–547

Hill TW, Kaefer E (2001) Improved protocols for aspergillus medium: trace elements and minimum medium salt stock solutions. Fungal Genet News Lett 48:20–21

Hu J, Lin X, Wang J, Dai J, Cui X, Chen R, Zhang J (2009) Arbuscular mycorrhizal fungus enhances crop yield and P-uptake of maize (*Zea mays* L.): A field case study on a sandy loam soil as affected by long-term P-deficiency fertilization. Soil Biol Biochem 41:2460–2465

Imaizumi-Anraku H, Takeda N, Charpentier M, Perry J, Miwa H, Umehara Y, Kouchi H, Murakami Y, Mulder L, Vickers K, Pike J, Downie JA, Wang T, Sato S, Asamizu E, Tabata S, Yoshikawa M, Murooka Y, Wu GJ, Kawaguchi M, Kawasaki S, Parniske M, Hayash M (2005) Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature 433:527–531

Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea J (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertility Soils 37:1–16

Kaldorf M, Koch B, Rexer KH, Kost G, Varma A (2005) Patterns of interaction between Populus Esch5 and *Piriformospora indica*: a transition from mutualism to antagonism. Plant Boil (Stuttg) 7:210–218

Kalia A, Gupta RP (2005) Proteomics: a paradigm shift. Crit Rev Biotechnol 25:173–198

Kamienski F (1881) Die Vegetationsorgane der Monotropa hypopitys L. Botanische Zeitung 39:457–461

Kanamori N, Madsen LH, Radutoiu S, Frantescu M, Quistgaard EM, Miwa H, Downie JA, James EK, Felle HH, Haaning LL, Jensen TH, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J (2006) A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. Proc Natl Acad Sci USA 103:359–364

Kirk PM, Cannon PF, Minter DW, Stalpers JA (2008) Dictionary of the fungi, 10th edn. CABI, Wallingford, UK, p 627

Knecht K, Seyffarth M, Desel C, Thurau T, Sherameti I, Lou B, Oelmuller R, Cai D (2010) Expression of BvGLP-1 encoding a germin-like protein from sugar beet in *Arabidopsis thaliana* leads to resistance against phytopathogenic fungi. Mol Plant Microbe Interact 23:446–457

Kogel KH, Franken P, Huckelhoven R (2006) Endophyte or parasite-what decides? Curr Opin Plant Biol 9:358–63

Koornneef A, Pieterse CM (2008) Cross talk in defense signaling. Plant Physiol 146:839–844

Kosuta S, Chabaud M, Lougnon G, Gough C, Denarie J, Barker DG, Becard G (2003) A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific *MtENOD11* expression in roots of *Medicago truncatula*. Plant Physiol 131:952–962

Kottke I, Beiter A, Weiss M, Haug I, Oberwinkler F, Nebel M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while Aneura pinguis (Metzgeriales) is associated with a *Tulasnella* species. Mycol Res 107:957–968

Kuhn H, Kuster H, Requena N (2009) Membrane steroid-binding protein 1 induced by a diffusible fungal signal is critical for mycorrhization in *Medicago truncatula*. New Phytol 185:716–133

Kumar M, Yadav V, Tuteja N, Johri AK (2009) Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. Microbiology 155:780–790

Kumari R, Kishan H, Bhoon YK, Varma A (2003) Colonization of cruciferous plants by *Piriformospora indica*. Curr Sci 85:1672–1674

Kumari R, Pham GH, Prasad R, Sachdev M, Srivastava A, Yadav V, Verma PK, Sharma S, Malla R, Singh A, Maurya AK, Prakash S, Pareek A, Rexer KH, Kost G, Garg AP, Oelmueller R, Sharma MC, Varma A (2004) *Piriformospora indica*: fungus of the millennium. In: Podila G, Varma A (eds) Basic research and applications: mycorrhizae. IK International-India/Kluwer academic, New York/Holland, pp 259–295

Kuo CG, Huang RS (1982) Effect of vesicular-arbuscular mycorrhizae on the growth and yield of rice-stubble cultured soybeans. Plant Soil 64:325–330

Ligrone R, Carafa A, Lumini E, Bianciotto V, Bonfante P, Duckett JG (2007) Glomeromycotean associations in liverworts: a molecular, cellular, and taxonomic analysis. Am J Bot 94:1756–1777

Markmann K, Giczey G, Parniske M (2008) Functional adaptation of a plant receptor- kinase paved the way for the evolution of intracellular root symbioses with bacteria. PLoS Biol 6 (e68):0497–0506. doi:10.1371/journal.pbio.0060068

Meena KK, Mesapogu S, Kumar M, Yandigeri MS, Singh G, Saxena AK (2010) Co-inoculation of the endophytic fungus *Piriformospora indica* with the phosphate-solubilising bacterium *Pseudomonas striata* affects population dynamics and plant growth in chickpea. Biol Fertil Soils 46:169–172

Messinese E, Mun J, Yeun L, Jayaraman D, Rougé P, Barre A, Lougnon G, Schornack S, Bono J, Cook D, Ané J (2007) A novel nuclear protein interacts with the symbiotic DMI3 calcium and calmodulin dependent protein kinase of *Medicago truncatula*. Mol Plant Microbe Interact 20:912–921

Navarro L, Bari R, Achard P, Lison P, Nemri A, Harberd NP, Jones JDG (2008) DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. Curr Biol 18:650–655

Navazio L, Moscatiello R, Genre A, Novero M, Baldan B, Bonfante P, Mariani P (2007) The arbuscular mycorrhizal fungus *Glomus intraradices* induces intracellular calcium changes in soybean cells. Caryologia 60:137–140

Oelmuller R, Sherameti I, Tripathi S, Varma A (2009) Piriformospora indica, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Parniske M (2004) Molecular genetics of the arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol 7:414–421

Paszkowski U (2006) Mutualism and parasitism: the yin and yang of plant symbioses. Curr Opin Plant Biol 9:364–370

Peskan-Berghofer T, Shahollari B, Giang PH, Hehl S, Markert C, Blanke V, Varma AK, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plant 122:465–477

Pham GH, Kumari R, Singh AN, Malla R, Prasad R, Sachdev M, Kaldorf M, Buscot F, Oelmuller R, Hampp R, Saxena AK, Rexer KH, Kost G, Varma A (2004) Axenic cultures of *Piriformospora indica*. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Germany, pp 593–613

Prajapati K, Yami KD, Singh A (2008) Plant growth promotional effect of *Azotobacter chroococcum*, *Piriformospora indica* and vermicompost on rice plant. Nepal J Sci Technol 9:85–90

Prasad R, Pham HG, Kumari R, Singh A, Yadav V, Sachdev M, Garg AP, Peskan T, Hehl S, Sherameti I, Oelmuller R, Varma A (2005) Sebacinaceae: culturable mycorrhiza-like endosymbiotic fungi and their interaction with non-transformed and transformed roots. In: Declerck S, Strullu DG, Fortin A (eds) *In vitro* culture of mycorrhizas, vol 4. Springer, Berlin, Heidelberg, pp 291–312

Rai MK, Singh A, Arya D, Varma A (2001) Positive growth responses of *Withania somnifera* and *Spilanthes calva* cultivated with *Piriformospora indica* in field. Mycorrhiza 11:123–128

Raj MK, Varma A, Pandey AK (2004) Antifungal potential of *Spilanthes calva* after inoculation of *Piriformospora indica*. Mycoses 47:479–81

Ray JG, Valsalakumar N (2010) Arbuscular mycorrhizal fungi and *Piriformospora indica* individually and in combination with *Rhizobium* on green gram. J Plant Nutr 33:285–298

Reinhardt D (2007) Programming good relations- development of the arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol 10:98–105

Rodriguez R, Redman R (2008) More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance *via* fungal symbiosis. J Exp Bot 59:1109–1114

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Saito K, Yoshikawa M, Yano K, Miwa H, Uchida H, Azamizu E, Sato S, Tabata S, Imaizumi-Anraku H, Umehara Y, Kouchi H, Murooka Y, Szczyglowski K, Downie A, Parniske M, Hayashi M, Kawaguchi M (2007) NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbiosis and seed production in *Lotus japonicus*. Plant Cell 19:610–624

Schafer P, Khatabi B, Kogel KH (2007) Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism. FEMS Microbiol Lett 275:1–7

Schafer P, Pfiffi S, Voll LM, Zajic D, Chandler PM, Waller F, Scholz U, Pons-Kuhnemann J, Sonnewald S, Sonnewald U, Kogel KH (2009) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. Plant J 59:461–474

Schafer P, Pfiffi S, Voll LM, Zajic D, Chandler PM, Waller F, Scholz U, Kühnemann JP, Sonnewald S, Sonnewald U, Kogel KH (2009) Phytohormones in plant root-*Piriformospora indica* mutualism. Plant Signal Behav 4:669–671

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajim M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J 31:279–292

Selosse MA, Bauer R, Moyerson B (2002) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol 155:183–195

Setaro S, Weib M, Oberwinkler F, Kottke I (2006) Sebacinales form ectendomycorrhizas with *Cavendishia nobilis*, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. New Phytol 169:355–365

Shahollari B, Vadasser J, Varma A, Oelmuller R (2007) A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 50:1–13

Shahollari B, Varma A, Oelmuller R (2005) Expression of a receptor kinase in Arabidopsis roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol 162:945–58

Sharma M, Schmid M, Rothballer M, Hause G, Zuccaro A, Imani J, Kampfer P, Domann E, Schafer P, Hartmann A, Kogel KH (2008) Detection and identification of bacteria intimately associated with fungi of the order Sebacinales. Cell Microbiol 10:2235–2246

Shaw SL, Long SR (2003) Nod factor elicits two separable calcium responses in *Medicago truncatula* root hair cells. Plant Physiol 131:976–984

Shende S, Bhagwat K, Wadegaonkar P, Rai M, Varma A (2006) *Piriformospora indica* as a new and emerging mycofertilizer and biotizer: potentials and prospects in sustainable agriculture. In: Rai MK (ed) Handbook of microbial biofertilizers. Haworth Press, Inc, NY, pp 447–496

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmuller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. J Biol Chem 280:26241–26247

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmüller R (2008) PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 54:428–439

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6:410–417

Shinozaki K, Yamaguchi-Shiozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227

Singh A, Varma A (2000) Orchidaceous mycorrhizal fungi. In: Mukerji KG, Chamola BP, Singh J (eds) Mycorrhizal biology. Kluwer/Plenum, New York, pp 265–288

Singh A, Singh A, Kumari M, Kumar S, Rai MK, Sharma AP, Varma A (2003) AMF-like-fungi: *Piriformospora indica*—a boon for plant industry. Biotechnology in sustainable biodiversity and food security. Proceedings of an International Conference, Kathmandu, Nepal, November 2000. In: Prasad BN (ed) Biotechnology in sustainable biodiversity and food security. Science Publishers Inc., Enfield, New Hampshire, pp 101–124

Singh G, Singh N, Marwaha TS (2009) Crop genotype and a novel symbiotic fungus influences the root endophytic colonization potential of plant growth promoting rhizobacteria. Physiol Mol Biol Plants 15:87–92

Stein E, Molitor A, Kogel KH, Waller F (2008) Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. Plant Cell Physiol 49:1747–1751

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stouggard J, Szczyglowski K, Parniske M (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417:959–962

Tarafdar JC, Rao AV (1996) Contribution of Aspergillus strains to acquisition of phosphorus by wheat (*Triticum aestivum* L.) and chick pea (*Cicer arietinum* L.) grown in a loamy soil. Appl Soil Ecol 3:190–114

Tirichine L, Imaizumi-Anraku H, Yoshida S, Murakami Y, Madsen LH, Miwa H, Nakagawa T, Sandal N, Albrektsen AS, Kawaguchi M, Downie JA, Sato S, Tabata S, Kouchi H, Parniske M, Kawasaki S, Stougaard J (2006) Deregulation of a Ca^{2+}/calmodulin dependent kinase leads to spontaneous nodule formation. Nature 441:1153–1156

Tisserant B, Gianinazzi PV, Gian IS, Gollotte A (1993) In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol Res 97:245–250

Urban A, Weiss M, Bauer R (2003) Ectomycorrhizas involving sebacinoid mycobionts. Mycol Res 107:3–14

Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B, Novak O, Stmad M, Ludwig-Muller J, Oelmuller R (2008) The role of auxins and cytokinins in the mutualistic interaction between *Arabidopsis* and *Piriformospora indica*. Mol Plant Microbe Interact 21:1371–1383

Vadassery J, Ranf S, Drzewiecki C, Mithofer A, Mazars C, Scheel D, Lee J, Oelmuller R (2009a) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J 59:193–206

Vadassery J, Tripathi S, Prasad R, Varma A, Oelmuller R (2009b) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and *Arabidopsis*. J Plant Physiol 166:1263–1274

Varma A, Franken P (1997) Patent number 97121440.8-2105. European Patent Office, Muenchen, Germany

Varma A, Singh A, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) The Mycota IX. Springer, Heidelberg, Germany, pp 123–150

Varma A (2008) Mycorrhiza-genetics and molecular biology, ecofunction, biotechnology, ecophysiology, structure and systematics, 3rd edn. Springer, Berlin, Heidelberg, p 797

Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Butehorn B, Fraken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root colonizing fungus. Mycologia 9:896–903

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Huckelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Waller F, Mukherjee K, Deshmukh SD, Achatz B, Sharma M, Schafer P, Kogel KH (2008) Systemic and local modulation of plant responses by *Piriformospora indica* and related Sebacinales species. J Plant Physiol 165:60–70

Wang D, Pajerowska-Mukhtar K, Culler AH, Dong X (2007) Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Curr Biol 17:1784–1790

Wardle DA, Bardgett RD, Klironomos JN, Setala H, van der Putten WH, Wall DH (2004) Ecological linkages between aboveground and belowground biota. Science 304:1629–1633

Weiss M, Selosse M-A, Rexer K-H, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in the phosphate transport to the host plant. J Biol Chem 285(34):26532–26544. doi:10.1074/jbc.M110.111021, PMID: 20479005; This is cited from URL http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2924090/

Yadav V, Verma PK, Varma A (2004) Phosphorus metabolism and regulation by symbiotic fungi. In: Podila GK, Varma A (eds) Basic research and applications of mycorrhizae. IK International Pvt Ltd, New Delhi, pp 111–139

Yano K, Yoshida S, Müller J, Singh S, Banba M, Vickers K, Markmann K, White C, Schuller B, Sato S, Asamizu E, Tabata S, Murooka Y, Perry J, Wang TL, Kawaguchi M, Imaizumi-Anraku H, Hayashi M, Parniske M (2008) CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc Natl Acad Sci USA 105:20540–20545

Yuan ZL, Zhang CL, Lin FC (2010) Role of diverse non-systemic fungal endophytes in plant performance and response to stress: progress and approaches. J Plant Growth Regul 29:116–126

Part II
Sebacinales Interaction with Different Plant Species

Chapter 5
Distinct but Closely Related Sebacinales form Mycorrhizae with Coexisting Ericaceae and Orchidaceae in a Neotropical Mountain Area

Sabrina Setaro, Juan Pablo Suárez, Paulo Herrera, Dario Cruz, and Ingrid Kottke

5.1 Introduction

Since the first description of *Sebacina vermifera* as intrahymenial fungus by Oberwinkler (1964) and the first identification of an orchid-mycorrhiza isolate as *S. vermifera* Oberwinkler by Warcup and Talbot (1967), Sebacinales were repeatedly retrieved as important mycorrhiza-forming fungi with members of diverse plant families (Warcup 1988; Glen et al. 2002; Selosse et al. 2002; Urban et al. 2003; Kottke et al. 2003; Allen et al. 2003; Setaro et al. 2006a, b; Suárez et al. 2008). Structurally, mycorrhizae involving Sebacinales are quite distinct, with specific structures largely depending on the individual plant family. Sebacinales form ectomycorrhizae with members of Fagales and Myrtales (Glen et al. 2002; Selosse et al. 2002; Urban et al. 2003), ericoid and cavendishioid mycorrhizae with Ericaceae (Allen et al. 2003; Setaro et al. 2006a, b), orchid mycorrhizae with Orchidaceae (Suárez et al. 2008), and jungermannioid mycorrhizae with liverworts (Kottke et al. 2003; Newsham and Bridge 2010). A molecular phylogeny revealed ectomycorrhiza-forming Sebacinales in a distinct clade together with *Sebacina incrustans*, separated from the other mycorrhiza-forming Sebacinales that cluster with *S. vermifera* (Weiß et al. 2004).

In a previous small-scale investigation comparing Sebacinales mycobionts of Ericaceae and Orchidaceae from the same sampling area in the tropical mountain rain forest of Southern Ecuador, we found molecular indications for a distinction

S. Setaro (✉)
Department of Biology, Wake Forest University, 1834 Wake Forest Road, Winston-Salem, NC 27106, USA
e-mail: setarosd@wfu.edu

J.P. Suárez • P. Herrera • D. Cruz • I. Kottke
Centro de Biología Celular y Molecular, Universidad Técnica Particular de Loja, San Cayetano Alto, s/n C.P. 11 01 608, Loja, Ecuador
e-mail: jpsuarez@utpl.edu.ec; piherrera@utpl.edu.ec; djcruz@utpl.edu.ec; ingrid.kottke@unibund.uni-tuebingen.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_5,

between these associations (Kottke et al. 2008). These results were based on sampling of roots from epiphytic orchids on stems of standing trees, but of hemiepiphytic Ericaceae growing in the humus fraction of soil and mineral soil (Kottke et al. 2008). Therefore, it was unclear whether the distinctiveness of Sebacinales clades was due to habitat differences or whether it was determined by the association with a distinct plant family. For the present study, a larger sample size was collected at the same locality. Sampling included epiphytic and terrestrial orchids on humus and mineral soil as well as terrestrial and hemiepiphytic ericads from pristine forest and disturbed habitats. In contrast to the 2008 study by Kottke et al., we sequenced not only the 28S of the rDNA but also included the more variable ITS (Nilsson et al. 2008) in order to get higher resolution on subgeneric levels. Phylogenetic inference, cluster analyses, and a phylogenetic-Bray-Curtis (PBC) transformation approach were used to address the following questions: (1) Is it possible to delimitate distinct clades of Sebacinales in the host plants under investigation? (2) Do Sebacinales show preference for either one of the two plant families independent of habitat and substrate? (3) In addition, since Sebacinales have been isolated from diverse geographic areas and display a worldwide distribution, the phylogenetic position of Sebacinales from the Neotropics was also of interest.

5.2 Materials and Methods

5.2.1 Study Sites and Sampling

Mycorrhizae from coexisting Ericaceae and Orchidaceae were collected in the Reserva Biológica San Francisco (3°58′S, 79º4′W), part of the Biosphere Reserve Podocarpus—El Condor, Southern Ecuador. The protected area contains pristine rain and cloud forest and sites that are disturbed due to anthropogenic impacts. Five different sites were selected for sampling of mycorrhizae of Ericaceae and Orchidaceae. Two sites were located inside undisturbed forest at 2,000 m (site 1) and 2,170 m (site 4), one in a 40-year-old regenerating part of the pristine forest at 2,170 m (site 3) and two on a 55-year-old anthropogenic landslide surrounded by mountain rain forest at 1,900 m (site 2 and 5). Detailed information on the area is given in Beck et al. (2008).

Roots from Ericaceae were collected in March 2007 and from Orchidaceae in February to May 2008. In total, 103 Orchidaceae, 17 of which were shown to be associated with Sebacinales and 19 Ericaceae were sampled. Mycorrhizae of Ericaceae were sampled from flowering individuals selected randomly by excavating the entire length of a root, beginning at the stem base and leading out to the finest rootlets. The rootlets of each host individual were pooled, cleaned under tap water, and freed from organic debris within 2 days after sampling. Fresh mycorrhizae were selected using a dissection microscope. A root cluster consisting

of two to eight rootlets was dried and stored in 1.5-ml microcentrifuge tubes with silica gel for DNA extraction.

Roots from orchids were collected from 2 or 3 orchid individuals in 56 permanently established plots of 1 m^2, 8 terrestrial or epiphytic plots per respective site. Only roots in contact with the tree bark were collected from epiphytic orchids. The roots of terrestrial orchids in the forest were sampled from the pure humus layer while on the landslide mycorrhizal roots were sampled from the mineral soil. Preliminary observations had shown the presence of orchid mycorrhizae in these microhabitats (Kottke et al. 2010). Roots were screened for fungal colonization of the cortical tissue the day of sampling by microscopic observation of freehand sections stained using methyl blue (0.05 % in lactic acid, Merck C.I. 42780). Well-colonized parts of roots were selected for DNA extraction and ultrastructural examination. DNA was conserved at −20 °C.

Ericaceae were identified to species level with the Flora of Ecuador (Luteyn 1996). In case where specimens could not be certainly identified, we consulted the experts of Neotropical Ericaceae, Jim Luteyn and Paola Pedraza. Orchid specimens were not sent to experts because of conservational issues and lack of flowering material available, so identification of Orchidaceae is on the level of morphospecies only. DNA barcoding of Orchidaceae allowed further identification on genus level.

5.2.2 *Light and Transmission Electron Microscopy*

Mycorrhizae of Orchidaceae were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) on the day of sampling. Samples were postfixed in 1 % osmium tetroxide and embedded in Spurr's plastic according to Bauer et al. (2006), but with prolonged infiltration steps of up to 12 h. Semi-thin sections were stained by crystal violet and observed for fungal colonization of the cortical tissue by use of light microscopy. Healthy looking colonized parts were selected for ultra-thin sectioning. Serial ultra-thin sections were prepared and examined using a ZEISS transmission electron microscope at 80 kV. Vouchers of the plastic embedded samples were deposited in the Herbarium Tubingense, Eberhard-Karls-University Tübingen.

Mycorrhizae of Ericaceae had already been collected and processed in the case of the Setaro et al. study 2006a, b in the study area. The preparation was analogous to those of Orchidaceae; for details see Setaro et al. (2006a, b).

5.2.3 *Processing of Fungal DNA Sequences*

For roots of Ericaceae, genomic DNA was isolated from dried mycorrhiza samples (~5 mm root length per host individual) using the DNAeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions but without the use of RNase A. To amplify part of the 18S, the ITS and portions of the 28S genes, we used the

universal forward primers NS23 (Gargas and Taylor 1992) and ITS1F (Gardes and Bruns 1993) as well as the Sebacinales-specific reverse primer MWS1 (kindly provided by Michael Weiß), in polymerase chain reactions (PCRs). In some cases, a nested PCR was required to obtain products suitable for cloning. The first amplification was conducted with the primers NS23/ITS1F and MWS1. This product was used as a template with final concentrations of 1:50, 1:500, or 1:5,000 for a second PCR with the primers NS23/ITS1F and the Sebacinales-specific primer MWS2 (kindly provided by Michael Weiß). We used the Phusion Master Mix with HF buffer (Finnzymes) as PCR reagents with a final volume of 20 µl. The template for the PCR reaction was the genomic DNA extract with a final concentration of 1:10. The PCR protocol was conducted according to the manufacturer's instructions, but with 30 cycles and an annealing temperature of either 60 °C, 63 °C, or 66.5 °C.

All positive PCR products were cloned with the Zero Blunt Topo PCR Cloning Kit (Invitrogen, Life Technologies) using a PCR product volume of 0.5 µl for the cloning reaction. Clones were checked for positive inserts by picking up to eight bacterial clones with a toothpick and placing them directly into a PCR reaction mixture. The reaction volume was 50 µl, with concentrations of 1.5 mM $MgCl_2$, 200 µM of each dNTP (Life Technologies, Invitrogen), 1 U of Taq polymerase (Life Technologies, Invitrogen), an amplification buffer (Life Technologies, Invitrogen), bovine serum albumin (0.004 %), and 0.5 µM of each primer (Biomers). We used M13F and M13R as the forward and reverse primer for this PCR reaction. One to ten inserts were amplified by rolling circle amplification with the TempliPhi Amplification Kit (GE Healthcare) and sequenced without prior purification on an ABI 3730 sequencing machine (GATC Biotech, Germany). Aside from M13F and M13R, we used several insert primers (NS23; ITS3Seb; kindly provided by M. Berbee; LR3: (Hopple and Vilgalys 1994); LR0R: (Hopple and Vilgalys 1994); SSS1: 5′-GTGAACCTGCGGAAGGATCATTA-3′; MWS1; ITS1F; NL4 reverse complement: (O'Donnell 1993); SSS2: 5′-TAGATG TTCTGGGCCGCACGC-3′; SSS3: 5′-GGAATAGGGAGAATCTGC-3′) to obtain the full sequence length in good quality.

For roots of Orchidaceae, DNA isolation, polymerase chain reaction, cloning and sequencing, a 1–2 cm long piece of root was cut for one DNA extraction per plant individual. The pieces were rinsed in sterile water and freed from the velamen. Genomic DNA was recovered using the DNAeasy Plant Mini Kit (Qiagen) according to the manufacturers' instructions. The whole ITS1-5.8S- ITS2 region and part of the 28S rDNA were amplified with the universal primers ITS1 (White et al. 1990) and TW14 (Cullings 1994) using the Phusion High-Fidelity PCR Mastermix (Finnzymes). PCR products were cloned with the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to the manufacturer's protocol (Stockinger et al. 2009). Twelve colonies per individual were selected for PCR amplification using modified M13F and M13R primers (Krüger et al. 2009). Success of PCR was tested in 1 % agarose gel. Eight colonies per orchid individual showing correct fragment size were grown in liquid LB Broth, MILLER (Difco) and plasmids were isolated with the S.N.A.P. miniprep kit (Invitrogen) according to manufacturers' instructions.

Plasmids were sequenced on an ABI 3730xl sequencing machine (Macrogen, Korea) using universal primers M13F and M13R.

All sequences were edited and congruent strands were combined using SEQUENCHER 4.6 (Gene Codes, Ann Arbor, MI, USA). BLAST (Altschul et al. 1990) against the NCBI nucleotide database (GenBank; http://www.ncbi.nlm.nih.gov/) was used to find published sequences with high similarity. Sequences that matched Sebacinales were compiled in a data set and checked for potential chimeric sequences. To do this, sequences were roughly aligned with POA (Grasso and Lee 2004) and analyzed with BELLEROPHON (Huber et al. 2004) and a window width of 200 base pairs. In addition, the alignment was partitioned with a window width of 400 base pairs and each partition was blasted separately. All sequences have been submitted to Genbank, the accession numbers are given in Table 5.1 and Fig. 5.2.

5.2.4 DNA Bar Coding of Orchidaceae

Genomic DNA of fresh or dried leaves was isolated using the DNAeasy Plant Mini Kit (Qiagen) according to the manufacturers' instructions. To amplify the chloroplast gene *mat*K, we used the primer combinations 19 F (Whitten et al. 2000)—881R (Pridgeon et al. 2001) and 731 F (Pridgeon et al. 2001)—*trnK*-2R (Johnson and Soltis 1995) in PCRs. We used the Phusion High-Fidelity Polymerase (Finnzyme) as PCR reagent in a final volume of 25 µl according to the manufacturers' instructions but with an annealing step for 20 s and annealing temperatures of 52 °C for the primers 19 F-881R and 58 °C for the primers 731 F-trnK2R. We added Bovine Serum Albumin (BSA-SIGMA) with a final concentration of 0.8 µg μl^{-1} to the reaction mix (Iotti and Zambonelli 2006). Every PCR was run with a negative control.

All positive PCR products were sequenced on an ABI 3730xl sequencing machine (Macrogen, Korea) using the same primers as for DNA amplification. The sequences were edited and congruent strands were combined using SEQUENCHER 4.6 (Gene Codes, Ann Arbor, MI, USA). BLAST (Altschul et al. 1990) against the NCBI nucleotide database (GenBank; http://www.ncbi.nlm.nih.gov/) was used to find published sequences with high similarity to identify the Orchidaceae to genus level.

5.2.5 Phylogenetic Analyses of Sebacinales

Phylogenetic analyses were performed with additional sequences of Sebacinales from NCBI in order to ascertain the phylogenetic position of Sebacinales from the sampling area in relation to Sebacinales from other hosts and locations all over the world. NCBI was searched for Sebacinales including ITS data and a minimum

Table 5.1 Sebacinales sequences with information about hosts and sampling localities

Accession number of Sebacinales	Host ID	Host species	Host genus	Host family	Location ID	Host life-form
FN663655	P04J05	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625938	P04J05	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625939	P04J05	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625940	P04J04	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625946	P04J04	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625947	P04J04	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625948	P04J07	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625949	P04J07	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625957	P04J05	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625958	P04J06	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625959	P04J06	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625960	P04J06	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	hemiepiphytic
EU625961	P04J05	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625963	P04J07	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625964	P04J07	*Cavendishia bracteata*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625969	P04A41	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	4	Hemiepiphytic
EU625970	P04A41	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	4	Hemiepiphytic
EU625971	P04A41	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	4	Hemiepiphytic
EU625972	P04A41	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	4	Hemiepiphytic
EU625987	P609	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	4	Hemiepiphytic
EU625988	P609	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	4	Hemiepiphytic
EU625941	P04J01	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625944	P04J01	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625945	P04J01	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625989	P563	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	5	Hemiepiphytic
EU625990	P573	*Cavendishia nobilis*	*Cavendishia*	Ericaceae	5	Hemiepiphytic

EU625925	P07Ma72	*Disterigma microphyllum*	*Disterigma*	Ericaceae	3	Hemiepiphytic
EU625929	P07Ma75	*Disterigma microphyllum*	*Disterigma*	Ericaceae	3	Hemiepiphytic
EU625930	P07Ma75	*Disterigma microphyllum*	*Disterigma*	Ericaceae	3	Hemiepiphytic
EU625935	P07Ma69	*Disterigma microphyllum*	*Disterigma*	Ericaceae	3	Hemiepiphytic
EU625936	P07Ma69	*Disterigma microphyllum*	*Disterigma*	Ericaceae	3	Hemiepiphytic
FN663736	P07Ma72	*Disterigma microphyllum*	*Disterigma*	Ericaceae	3	Hemiepiphytic
FN663737	P07Ma72	*Disterigma microphyllum*	*Disterigma*	Ericaceae	3	Hemiepiphytic
EU626003	P07Ma72	*Disterigma microphyllum*	*Disterigma*	Ericaceae	3	Hemiepiphytic
FN663656	P04A07	*Disterigma microphyllum*	*Disterigma*	Ericaceae	4	Hemiepiphytic
EU625984	P04J12	*Disterigma microphyllum*	*Disterigma*	Ericaceae	4	Hemiepiphytic
EU625985	P04J12	*Disterigma microphyllum*	*Disterigma*	Ericaceae	4	Hemiepiphytic
EU625973	P07A51	*Gaultheria erecta*	*Gaultheria*	Ericaceae	5	Terrestrial
EU625974	P07A51	*Gaultheria erecta*	*Gaultheria*	Ericaceae	5	Terrestrial
EU625933	P07A35	*Gaultheria reticulata*	*Gaultheria*	Ericaceae	5	Terrestrial
EU625934	P07A35	*Gaultheria reticulata*	*Gaultheria*	Ericaceae	5	Terrestrial
EU625962	P07A35	*Gaultheria reticulata*	*Gaultheria*	Ericaceae	5	Terrestrial
HM451828, HM451835	2TF2	Orchid sp. 1	cf. *Maxillaria*	Orchidaceae	2	Terrestrial
HM451800	2TF2	Orchid sp. 1	cf. *Maxillaria*	Orchidaceae	2	Terrestrial
HM451801	2TF2	Orchid sp. 1	cf. *Maxillaria*	Orchidaceae	2	Terrestrial
HM451827, HM451834	2TE1	Orchid sp. 10	*Prosthechea*	Orchidaceae	2	Terrestrial
HM451798	2TE1	Orchid sp. 10	*Prosthechea*	Orchidaceae	2	Terrestrial
HM451799	2TE1	Orchid sp. 10	*Prosthechea*	Orchidaceae	2	Terrestrial
HM451802	2TH1	Orchid sp. 11	*Prosthechea*	Orchidaceae	2	Terrestrial
HM451829, HM451836	2TH1	Orchid sp. 11	*Prosthechea*	Orchidaceae	2	Terrestrial
HM451803	2TH1	Orchid sp. 11	*Prosthechea*	Orchidaceae	2	Terrestrial
HM451804	2TH1	Orchid sp. 11	*Prosthechea*	Orchidaceae	2	Terrestrial
HM451805	2TH1	Orchid sp. 11	*Prosthechea*	Orchidaceae	2	Terrestrial
HM451806	2TH1	Orchid sp. 11	*Prosthechea*	Orchidaceae	2	Terrestrial

(continued)

Table 5.1 (continued)

Accession number of Sebacinales	Host ID	Host species	Host genus	Host family	Location ID	Host life-form
HM451813	3EH1	Orchid sp. 12	*Scaphyglottis*	Orchidaceae	3	Epiphytic
HM451830, HM451837	3EH1	Orchid sp. 12	*Scaphyglottis*	Orchidaceae	3	Epiphytic
HM451831, HM451838	3EH1	Orchid sp. 12	*Scaphyglottis*	Orchidaceae	3	Epiphytic
HM451792	1 EB1	Orchid sp. 13	*Stelis*	Orchidaceae	1	Epiphytic
HM451793	1 EB1	Orchid sp. 13	*Stelis*	Orchidaceae	1	Epiphytic
HM451794	1 EB1	Orchid sp. 13	*Stelis*	Orchidaceae	1	Epiphytic
HM451809	3EC2	Orchid sp. 14	*Stelis*	Orchidaceae	3	Epiphytic
HM451826, HM451833	1ED1	Orchid sp. 15	*Stelis*	Orchidaceae	1	Epiphytic
HM451824	4EG2	Orchid sp. 15	*Stelis*	Orchidaceae	4	Epiphytic
HM451832, HM451839	4EE2	Orchid sp. 16	*Stelis*	Orchidaceae	4	Epiphytic
HM451820	4EE2	Orchid sp. 16	*Stelis*	Orchidaceae	4	Epiphytic
HM451821	4EE2	Orchid sp. 16	*Stelis*	Orchidaceae	4	Epiphytic
HM451822	4EE2	Orchid sp. 16	*Stelis*	Orchidaceae	4	Epiphytic
HM451823	4EE2	Orchid sp. 16	*Stelis*	Orchidaceae	4	Epiphytic
HM451807	3EA2	Orchid sp. 2	Not known	Orchidaceae	3	Epiphytic
HM451808	3EA2	Orchid sp. 2	Not known	Orchidaceae	3	Epiphytic
HM451811	3EG2	Orchid sp. 3	*Elleanthus*	Orchidaceae	3	Epiphytic
HM451812	3EG2	Orchid sp. 3	*Elleanthus*	Orchidaceae	3	Epiphytic
HM451817	3TH2	Orchid sp. 4	*Maxillaria*	Orchidaceae	3	Humus epiphytes
HM451825	4TF2	Orchid sp. 5	*Maxillaria*	Orchidaceae	4	Humus epiphytes
HM451795	1TG2	Orchid sp. 6	Not known	Orchidaceae	1	Terrestrial
HM451796	1TG2	Orchid sp. 6	Not known	Orchidaceae	1	Terrestrial
HM451797	1TG2	Orchid sp. 6	Not known	Orchidaceae	1	Terrestrial
HM451810	3EG1	Orchid sp. 7	*Epidendrum*	Orchidaceae	3	Epiphytic
HM451814	3 TB1	Orchid sp. 8	Not known	Orchidaceae	3	Humus epiphytes
HM451815	3 TB1	Orchid sp. 8	Not known	Orchidaceae	3	Humus epiphytes

HM451816	3 TB1	Orchid sp. 8	Not known	Orchidaceae	3	Humus epiphytes
HM451818	4EA1	Orchid sp. 9	*Pleurothallis*	Orchidaceae	4	Epiphytic
HM451819	4EA1	Orchid sp. 9	*Pleurothallis*	Orchidaceae	4	Epiphytic
FN663658	P07A61	*Psammisia guianensis*	*Psammisia*	Ericaceae	5	Hemiepiphytic
FN663689	P07A61	*Psammisia guianensis*	*Psammisia*	Ericaceae	5	Hemiepiphytic
FN663728	P07A61	*Psammisia guianensis*	*Psammisia*	Ericaceae	5	Hemiepiphytic
FN663732	P07A61	*Psammisia guianensis*	*Psammisia*	Ericaceae	5	Hemiepiphytic
FN663733	P07A61	*Psammisia guianensis*	*Psammisia*	Ericaceae	5	Hemiepiphytic
EU625978	P04A35	*Sphyrospermum cordifolium*	*Sphyrospermum*	Ericaceae	5	Hemiepiphytic
FN663670	P04A35	*Sphyrospermum cordifolium*	*Sphyrospermum*	Ericaceae	5	Hemiepiphytic
FN663678	P04A35	*Sphyrospermum cordifolium*	*Sphyrospermum*	Ericaceae	5	Hemiepiphytic

length of 400 base pairs in order to exclude excessively short sequences. The NCBI sequences that met these criteria were added to our data set. Sequences were roughly aligned with POA and alignment columns upstream the 5′ direction of the ITS1F primer region were excluded from the data set. Furthermore, all sequences that were not aligned across the 5.8S region were excluded as well as were all sequences with more than 65 % of leading and trailing gaps. Because of these preconditions, not all Sebacinales from Genbank could be included in the data set.

To prune the data set, single linkage clustering with a threshold of 0.5 % was performed with OPTSIL (Göker et al. 2009). All Sebacinales with identical names or isolated from the same host plant, and affiliated to the same cluster, were excluded with the exception of one representative. The remaining sequences were joined with our data set of Sebacinales from Ecuadorian Ericaceae and Orchidaceae.

All gaps and missing data were removed and the sequences were aligned with MAFFT v6.602b (Katoh et al. 2002) and the option linsi as well as with POA (Grasso and Lee 2004). The most reliable of both alignments was chosen with TRIMAL and the option comparesets (Capella-Gutiérrez et al. 2009). The best alignment was used to perform a Maximum Likelihood analysis with 1,000 rapid bootstrap replicates (Stamatakis et al. 2008) in RAXML 7.0.4 sequential version using the GTRmix model (Stamatakis 2006).

After the analysis, the ML tree was rooted with the midpoint method in FIGTREE (Rambaut 2009). To enhance visualization of the tree, a large clade (in total 100 sequences) containing sequences of Sebacinales group A (Weiß et al. 2004) and related taxa was collapsed. None of the Sebacinales associated with Ericaceae and Orchidaceae from this study were in the collapsed clade.

5.2.6 *Cluster Analysis of Ericaceae and Orchidaceae*

We used the UNIFRAC metric (Lozupone and Knight 2005) in combination with hierarchical clustering to determine whether Ericaceae and Orchidaceae are significantly associated with different guilds of Sebacinales. The UNIFRAC metric measures the difference between two environments (in our case host species) in terms of the branch length that is unique to one host or the other (Lozupone and Knight 2005).

All sequences of Sebacinales were aligned with MAFFT v6.602b (Katoh et al. 2002) and the option linsi as well as with POA (Grasso and Lee 2004). The most reliable of both alignments was chosen with TRIMAL and the option comparesets (Capella-Gutiérrez et al. 2009). A Maximum Likelihood (ML) tree was reconstructed in RAXML 7.0.4 sequential version using the GTRmix model (Stamatakis 2006). The ML tree was rooted using the midpoint method in FIGTREE (Rambaut 2009) and analyzed with FASTUNIFRAC (Hamady et al. 2010) clustering and 1,000 jackknife replications.

5.2.7 *Distance Transformation and Neighbor-Net Analysis of Ericaceae and Orchidaceae*

Another approach to determine whether Ericaceae and Orchidaceae are associated with different guilds of Sebacinales is the PBC transformation approach (Göker and Grimm 2008) in combination with Neighbor-Net analysis. This novel approach transforms phylogenetic distance matrices of the "associates" to distance matrices of the "host" using Sørensen's distance coefficient. In contrast to the Sørensen distances, PBC does not require knowledge of the taxonomic affiliation of the sampled species in advance. Rather, it determines the most similar specimens between two hosts automatically when computing pair-wise distances from the reduced matrix (Göker and Grimm 2008).

The same alignment was used as for the UniFrac distance measure to compute a sequence distance matrix (GTR model of evolution) with Paup 4.0b10 (Swofford 2002). The distance matrix was used as "associate" distances and the respective host species as "hosts." The distance transformation was calculated with pbc (Göker and Grimm 2008) and the option—minimum one associate per host. A Neighbor-Net analysis of the PBC distance matrix was conducted with Splitstree v4 (Huson and Bryant 2006) to visualize the outcome of the distance transformation. To test the treelikeness of the PBC network, delta scores were computed in Splitstree v4. Delta scores are based on quartets of taxa and range between zero (maximum treelikeness) to one and are a reliable predictor of phylogenetic accuracy (Holland et al. 2002; Auch et al. 2006).

5.3 Results

5.3.1 *Structural Differences in Colonization of Roots of Neotropical Ericaceae and Orchidaceae*

Light and transmission electron microscopic observations revealed Sebacinales as the dominant mycobionts of Ericaceae in the area under investigation. Hyphae of Sebacinales were recognized by the typical septal pore structure (Fig. 5.1b). While species belonging to the Andean-clade of Ericaceae (Kron et al. 2002) displayed ectendomycorrhizas (cavendishioid mycorrhizae - Setaro et al. 2006a, b) with a hyphal sheath, Hartig net, and intracellular colonization (Fig. 5.1c), the non-Andean clade ericads showed only intracellular colonization (Fig. 5.1a) characterizing ericoid mycorrhizae (Smith and Read 2008). Sebacinales in orchid roots formed the typical pelotons in the multilayered cortical tissue that are encased by cell wall material after degeneration (Fig. 5.1d). Hyphae were also found in the dead cells of the velamen entering through passage cells of the exodermal layer into the cortical layer (Kottke and Suárez 2009). Sebacinales were only rarely observed

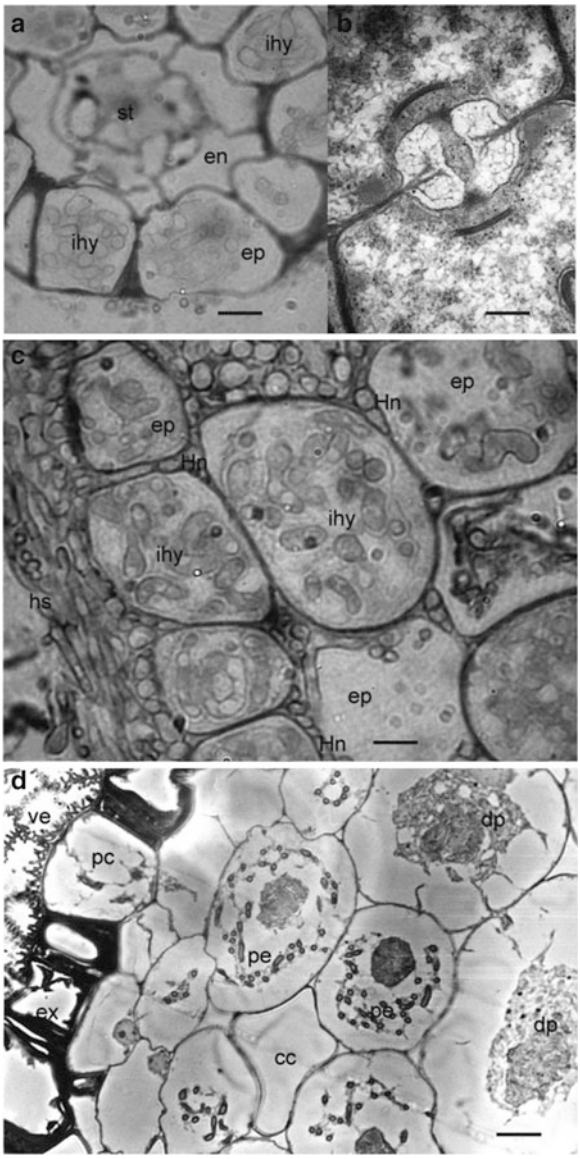

Fig. 5.1 Structural differences of the mycorrhizal types (**a**) Light microscopic micrograph of a square section through the ericoid mycorrhiza of a species of Sebacinales in *Gaultheria erecta*; hyphae colonizing the single layer of epidermal cells that surrounds the endodermis and stele; *scale bar* 6.3 μm. (**b**) Transmission electron micrograph of a hyphal septum of a species of Sebacinales in epidermal cells of Ericaceae displaying dolipore structure and continuous parenthesome; scale bar 0.15 μm. First published in Setaro et al. (2006a) (**c**) Light micrograph of tangential section through the epidermal cells of a mycorrhiza of *Semiramisia speciosa* and a species of Sebacinales (cavendishioid mycorrhiza); hyphal sheath, Hartig net, and enlarged intracellular hyphae visible; scale bar 4 μm. (**d**) Light micrograph of a square section through

in the tissue of the orchids under investigation. The most frequent fungi of orchids observed in this study were Tulasnellales, followed by Atractiellomycetes (Kottke et al. 2010).

5.3.2 *Phylogeny of Sebacinales Associated with Neotropical Ericaceae and Orchidaceae*

Detailed information of Sebacinales with respect to hosts, sampling localities, and life-forms is given in Table 5.1. Sebacinales associated with Neotropical Ericaceae and Orchidaceae from the present study fall in seven major clades (clades I, II, III, IV, V, VI, VII) with high bootstrap support (Figs. 5.2a, b). All of these clades contain Sebacinales either associated with Ericaceae or Orchidaceae, but not with both host families. Clade I and VI contain Sebacinales from tropical and temperate Ericaceae as well as undefined Sebacinales from soil (Fig. 5.2a, b). The remaining clades (II, III, IV, V, and VII) only contain Sebacinales associated with Neotropical Orchidaceae from the present study. Orchid clade III is closely related (96 % bootstrap support) to *S. vermifera* isolated from an Australian orchid, *Eriochilus cucullatus*. The Orchidaceae clades II, III, IV, and V are closely related (100 % bootstrap support) to clade I containing Sebacinales associated with Ericaceae (Fig. 5.2a). A sequence of Sebacinales associated with *Phleum pratense* (EU910901) falls within that group. Sebacinales in clade VI are related to *S. vermifera* (DQ520096, EU625995) and to Sebacinales associated with grasses, herbs, trees, and an arbutoid mycorrhizal Ericaceae (*Pyrola rotundifolia* EU668934) (Fig. 5.2b). Clade VII is rather isolated, as its close relationship to clade VI is not supported. However, all seven clades are more closely related to each other than to Sebacinales of group A sensu Weiß et al. (2004) as they cluster together with 100 % bootstrap support (Fig. 5.2a, b).

5.3.3 *UNIFRAC Cluster and PBC-Neighbor-Net Analyses*

The Unifrac clustering (UC) of host individuals based on phylogenetic distances of their associates (Sebacinales) shows two major clades and one isolated sequence (*Sphyrospermum cordifolium*—Fig. 5.3). Clade 2 has 83 % jackknife support, clade

Fig. 5.1 (continued) the orchid mycorrhiza of *Sobralia rosea* and a species of Sebacinales; velamen, exodermal layer with passage cell containing hyphae and multilayered cortical cells with hyphal pelotons and degenerating pelotons are displayed; *scale bar* 20 μm. Abbreviations: *cc* cortical cell, *ep* epidermis, *dp* degenerating pelotons, *en* endodermis, *ex* exodermis, *ihy* intracellular hyphae, *Hn* Hartig net, *hs* hyphal sheath, *pc* passage cell, *pe* pelotons, *hc* hyphal coils, *ve* velamen

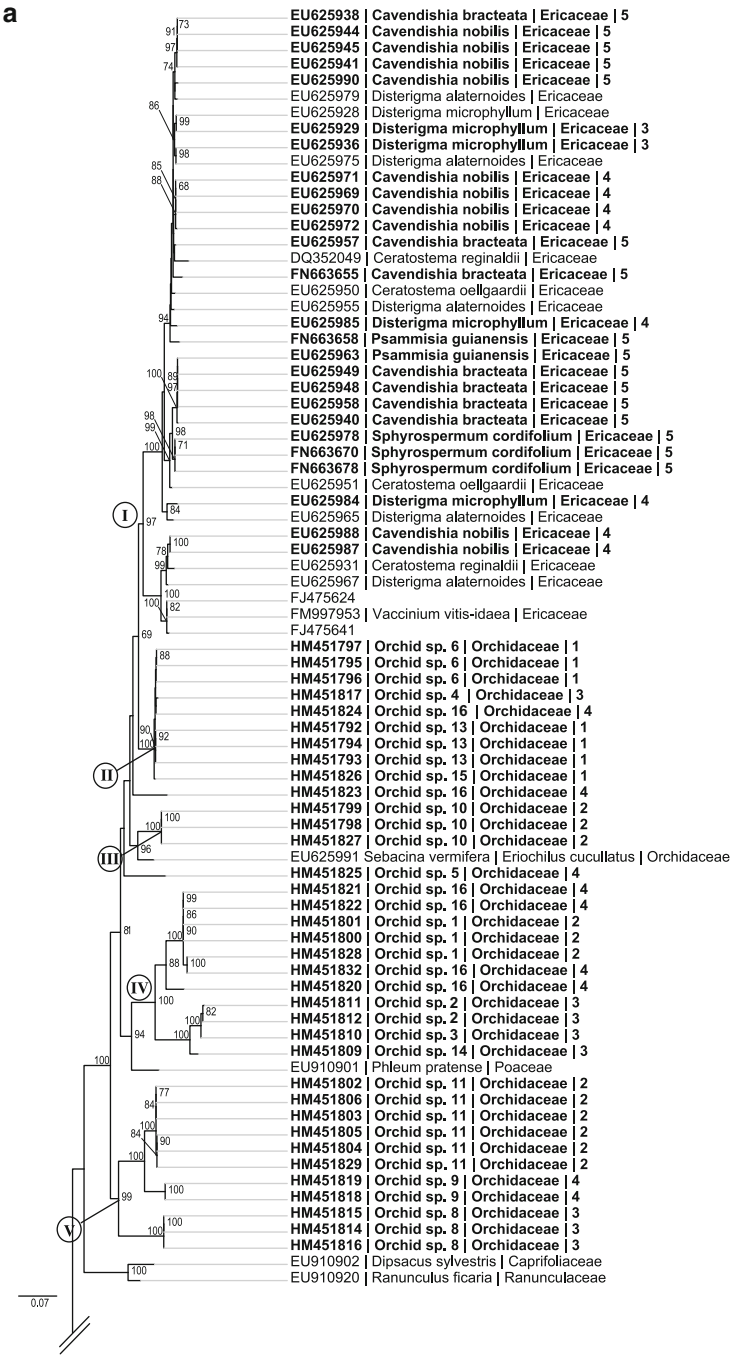

Fig. 5.2 (continued)

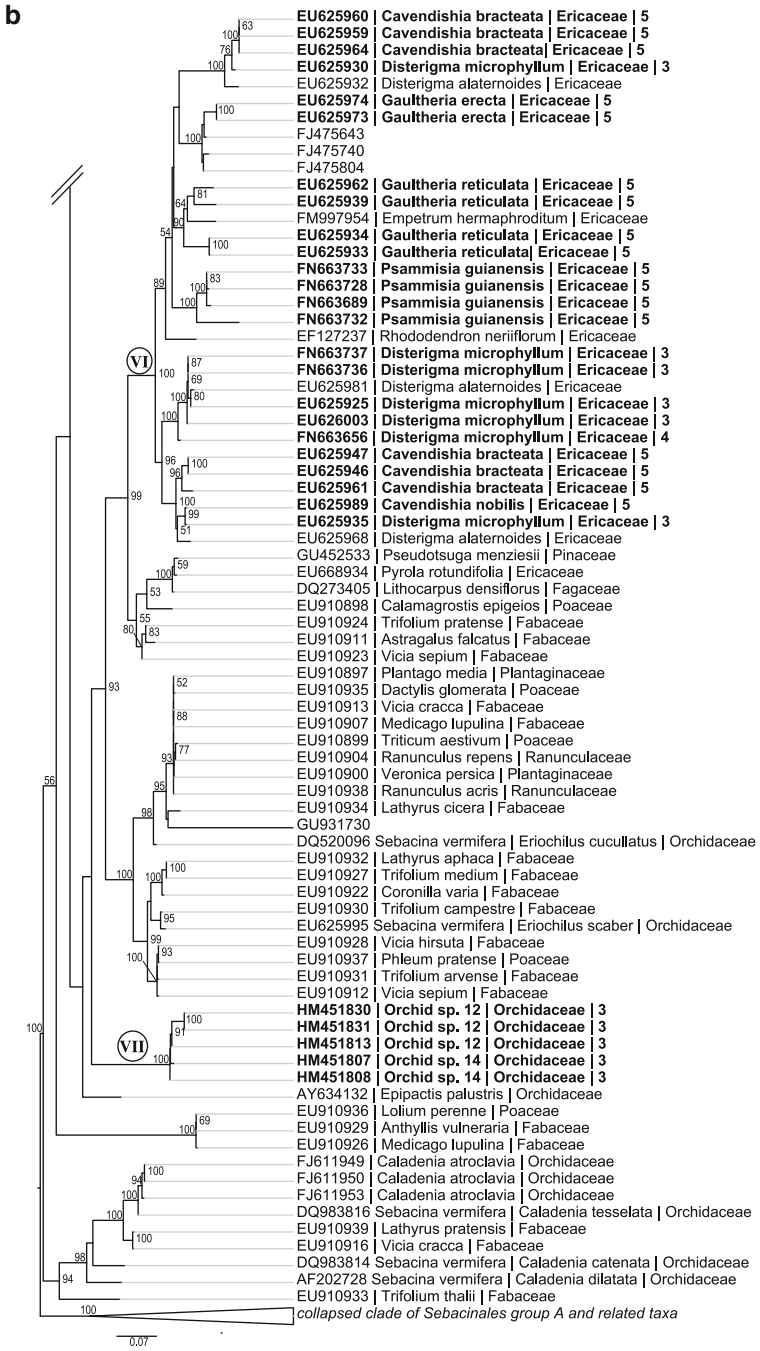

Fig. 5.2 Maximum Likelihood tree of Sebacinales associated with Ericaceae and Orchidaceae inferred from ITS and 28S data. (**a**) Upper part of the tree, (**b**) lower part of the tree. Maximum Likelihood analysis was performed with RAXML and 500 rapid bootstrap replicates. The tree was

1 is not supported. Clade 2 contains all hosts of Orchidaceae with the exception of Orchid sp. 12 and Orchid sp. 2 that fall in a supported subclade (subclade 2) of clade 1. In clade 2 there are three subclades (# 3, 4, 5) that each consist of epiphytic as well as terrestrial Orchidaceae from different sampling localities (Table 5.1). The positions of the species of Ericaceae are mainly unsupported, with the exception of the two terrestrial species, *Gaultheria erecta* and *Gaultheria reticulata* that form a subclade (# 2) with moderate support (72 %) (Fig. 5.3).

The PBC-Neighbor-Net analysis (PBC) also shows a general division of Sebacinales from Ericaceae and Orchidaceae and the distinction of clade 2 from clade 1 (Fig. 5.4). The delta score of the PBC-Neighbor-Net is 0.31, indicating rather high treelikeness of the network. The unsupported clade 1 from the UC analysis appear also in the PBC analysis but large box-like structures between the hosts indicate a high amount of reticulation between host species of clade 1. All subclades in the UC analysis appear also in the PBC analysis (Figs. 5.3 and 5.4). The orchid species of sublcade 2 and the isolated ericad species *S. cordifolium* are intersectional species as they are in between ericad and orchid species in the PBC network (Fig. 5.4). Orchid species of subclade 2 are closer to ericad species than to orchid species, whereas *S. cordifolium* is in between both clades (# 1 and 2).

5.4 Discussion

5.4.1 Distinct guilds of Sebacinales in Neotropical Ericaceae and Orchidaceae

Comparative investigations of mycobionts of Ericaceae and Orchidaceae were carried out over several years (2003–2009) in the tropical mountain rain forest of the Northern Andes in South Ecuador. We showed that these two plant families harbor Sebacinales as mycobionts. The involved Sebacinales appeared to be closely related but are probably not identical species (Kottke et al. 2008; Suárez et al. 2008). This behavior distinguishes Sebacinales from other fungal orders, since Sebacinales are the only order that contains fungi forming mycorrhiza with chlorophyllous species of both host families (Taylor et al. 2003). Data of the 28S rDNA (Kottke et al. 2008; Suárez et al. 2008) and 5.8S-ITS2 (Suárez et al. 2008) were used in previous investigations to compare Sebacinales mycobionts of epiphytic Orchidaceae and mostly hemiepiphytic Ericaceae. We found that the investigated Orchidaceae and Ericaceae were associated with distinct guilds of Sebacinales,

Fig. 5.2 (continued) midpoint rooted. Sequences marked in *bold* are from the present data set. The labels correspond to Genbank number | host species | host family | sampling locality. The remaining sequences are from Genbank and the labels indicate Genbank number and fungal species (if provided on NCBI) | host species (if provided on NCBI) | host plant family. Roman numbers indicate clade names

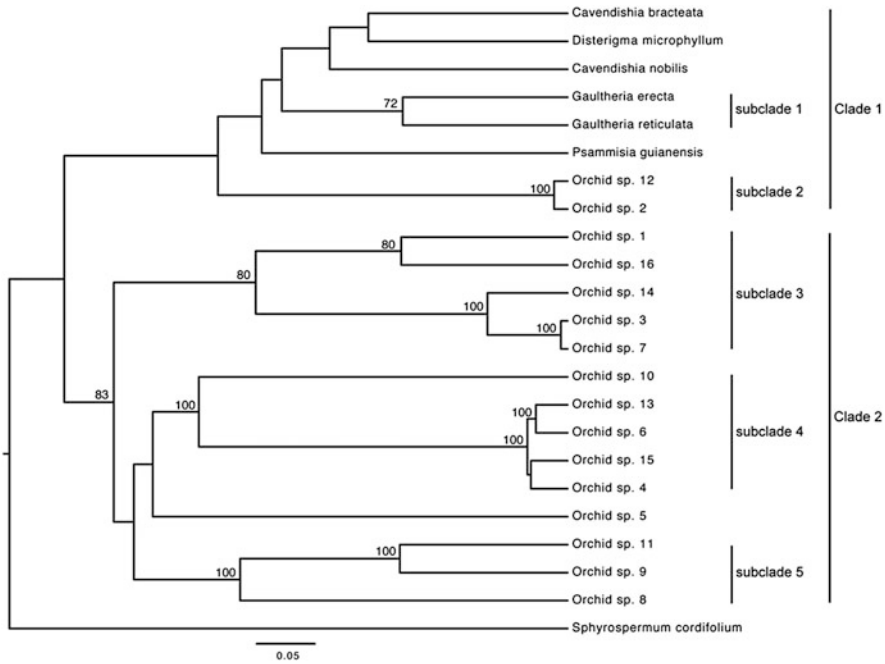

Fig. 5.3 UPGMA cluster of Ericaceae and Orchidaceae associated with Sebacinales. Analysis was performed with FASTUNIFRAC and 1,000 jackknife replicates. Labels indicate names of host species, if known. For more information about hosts, please see Table 5.1

inferred from the absence of well-supported clades on a lower taxonomic level. The overall support values of the 28S rDNA and 5.8S-ITS2 analyses were moderate, with good support values only for some ericad and orchid clades (Kottke et al. 2008; Suárez et al. 2008). The phylogenetic relationships of Sebacinales in the unsupported groups were unclear (Kottke et al. 2008; Suárez et al. 2008).

The present study is more extensive, including Sebacinales associated with epiphytic and terrestrial Orchidaceae and terrestrial and hemiepiphytic Ericaceae from the pristine forest and disturbed habitats. Furthermore, we sequenced ITS (ITS1-5.8S-ITS2) and the D1/D2 region of the 28S rDNA in order to infer phylogenetic relationships of Sebacinales based on a combined dataset and to test the hypothesis that Ericaceae and Orchidaceae are associated with distinct guilds of Sebacinales.

The results of the ML (Fig. 5.2a, b), UN (Fig. 5.3), and PBC analyses (Fig. 5.4) presented here give further indication that the coexisting Ericaceae and Orchidaceae in the tropical mountain rain forest in Southern Ecuador form mycorrhizae with distinct guilds of Sebacinales. The ML analysis shows that Sebacinales in Ericaceae and Orchidaceae form seven distinct clades, which are all well supported (Fig. 5.2a, b). None of the seven clades contain Sebacinales sequences from both host families. The orchid clades (# II, III, IV, V, VII) contain

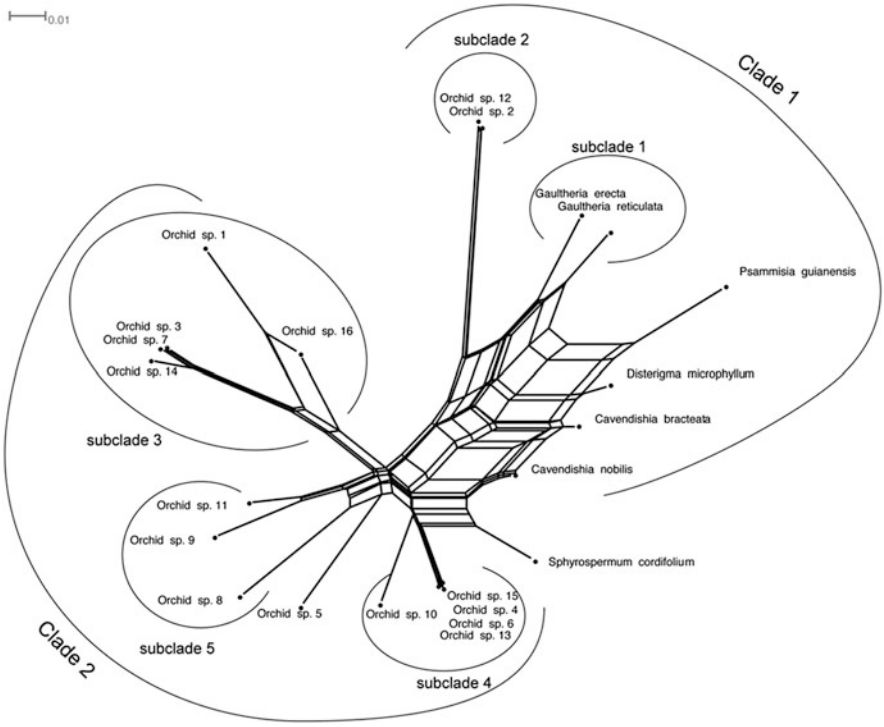

Fig. 5.4 PBC-Neighbor-Net Analysis of Ericaceae and Orchidaceae associated with Sebacinales. Analysis was performed with PBC and Neighbor-Net construction with SPLITSTREE. Delta value is 0.31. Labels indicate names of host species, if known. Large box-like structures indicate reticulation between respective hosts. For more information about hosts, please see Table 5.1

closely related Sebacinales associated with epiphytic as well as terrestrial hosts and from different sites. The same is true for Sebacinales associated with Ericaceae (clades I, VI). This suggests that the association with either Ericaceae or Orchidaceae is more important than the host's life-form or the sampling site of Sebacinales in the study area.

Ericaceae and Orchidaceae have different mycorrhizal types (Smith and Read 2008), with Orchidaceae forming orchid mycorrhizae (Fig. 5.1d, Burgeff 1909) and Ericaceae forming either monotropoid (Duddridge and Read 1982), arbutoid (Molinia and Trappe 1982), ericoid (Fig. 5.1a, Pearson and Read 1973; Read 1996), or cavendishioid (Fig. 5.1c; Setaro et al. 2006a, b) mycorrhizae. In contrast to orchid mycorrhiza, ericoid and cavendishioid mycorrhizae can be formed by the same Sebacinales phylotype, indicating that the mycorrhizal features are induced by signals from the plant rather than the fungi (Kottke et al. 2008). The finding that the same fungi are able to form ericoid and cavendishioid mycorrhizae corresponds with the similarity of both mycorrhizal associations (Setaro et al. 2006a, b). The root anatomy of both ericoid and cavendishioid mycorrhizae display a small stele,

the endodermis, and one further cell layer, the epidermis (identified by some authors as epidermis e.g., Peterson and Massicotte 2004 and by others as cortical layer e.g., Bonfante-Fasolo and Gianinazzi-Pearson 1979). The host plants forming ericoid and cavendishioid mycorrhizae are closely related and the associated fungi in both mycorrhizal associations are Sebacinales and ascomycetes, mainly Helotiales (Allen et al. 2003; Hambleton and Sigler 2005; Setaro et al. 2006a, b). However, the UN and PBC analysis both indicate that ericoid and cavendishioid mycorrhizal Ericaceae in this study are associated with distinct fungal guilds, although the fungi are closely related (Figs. 5.3 and 5.4).

Orchid mycorrhiza is distinct from ericoid and cavendishioid mycorrhizae and also from other mycorrhizal associations regarding the anatomy of the mycorrhizal interaction and the fungal taxonomy (Taylor et al. 2003). Pelotons are formed in the cortical cells of the orchid root that are digested by the plant shortly after their formation (Smith and Read 2008). In addition to Sebacinales, basidiomycetes belonging to Tulasnellales (Warcup 1981; Suárez et al. 2006), Ceratobasidiales (Warcup 1981) and Atractiellomycetes (Kottke et al. 2010) are orchid-mycorrhiza-forming fungi. The differences between orchid mycorrhiza and ericoid/cavendishioid mycorrhiza corresponds to the finding that Neotropical Orchidaceae and Ericaceae are associated with distinct Sebacinales.

5.4.2 *Phylogenetic Position and Biogeography of Sebacinales Associated with Neotropical Ericaceae and Orchidaceae*

In general, Sebacinales have a broad mycorrhizal potential as exemplified in the introduction, but include also species of supposedly endophytic lifestyle. In our present phylogenetic analysis, we included all currently available sequences for Sebacinales that contain ITS data and that were at least 400 base pairs long. Many of these Sebacinales were isolated from roots of herbaceous plants for which an endophytic lifestyle is suggested (Selosse et al. 2009) but with no indication of mutualisms so far. The ML analysis (Fig. 5.2a,b) shows that Neotropical Sebacinales do not have a monophyletic origin and have close relatives in the Northern hemisphere (e.g., FM997953—clade I, EU910901—clade IV, FM997954—clade VI) and Australia (EU625991—clade III). Close relatives of the Neotropical Sebacinales are species belonging to the *Sebacina vermifera* complex. This corroborates previous studies of Sebacinales that were based on a different taxon sampling and on 28S rDNA data (Weiß et al. 2004; Setaro et al. 2006a; Selosse et al. 2007; Suárez et al. 2008; Kottke et al. 2008).

The phylogenetic relationships of the seven clades of Sebacinales show that orchid clades II, III, and IV are more closely related to ericad clade I than to orchid clades V and VII (Fig. 5.2a, b) and thus indicate that the mycorrhizal association with Neotropical Ericaceae and Orchidaceae evolved several times in Sebacinales. In the close relationship of clades I, II, III, IV, and V are relatively few sequences

from other places besides the Neotropics (Fig. 5.2a, b). However, clades VI and VII are closely related to Sebacinales associated with many different hosts (grasses, trees, Fabaceae, Ericaceae, and Orchidaceae) from several regions of the world. This indicates that Sebacinales of clades I, II, III, IV and V have a different evolutionary history than those of clade VI and VII (Fig. 5.2a, b). Clade I and II have relatively short branch lengths indicating that these clades are rather young, which corresponds to the relatively recent uplift of the Andes (Gregory-Wodzicki 2000) and the subsequent diversification of Orchidaceae (Gustafsson et al. 2010) and Neotropical Ericaceae due to adaptive radiation (Luteyn 2002; Kron and Luteyn 2005). However, shorter branch lengths could also be the result of lower substitution rates (Takezaki et al. 1995). In general, Sebacinales are considered as rather old because of their relatively basal position in the Agaricomycotina (Weiß et al. 2004; Hibbett and Matheny 2009), but so far there are no detailed studies about age estimation in Sebacinales.

Knowledge of the ecology of the ancestors of Sebacinales from Neotropical Ericaceae and Orchidaceae would be important to elucidate whether the different mycorrhizal states evolved from endophytic/saprophytic or mycorrhizal precursors. Furthermore, the ancestors of Sebacinales associated with Neotropical Ericaceae and Orchidaceae could have been introduced to the area together with their host plants or could have been adapted from the local Sebacinales community. Hibbet and Matheny (2009) performed Bayesian relaxed molecular clock analyses in order to infer the ecological lifestyle of the ancestors of ectomycorrhizal basidiomycetes with the result that the ectomycorrhizal state in basidiomycetes evolved independently several times from saprophytic ancestors (Hibbett and Matheny 2009). This approach is also conceivable for Neotropical Sebacinales in the future but a more extensive sampling of Sebacinales from other hosts, especially herbaceous plants, or soil of the same area would be crucial.

5.4.3 Suitability of Unifrac and PBC-Neighbor-Net Analyses for Ecological Analyses of Sebacinales

Most of the Sebacinales lack distinguishing morphological characters to identify them to species level (Weiß et al. 2004). Therefore, conventional ecological approaches based on species entities (e.g., cluster analysis and ordination techniques—Legendre and Legendre 1998) are not feasible to characterize communities of Sebacinales. The Unifrac and PBC approaches are a useful alternative, since they both use phylogenetic data without the need to delimitate fungal sequences to species or molecular operational taxonomic units (Lozupone and Knight 2005; Göker and Grimm 2008). Especially the Unifrac approach has been used for a variety of organisms for which the same species delimitation problems exist as for Sebacinales (e.g., Schloss 2008; Chu et al. 2010; Dimitriu and Grayston 2010; Doherty et al. 2010). The Unifrac metric measures the difference between

two environments (in our case host species) in terms of the branch length that is unique to one host or the other (Lozupone and Knight 2005). The PBC approach was published in 2008 (Göker and Grimm 2008) and used to detect hybrids in *Acer* spp. and also discussed in the context of a broad applicability to ecological questions where "host" and "associate" data are available (Göker and Grimm 2008). This novel approach transforms phylogenetic distance matrices of the "associates" to distance matrices of the "host" using Sørensen's distance coefficient and determines the most similar specimens between two hosts automatically when computing pair-wise distances from the reduced matrix (Göker and Grimm 2008). Subsequently, phylogenetic analyses like Neighbor-Net analysis allows visualization of the transformed distance matrix and Delta values can be computed to statistically evaluate phylogenetic accuracy (Göker and Grimm 2008). For Sebacinales, this is the first study that uses the PBC approach to investigate fungal communities in different host species.

Both analyses showed the general division of Sebacinales associated with Ericaceae or Orchidaceae since almost all orchid species sampled from the study site group together in a well-supported clade in the UN cluster analysis (Fig. 5.3) and appear together in the PBC-Neighbor-Net analysis (Fig. 5.4). Clade 1 has no jackknife support in the Unifrac (Fig. 5.3) and a relatively high occurrence of reticulation in the PBC analysis (Fig. 5.4). The jackknife statistic implemented in FastUnifrac indicates whether the result of the analysis is robust in terms of sample size and evenness (Hamady et al. 2010). Thus, the missing support values for most of the Ericaceae indicate that species of Ericaceae are associated with a more diverse community of Sebacinales than are Orchidaceae.

Findings from both ecological analyses, UN (Fig. 5.3) and PBC (Fig. 5.4), are congruent and correspond with results from the phylogenetic ML analysis of the associated Sebacinales (Fig. 5.2a, b). The results show that both analyses performed well. The UN approach has the advantage that it is already well established in ecology as exemplified above, and resampling statistics that support the outcome of the analyses are integrated in the program (Lozupone and Knight 2005; Hamady et al. 2010). However, the PBC approach gives the user more freedom how to analyze and visualize the data so that even networks can be computed (Göker and Grimm 2008). Networks have the advantage that data can be displayed that are not necessarily treelike (Huson and Bryant 2006), what might be the case for many host species and was true for most of the Ericaceae in this study. We think that both approaches are a suitable tool for dealing with ecological questions in Sebacinales, especially when many hosts and localities are to be investigated.

References

Allen T, Millar T, Berch S, Berbee M (2003) Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. New Phytol 174:864–878

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Auch A, Henz S, Holland B, Göker M (2006) Genome BLAST distance phylogenies inferred from whole plastid and whole mitochondrion genome sequences. BMC Bioinformatics 7:350

Bauer R, Begerow D, Sampaio JP, Weiß M, Oberwinkler F (2006) The simple-septate basidiomycetes: a synopsis. Mycol Progr 5:41–66

Beck E, Bendix J, Kottke I, Makeschin F, Mosandl R (2008) Gradients in a tropical mountain ecosystem of Ecuador. Ecological Studies 198. Springer

Burgeff H (1909) Die Wurzelpilze der Orchideen. Gustav Fischer, Jena, Germany

Bonfante-Fasolo P, Gianinazzi-Pearson V (1979) Ultrastructural aspects of Endomycorrhiza in the Ericaceae I. Naturally infected hair roots of *Calluna vulgaris* L. Hull. New Phytol 83:739–744

Capella-Gutiérrez S, Silla-Martinez JM, Gabaldón T (2009) trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25:1972–1973

Chu H, Fierer N, Lauber C, Caporaso J, Knight R, Grogan P (2010) Soil bacterial diversity in the Arctic is not fundamentally different from that found in other biomes. Environ Microbiol. doi:10.1111/j.1462-2920.2010.02277.x

Cullings K (1994) Molecular phylogeny of the Monotropoideae (Ericaceae) with a note on the placement of the Pyroloideae. J Evolut Biol 7:501–516

Dimitriu PA, Grayston SJ (2010) Relationship between soil properties and patterns of bacterial β-diversity across reclaimed and natural boreal forest soils. Microb Ecol 59:563–573

Doherty M, Tamura M, Costas BA, Ritchie ME, McManus GB, Katz LA (2010) Ciliate diversity and distribution across an environmental and depth gradient in Long Island Sound, USA. Environ Microbiol 12:886–898

Duddridge JA, Read D (1982) An ultrastructural analysis of the development of mycorrhizas in Monotropa hypopitys L. New Phytol 92:203–214

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gargas A, Taylor JW (1992) Polymerase Chain Reaction (PCR) primers for amplifying and sequencing nuclear 18S rDNA from lichenized fungi. Mycologia 84:589–592

Glen M, Tommercup IC, Bougher NL, O'Brien PA (2002) Are Sebacinaceae common and widespread ectomycorrthizal associates of Eucalyptus species in Australian forests? Mycorrhiza 12:243–247

Göker M, Grimm G (2008) General functions to transform associate data to host data, and their use in phylogenetic inference from sequences with intra-individual variability. BMC Evol Biol 8:86

Göker M, García-Blázquez G, Voglmayr H (2009) Molecular taxonomy of phytopathogenic fungi: a case study in Peronospora. PLoS One 4:e6319

Grasso C, Lee C (2004) Combining partial order alignment and progressive multiple sequence alignment increases alignment speed and scalability to very large alignment problems. Bioinformatics 20:1546–1556

Gregory-Wodzicki K (2000) Uplift history of the Central and Northern Andes: a review. GSA Bulletin 112:1091–1105

Gustafsson AL, Verola CF, Antonelli A (2010) Reassessing the temporal evolution of orchids with new fossils and a Bayesian relaxed clock, with implications for the diversification of the rare South American genus Hoffmannseggella (Orchidaceae: Epidendroideae). BMC Evol Biol 10:177

Hamady M, Lozupone C, Knight R (2010) Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. ISME J 4:17–27

Hambleton S, Sigler L (2005) Meliniomyces, a new anamorph genus for root-associated fungi with phylogenetic affinities to *Rhizoscyphus ericae* (≡ *Hymenoscyphus ericae*), Leotiomycetes. Stud Mycol 53:1–27

Hibbett D, Matheny P (2009) The relative ages of ectomycorrhizal mushrooms and their plant hosts estimated using Bayesian relaxed molecular clock analyses. BMC Biol 7:13

Holland B, Huber K, Dress A, Moulton V (2002) ∂ Plots: a tool for analyzing phylogenetic distance data. Mol Biol Evol 19:2051

Hopple J, Vilgalys R (1994) Phylogenetic relationships among coprinoid taxa and allies based on data from restriction site mapping of nuclear rDNA. Mycologia 86:96–107

Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20:2317–2319

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267

Iotti M, Zambonelli A (2006) A quick and precise technique for identifying ectomycorrhizas by PCR. Mycol Res 110:60–65

Johnson LA, Soltis DE (1995) Phylogenetic inference in Saxifragaceae sensu stricto and Gilia (Polemoniaceae) using *mat*K sequences. Ann Missouri Bot Gard 82:149–175

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066

Kottke I, Beiter A, Weiß M, Haug I, Oberwinkler F, Nebel M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while *Aneura pinguis* (Metzgeriales) is associated with a Tulasnella species. Mycol Res 107:957–968

Kottke I, Haug I, Setaro S, Suárez J, Weiß M, Preussing M, Nebel M, Oberwinkler F (2008) Guilds of mycorrhizal fungi and their relation to trees, ericads, orchids and liverworts in a neotropical mountain rain forest. Basic Appl Ecol 9:13–23

Kottke I, Suárez JP (2009) Mutualistic, root-inhabiting fungi of orchids – identification and functional types. In: Pridgeon AM, Suárez JP (eds) Proceedings of the Second Scientific Conference on Andean Orchids. Universidad Técnica Particular de Loja, Loja, Ecuador, pp 84–99. ISBN 978-9942-00-502-1

Kottke I, Suárez J, Herrera P, Cruz D, Bauer R, Haug I, Garnica S (2010) Atractiellomycetes belonging to the 'rust' lineage (Pucciniomycotina) form mycorrhizae with terrestrial and epiphytic neotropical orchids. Proc R Soc B 277:1289–1298

Kron KA, Judd WS, Stevens PF, Crayn DM, Anderberg AA, Gadek PA, Quinn CJ, Luteyn JL (2002) Phylogenetic classification of Ericaceae: molecular and morphological evidence. Bot Rev 68:335–423

Kron KA, Luteyn JL (2005) Origins and biogeographic patterns in Ericaceae: new insights from recent phylogenetic analyses. Biol Skr 55:479–500

Krüger M, Stockinger H, Krüger C, Schüßler A (2009) DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol 183:212–223

Legendre P, Legendre L (1998) Numerical ecology, 2nd edn. Elsevier Science B. V, Amsterdam

Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71:8228–8235

Luteyn JL (1996) Ericaceae. In: Flora of Ecuador, Vol. 54. Department of Systematic Botany, University of Göteborg, Stockholm, Sweden, pp 1–404

Luteyn JL (2002) Diversity, Adaptation, and Endemism in Neotropical Ericaceae: Biogeographical Patterns in the Vaccinieae. Bot Rev 68:55–87

Molinia R, Trappe JM (1982) Lack of mycorrhizal specificity by the ericaceous hosts *Arbutus menziesii* and *Arctostaphylos uva-ursi*. New Phytol 90:495–509

Newsham KK, Bridge PB (2010) Sebacinales are associates of the leafy liverwort *Lophozia excisa* in the southern maritime Antarctic. Mycorrhiza 20:307–313

Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson KH (2008) Intraspecific ITS variability in the kingdom fungi as expressed in the international sequence databases and its implications for molecular species identification. Evol Bioinform 4:193–201

Oberwinkler F (1964) Intrahymeniale Heterobasidiomyceten. Fruchtkörperlose Sebacina-Sippen und ihre systematische Stellung. Nova Hedwigia 7:489–498

O'Donnell K (1993) Fusarium and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International, Washington, DC, USA, pp 225–233

Pearson V, Read DJ (1973) The biology of mycorrhiza in the Ericaceae: I. The isolation of the endophyte and synthesis of mycorrhizas in aseptic culture. New Phytol 72:371–379

Peterson RL, Massicotte HB (2004) Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. Can J Bot 82:1074–1088

Pridgeon AM, Solano R, Chase MW (2001) Phylogenetic relationships in Pleurothallidinae (Orchidaceae): combined evidence from nuclear and plastid DNA sequences. Am J Bot 88:2286–2308

Rambaut A (2009) FigTree Tree Figure Drawing Tool Version 1.3.1. http://tree.bio.ed.ac.uk/

Read DJ (1996) The structure and function of the Ericoid mycorrhizal root. Ann Bot 77:365–374

Schloss P (2008) Evaluating different approaches that test whether microbial communities have the same structure. ISME J 2:265–275

Selosse MA, Bauer R, Moyersoen B (2002) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol 155:183–195

Selosse MA, Dubois MP, Alvarez N (2009) Do Sebacinales commonly associate with plant roots as endophytes? Mycol Res 113:1062–1069

Selosse MA, Setaro S, Glatard F, Richard F, Urcelay C, Weiß M (2007) Sebacinales are common mycorrhizal associates of Ericaceae. New Phytol 174:864–878

Setaro S, Weiß M, Oberwinkler F, Kottke I (2006a) Sebacinales form ectendomycorrhizae with *Cavendishia nobilis*, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. New Phytol 169:355–365

Setaro S, Kottke I, Oberwinkler F (2006b) Anatomy and ultrastructure of mycorrhizal associations of neotropical Ericaceae. Mycol Prog 5:243–254

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Elsevier, Great Britain

Stamatakis A (2006) RaxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690

Stamatakis A, Hoover P, Rougemont J (2008) A rapid Bootstrap algorithm for the RAxML Web Servers. Syst Biol 57:758

Stockinger H, Walker C, Schüßler A (2009) '*Glomus intraradices* DAOM197198', a model fungus in arbuscular mycorrhiza research, is not *Glomus intraradices*. New Phytol 183:1176–1187

Suárez JP, Weiß M, Abele A, Garnica S, Oberwinkler F, Kottke I (2006) Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. Mycol Res 110:1257–1270

Suárez JP, Weiß M, Abele A, Oberwinkler F, Kottke I (2008) Members of Sebacinales subgroup B form mycorrhizae with epiphytic orchids in a neotropical mountain rain forest. Mycol Prog 7:75–85

Swofford DL (2002) Paup* Phylogenetic Analysis Using Parsimony (* and other Methods). Version 4. Sinauer Associates, Sunerland, Massachusetts

Takezaki N, Rzhetsky A, Nei M (1995) Phylogenetic test of the molecular clock and linearized trees. Mol Biol Evol 12:823–833

Taylor DL, Bruns TD, Leaky JR, Read DJ (2003) Mycorrhizal specificity and function in Myco-heterotrophic plants. In: Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Berlin, pp 375–414

Urban A, Weiß M, Bauer R (2003) Ectomycorrhizae involving sebacinoid mycobionts. Mycol Res 107:3–14

Warcup J, Talbot P (1967) Perfect states of rhizoctonias associated with orchids. New Phytol 66:631–641

Warcup J (1981) The mycorrhizal relationships of Australian orchids. New Phytol 87:371–381

Warcup JH (1988) Mycorrhizal associations of isolates of *Sebacina vermifera*. New Phytol 110:227–231

Weiß M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes. In: PCR protocols: a guide to method and applications, pp 315–322

Whitten WM, Williams NH, Chase MW (2000) Subtribal and generic relationships of Maxillarieae (Orchidaceae) with emphasis on Stanhopeinae: combined molecular evidence. Am J Bot 87:1842–1856

Chapter 6
Impact of *Piriformospora indica* on Tomato

Diana Rocio Andrade-Linares, Anja Müller, Ahmad Fakhro, Dietmar Schwarz, and Philipp Franken

6.1 Introduction

Among the heterobasidiomycetous fungi, a new order was proposed 8 years ago based on molecular phylogeny studies, the Sebacinales (Weiss et al. 2004). This order contains species forming all kind of mycorrhizal associations except arbuscular mycorrhiza which is restricted to the phylum Glomeromycota (Schüssler et al. 2001). Some Sebacinales are even able to form different types of mycorrhiza thereby connecting plants from very diverse plant families (Kottke et al. 2008). In a recent study with 39 plant species among monocot and dicot families, Sebacinales have been detected whenever sequences were obtained (Selosse et al. 2009) and a world-wide screen revealed that members of this order are common in all terrestrial ecosystems (Weiss et al. 2011). This suggest that in addition of being ecto-, ericoid and orchid mycorrhizal fungi, this order seems to contain species which belong to the group of the non-mycorrhizal root endophytes. It was in retrospect therefore not surprising that *Piriformospora indica* firstly isolated in the Thar Desert of India (Verma et al. 1998) later turned out to form orchid mycorrhizal structures (Blechert et al. 1998) and was able to colonise a number of other plant species (Varma et al. 1999). This fungus (together with some isolates of the closely related species *Sebacina vermifera*) is able to establish associations with roots of many different plants. Moreover, it influences whole plant physiology often improving vegetative growth, inducing resistance against pathogens and increasing yield (Table 6.1). The fact that the fungus can be cultivated on different media without a host (Verma et al. 1998) and that large biomass quantities can be easily obtained, let researchers from the beginning propose its application in future plant production systems (Varma et al. 1999). Important crop plants among the monocots (maize, barley, wheat) have

D.R. Andrade-Linares • A. Müller • A. Fakhro • D. Schwarz • P. Franken (✉)
Department for Plant Nutrition, Leibniz-Institute of Vegetable and Ornamental Crops, Theodor-Echtermeyer-Weg 1, 14979 Grossbeeren, Germany
e-mail: andrade@igzev.de; Mueller@igzev.de; azfakhro@yahoo.de; schwarz@igzev.de; Franken@igzev.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_6,

Table 6.1 Plant hosts of *Piriformospora indica*. The results of experiments with different plant species and various methods of inoculation are shown, where statistical analyses of the effect on plant performance were carried out

Species	Inoculation	Increases	Reference
Zea mays	In pots	Vegetative growth[a]	Varma et al. (1999)
Petroselinum crispum	In pots	Vegetative growth[a]	Varma et al. (1999)
Artemisia annua	In pots	Vegetative growth[a]	Varma et al. (1999)
Bacopa monnieri	In pots	Vegetative growth[a]	Varma et al. (1999)
Nicotiana tabacum	In pots	Vegetative growth[a]	Varma et al. (1999)
Populus tremula	In pots	Vegetative growth[a]	Varma et al. (1999)
Nicotiana tabacum	Of calli	Vegetative growth[a]	Varma et al. (1999)
Withania somnifera	Of seeds for field	Vegetative growth and flowering	Rai et al. (2001)
Spilanthes calva	Of seeds for field	Vegetative growth and flowering	Rai et al. (2001)
Arabidopsis thaliana	In pots	Vegetative growth	Peskan-Berghofer et al. (2004)
Nicotiana attenuata	Of seeds[b]	Vegetative growth	Barazani et al. (2005)
Nicotiana attenuata	Of seeds[b]	Seed production	Barazani et al. (2005)
Nicotiana attenuata	Of seeds[b]	Susceptibility for herbivores	Barazani et al. (2005)
Hordeum vulgare	Of seedlings	Vegetative growth	Waller et al. (2005)
Hordeum vulgare	Of seedlings	Disease resistance	Waller et al. (2005)
Hordeum vulgare	Of seedlings	Salt tolerance	Waller et al. (2005)
Hordeum vulgare	Pots in the field	Yield	Waller et al. (2005)
Pelargonium x hortorum	Of cuttings	Enhanced rooting	Drüge et al. (2007)
Euphorbia pulcherrima	Of cuttings	Enhanced rooting	Drüge et al. (2007)
Petunia x *hybrida*	Of cuttings	No effect	Drüge et al. (2007)
Triticum aestivum	In pots	Vegetative growth	Serfling et al. (2007)
Triticum aestivum	In pots	Disease resistance	Serfling et al. (2007)
Triticum aestivum	In the field	Only straw, not yield	Serfling et al. (2007)
Triticum aestivum	In the field	Partial disease resistance	Serfling et al. (2007)
Arabidopsis thaliana	In pots	Seed production	Shahollari et al. (2007)
Arabidopsis thaliana	In Petri plates	Drought tolerance	Sherameti et al. (2008)
Arabidopsis thaliana	Of seedlings	Disease resistance	Stein et al. (2008)
Linum album	CF[c] to cells	Lignan production	Baldi et al. (2008)
Cicer arietinum	Of seeds	Yield	Meena et al. (2010)

[a]No statistical analysis, but large differences between controls and inoculated plants and low standard deviations
[b]Effects also achieved with *Sebacina vermifera*
[c]Culture filtrate

been already investigated, but analysis of dicotyledonous plants was up to 2010, when the chapter was compiled, restricted to models like *Arabidopsis* (discussed in other chapters of the book) or to plants with less economical importance (Table 6.1).

Among the dicotyledonous plants, the nightshade family (*Solanaceae*) includes important crops and model plants as tomato, potato, tobacco, eggplant, pepper, cape gooseberry or petunia, but only two *Nicotiana* species have been shown up to now to be responsive to Sebacinales (Varma et al. 1999; Barazani et al. 2005). We are using *Solanum lycopersicon* at the Leibniz-Institute of Vegetable and Ornamental Crops (IGZ) for many research topics as it is not only a model plant. Tomato is the most commonly grown fresh market vegetable worldwide and therefore of high importance for global nutrition (faostat.fao.org). In this chapter, we will summarise the work which has been carried out at the IGZ concerning the impact of *P. indica* on tomato.

6.2 Conditions for Analysing *Piriformospora indica* – Tomato Interactions

Tomatoes are commercially cultivated in the soil under field or under protected conditions. In greenhouses or under plastic cover, soilless cultivation using different substrates became more and more important during the last 30 years (Savvas 2003). It was therefore necessary to find out the optimal inoculation conditions (mode of inoculum production, inoculum amounts, time point of inoculation) in a soilless system. Such a system had not been used for plant inoculation with *P. indica*, but it turned out that the fungus is able to successfully colonise tomato roots in nutrient solution (Fig. 6.1) and also influences growth characteristics of tomato similar as in horticultural substrate or sand (Fakhro et al. 2010).

Further analyses were carried out to reveal the optimal experimental conditions for inoculation of the plants. At first, the optimal time point of inoculation was determined as being at 4 weeks after sowing (Fakhro et al. 2010). Secondly, different methods of inoculum production were compared. On the one hand, the fungus was cultivated on agar plates, and spores were harvested after 2 weeks; on the other hand, liquid medium was inoculated with such spores, and complete mycelium with spores was obtained after 4 weeks. Two different media were used, potato dextrose (PD) or complete medium (CM, Pontecorvo et al. 1953). All four types of inocula (CM or PD spores or complete mycelium) were used to inoculate tomato plants in a hydroponic system. Six weeks later, colonisation intensity and shoot fresh weights were estimated (Fig. 6.2).

These results indicate that the mode of inoculum production has an influence on the level of colonisation. If spores were used, fungal spread in the root was high, while inoculation with total mycelium resulted in lower colonisation intensities. Interestingly, impact on plant growth was negative if *P. indica* inocula were produced on PD and colonisation levels were inversely correlated with shoot fresh weights. The latter indicates that at least in tomato, *P. indica* can perform negative effects if it occupies a too large area of the root. This is supported by experiments with different amounts of inocula in nutrient solution, sand or substrate, where positive effects were achieved with 3×10^5 cfu/ml, but *P. indica*

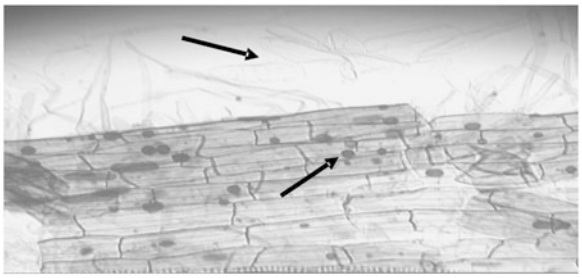

Fig. 6.1 *Piriformospora indica* colonisation of tomato roots. Tomato plants (cv. Hildares) were grown in a hydroponic system and inoculated with spores and hyphae from *P. indica*. Eight weeks after inoculation, roots were harvested and stained with trypan blue. *Arrows* indicate fungal hyphae outside and chlamydospores inside of the root

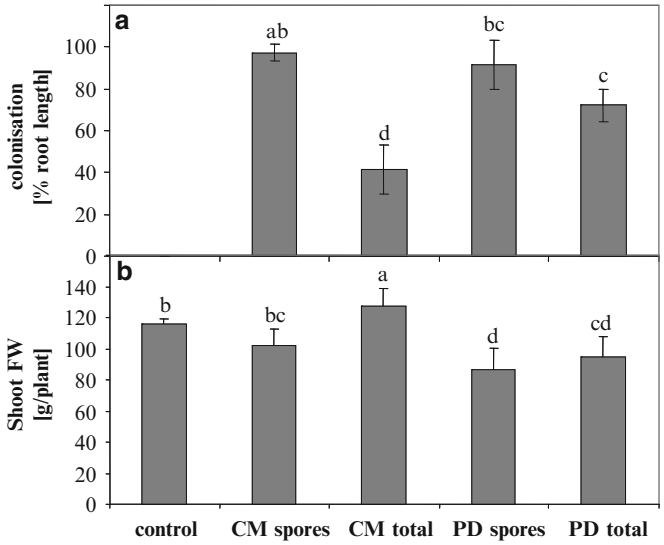

Fig. 6.2 *Piriformospora indica* colonisation intensity and plant growth promotion. *P. indica* was cultivated on complex medium (CM) or on potato dextrose (PD) in liquid medium or on agar plates. Spores were harvested from agar plates (spores) or hyphae and spores from liquid medium (total) and used for inoculating of tomato plants (cv. Hildares) grown in a hydroponic system. Six weeks after inoculation, roots were harvested and stained with trypan blue, and colonisation intensity was estimated by calculating the percentage of root length occupied by fungal chlamydospores. (**a**) In parallel fresh weights of shoots were determined. (**b**) Significant differences between treatments are indicated by different letters above the columns (one-way ANOVA; $P = 0.05$; $n = 3 \times 10$)

impact was negative at 9×10^5 (Fakhro et al. 2010). Such effects were even more drastic using soil fertilised with low amounts of nitrogen or phosphorous (Fig. 6.3).

At optimal fertilisation, positive effects of *P. indica* disappeared at high inoculum concentrations. No growth promotion was observed at low nitrogen but was

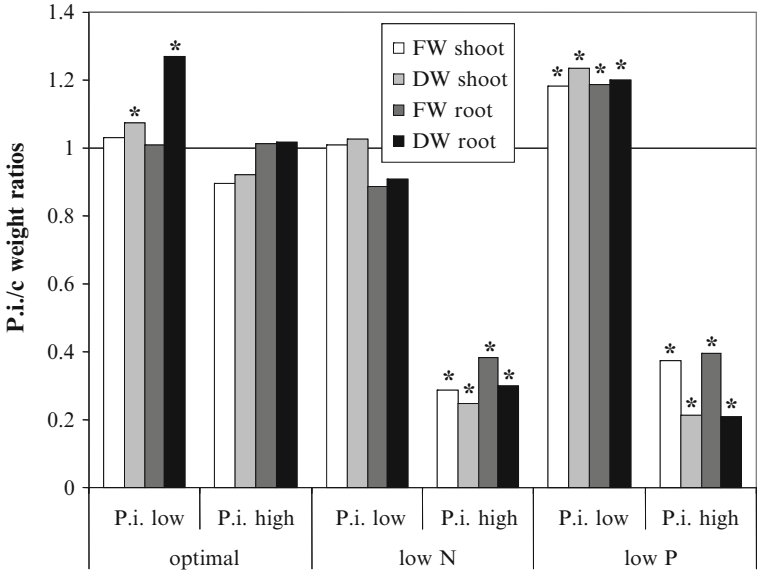

Fig. 6.3 *Piriformospora indica* inoculum amounts and fertilisation. Roots of tomato plantlets (cv. Liberto) were dipped in a suspension of 5×10^5 spores ml^{-1} (P.i. low and high) and transferred to pots with a phosphorous-poor soil where half of the plants were additionally inoculated with 10 g mycelium per 250 ml substrate (P.i. high) or not (P.i. low). The soil was optimally fertilised for tomato growth (De Kreij et al. 1997) or with only 10 % nitrogen (low N) or 0 % phosphate (low P). Plants were harvested 4 weeks after inoculation, and fresh and dry weights of shoots and roots were measured. Ratios of values obtained for inoculated plants to the values of the controls are shown. Significant differences between inoculated and non-inoculated plants are indicated by *asterisks* (two-way ANOVA; $P = 0.05$; $n = 5$). Interaction between the factors inoculation and fertilisation was significant

significant for all four growth characteristics at low phosphate fertilisation. This might indicate that the fungus supports phosphate supply of the plant. Such a role in plant nutrition could not be observed in other plants, such as tobacco and barley (Barazani et al. 2005; Achatz et al. 2010), but a phosphate transporter was recently isolated from *P. indica* and suppressing its expression resulted in a disappearance of plant growth promotion abilities in maize (Yadav et al. 2010). Interestingly, when plants grown under such nutrient-limiting conditions were inoculated with high amounts of *P. indica*, severe negative effects could be observed. Endophyte inoculation reduced biomasses by 60–80 %.

It became clear from these studies that *P. indica* interacts with tomato in soil, in different substrates and also in hydroponic cultures. The outcome of this interaction, however, depends on the type and the amount of inoculum being used, the time point of inoculation and the nutrient conditions in the environment. The basis for negative effects could be the unique mode of root colonisation: the fungus induces programmed cell death as it has been shown at least in barley (Deshmukh et al. 2006) which leads to higher percentage of dead root cells as in the corresponding

controls (Franken et al. 2000). Why the negative effects are so severe under low nutrient conditions is not clear. It is, however, interesting to note that such strong negative effects were also observed when the fungus was cultivated on ammonium instead of nitrate as sole N source (Kaldorf et al. 2005). The authors suggested the production of toxins by the fungus under certain growth conditions. Further analysis will be necessary to prevent such phenomena.

6.3 Impact on Vegetative Growth

In contrast to the results obtained in other plants (Table 6.1), the effects of *P. indica* on vegetative development are marginal. There are some enhancements of biomasses between 4 and 10 weeks after inoculation (Fig. 6.2 and 6.3; Fakhro et al. 2010), but they never exceed a value of 30 % and rarely concern the whole plant. Moreover, this enhancement could not be observed in older plants. Already 10 weeks after inoculation, plants inoculated with *P. indica* showed no difference to the control plants (Table 6.2), and also in a long-term experiment carried out to monitor yield (see next paragraph), biomasses of leaves in plants with colonised or non-colonised roots were nearly identical (Fig. 6.4). This indicates that *P. indica* accelerates development in the beginning as it has been already discussed for barley (Achatz et al. 2010) but that this advantage is later being caught up by non-colonised plants. This can only be explained by a negative effect of *P. indica* because tomato shoot growth is under the applied conditions not limited by other factors. If root growth is slowed down while colonisation is further progressing, the increasing percentage of dead cells as discussed above might explain such a negative effect at later stages of the interaction.

6.4 Impact on Generative Development and Yield

Comparing plants colonised with *P. indica* and the corresponding controls, differences in flowering time could be observed in many experiments. Colonised plants developed inflorescences already 12 days after inoculation where flowers could not be observed at control plants (Fig. 6.5). After 3 weeks, all plants bore flowers, but the number still was different. Plants inoculated with the fungus had significant higher numbers of flowers than controls. Similar to the vegetative growth, however, these differences became non-significant with increasing plant age (Fig. 6.6).

Because flower numbers were different at the early dates, it was not surprising that biomasses of fruits at the early harvests also showed significant differences. Hence, at an early harvest, fruit fresh weights were double in plants colonised by the fungus compared to the corresponding controls (Fakhro et al. 2010). Repeating the experiments showed at the first two harvests 30 % and 70 % increase in fruit fresh

Table 6.2 *Piriformospora indica* impact on vegetative growth 10 weeks after inoculation. Tomato plants (cv. Hildares) in pot cultures with commercial garden substrate were inoculated (P.i.) or not (C) in three consecutive experiments. After 10 weeks plants were harvested, and fresh weights (FW) of shoots and roots, dry weights (DW) of roots, leaf numbers and leave areas were measured

	Experiment 1		Experiment 2		Experiment 3	
	C	P.i.	C	P.i.	C	P.i.
Leaf number	12.5	12.5	**13**[a]	**13.8**	14	14.2
Leaf area [cm^2]	1262.1	1212.5	1720.1	1786.8	2148.1	2129.1
Shoot FW [g]	53.1	51.7	80.2	82.2	105.5	108.9
Shoot DW [g]	5.21	5.03	7.58	7.81	9.04	9.18
Root FW [g]	16.9	14.5	10.9	12.2	13.0	12.2

[a]Significant differences are shown in bold (one-way ANOVA; $P = 0.05$; $n = 6$)

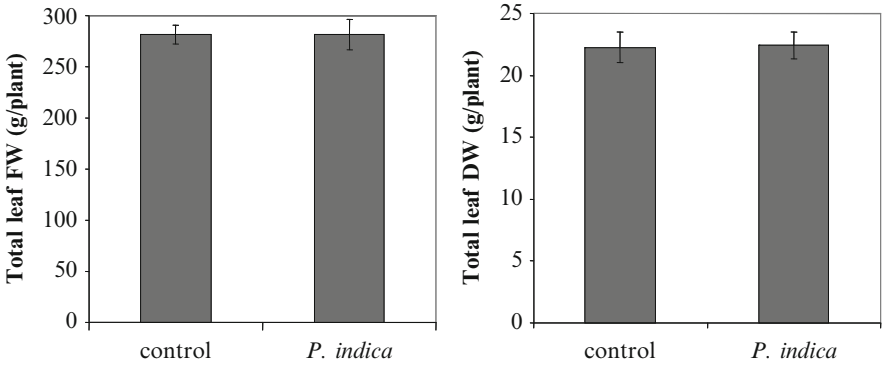

Fig. 6.4 *Piriformospora indica* impact on vegetative biomass after 4 months. Tomato plants (cv. Hildares) inoculated or not with *P. indica* were cultivated in a hydroponic system (three gullies with each ten plants). At the end of the experiment (4 months after inoculation), total leaf fresh weight (FW) and dry weight (DW) were measured. Significant differences between inoculated plants and controls were not detected (one-way ANOVA; $P = 0.05$; $n = 3 \times 10$)

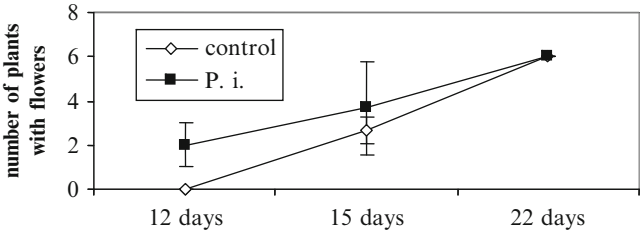

Fig. 6.5 *Piriformospora indica* impact on flowering time. Tomato plants (cv. Hildares) inoculated or not with *P. indica* were cultivated in substrate. Plants with flowers were counted 12, 15 and 22 days after inoculation. Significant differences between inoculated plants and controls were only detected at the first date (one-way ANOVA; $P = 0.05$; $n = 6$)

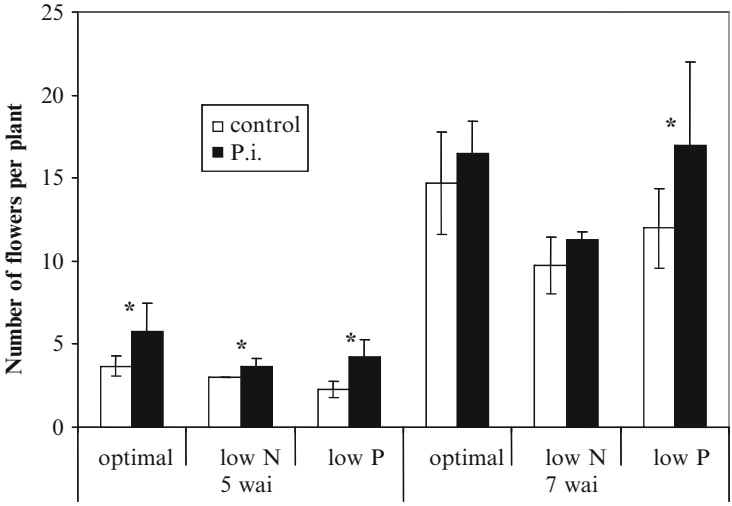

Fig. 6.6 *Piriformospora indica* impact on flower number. Tomato plants (cv. Liberto) inoculated or not with *P. indica* were cultivated in soil. The number of flowers was counted 5 or 7 weeks after inoculation. Significant differences between inoculated plants and controls are indicated by *asterisks* above the columns (one-way ANOVA; $P = 0.05$; $n = 5$)

weights, but thereafter colonised plants did not bear more tomatoes than the corresponding controls (Fig. 6.7).

Analysing the fruits of controls and *P. indica*-colonised plants revealed that the surplus of tomatoes at the early dates possessed blossom-end rot (BER). This disorder of many vegetable plants is based on Ca^{2+} deficiency at the affected sites and occurs often in vigorous growing plants (Olle and Bender 2009). Different cultivation techniques are being used to reduce this disorder, and one is the application of gibberellin biosynthesis inhibitors. In this respect it is interesting to note that *P. indica* up-regulates genes involved in the biosynthesis of this phytohormone (Schäfer et al. 2009). Subtracting the fruits from total yield resulted in similar amounts of marketable fruits harvested from plants being colonised by the endophyte and from control plants (Fig. 6.8).

6.5 Conclusion

Piriformospora indica seems to accelerate vegetative and generative development in tomato plants. This has been already observed in barley (Achatz et al. 2010). While this accelerated development results in increased yields in certain cultivars of this cereal crop (Waller et al. 2005) without any losses in quality (Achatz et al. 2010), this was not the case with tomato. It has, however, to be mentioned that *P. indica* does reduce disease symptoms in case of the fungal pathogen *Verticillium*

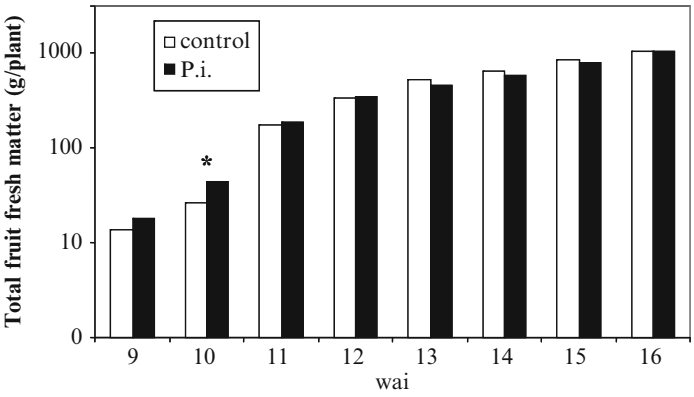

Fig. 6.7 *Piriformospora indica* impact on fruit biomasses. Tomato plants (cv. Hildares) inoculated or not with *P. indica* were cultivated in hydroponic cultures. Fruits were harvested between 9 and 16 weeks after inoculation (wai), and fresh weights were measured. Significant differences between inoculated plants and controls are indicated by *asterisks* above the columns (one-way ANOVA; $P = 0.05$; $n = 4 \times 10$)

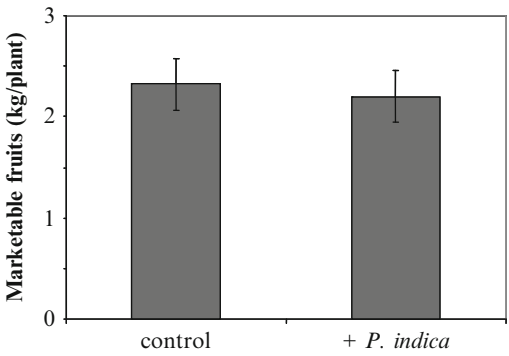

Fig. 6.8 *Piriformospora indica* impact on yield. Tomato plants (cv. Hildares) inoculated or not with *P. indica* were cultivated in hydroponic cultures. Fruits were harvested between 9 and 16 weeks after inoculation, and those possessing blossom-end rot disorder were removed. Fresh weight sums of marketable fruits from all harvests are shown. No significant differences between inoculated plants and controls were detected (one-way ANOVA; $P = 0.05$; $n = 4 \times 10$)

dahliae and confines the spread of *Pepino mosaic virus* (Fakhro et al. 2010). The application of this endophyte can therefore be recommended although yield seems not to be increased. Moreover, it will be interesting to investigate if the fungus also helps tomato plants to overcome abiotic stresses as it has been shown for higher salt concentrations during cultivation of barley and *Arabidopsis* (Baltruschat et al. 2008; Sherameti et al. 2008). Finally, it might be possible that yield increases can be achieved under particular conditions, e.g. at low nutrient availability or with other cultivars which have not been tested yet.

Acknowledgements This research was supported by the Ministries of Consumer Protection, Food and Agriculture of the Federal Republic of Germany, of the Land Brandenburg and of the Land Thüringen. D. R. Andrade-Linares was supported by the German Academic Exchange Service (DAAD) and A. Fakhro by a scholarship of the Al-Furat University (Syria).

References

Achatz B, von Rüden S, Andrade-Linares DR, Neumann E, Pons-Kuhnemann J, Kogel K-H, Franken P, Waller F (2010) Root colonization by *Piriformospora indica* enhances grain yield in barley under diverse nutrient regimes by accelerating plant development. Plant Soil 333:59–70

Baldi A, Jain A, Gupta N, Srivastava AK, Bisaria VS (2008) Co-culture of arbuscular mycorrhiza-like fungi (*Piriformospora indica* and *Sebacina vermifera*) with plant cells of *Linum album* for enhanced production of podophyllotoxins: a first report. Biotechnol Lett 30:1671–1677

Baltruschat H, Fodor J, Harrach BD, Niemczyk E, Barna B, Gullner G, Janeczko A, Kogel KH, Schäfer P, Schwarczinger I, Zuccaro A, Skoczowski A (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytol 180:501–510

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. Oecologia 146:234–243

Blechert O, Kost G, Hassel A, Rexer K-H, Varma A (1998) First remarks on the symbiotic interaction between *Piriformospora indica* and terrestrial orchids. In: Varma A, Hock B (eds) Mycorrhiza. Springer, Heidelberg, pp 683–688

De Kreij C, Voogt W, van den Bos AL, Baas R (1997) Voedingsoplossingen voor de teelt van tomaat in gesloten teeltsystemen. Brochure VG Tomaat, PBG Naaldwijk, The Netherlands

Deshmukh S, Hueckelhoven R, Schäfer P, Imani J, Sharma M, Weiss M, Waller F, Kogel KH (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Drüge U, Baltruschat H, Franken P (2007) *Piriformospora indica* promotes adventitious root formation in cuttings. Sci Hortic 112:422–426

Fakhro A, Andrade-Linares DR, von Bargen S, Bandte M, Buttner C, Grosch R, Schwarz D, Franken P (2010) Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens. Mycorrhiza 20:191–200

Franken P, Requena N, Bütehorn B, Krajinski F, Kuhn G, Lapopin L, Mann P, Rhody D, Stommel M (2000) Molecular analysis of the arbuscular mycorrhiza symbiosis. Arch Agron Soil Sci 45:271–286

Kaldorf M, Koch B, Rexer KH, Kost G, Varma A (2005) Patterns of interaction between *Populus* Esch5 and *Piriformospora indica*: a transition from mutualism to antagonism. Plant Biol 7:210–218

Kottke I, Haug I, Setaro S, Suarez JP, Weiss M, Preussing M, Nebel M, Oberwinkler F (2008) Guilds of mycorrhizal fungi and their relation to trees, ericads, orchids and liverworts in a neotropical mountain rain forest. Basic Appl Ecol 9:13–23

Meena KK, Mesapogu S, Kumar M, Yandigeri MS, Singh G, Saxena AK (2010) Co-inoculation of the endophytic fungus *Piriformospora indica* with the phosphate-solubilising bacterium *Pseudomonas striata* affects population dynamics and plant growth in chickpea. Biol Fertil Soils 46:169–174

Olle M, Bender I (2009) Causes and control of calcium deficiency disorders in vegetables: a review. J Hortic Sci Biotechnol 84:577–584

Peskan-Berghofer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plantarum 122:465–477

Pontecorvo G, Roper JA, Hemmons LM, MacDonald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141–238

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128

Savvas D (2003) Hydroponics: a modern technology supporting the application of integrated crop management in greenhouse. J Food Agric Environ 1:80–86

Schäfer P, Pfiffi S, Voll LM, Zajic D, Chandler PM, Waller F, Scholz U, Pons-Kuhnemann J, Sonnewald S, Sonnewald U, Kogel KH (2009) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. Plant J 59:461–474

Schüssler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1421

Selosse MA, Dubois MP, Alvarez N (2009) Do Sebacinales commonly associate with plant roots as endophytes? Mycol Res 113:1062–1069

Serfling A, Wirsel SGR, Lind V, Deising HB (2007) Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field conditions. Phytopathology 97:523–531

Shahollari B, Vadassery J, Varma A, Oelmüller R (2007) A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 50:1–13

Sherameti I, Tripathi S, Varma A, Oelmüller R (2008) The root-colonizing endophyte *Pirifomospora indica* confers drought tolerance in Arabidopsis by stimulating the expression of drought stress-related genes in leaves. Mol Plant Microbe Interact 21:799–807

Stein E, Molitor A, Kogel KH, Waller F (2008) Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. Plant Cell Physiol 49:1747–1751

Varma A, Verma S, Sudah, Sahay N, Franken P (1999) *Piriformospora indica*, a cultivable plant growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Verma S, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. nov. sp. nov., a new root-colonizing fungus. Mycologia 90:896–903

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Weiss M, Sykorova Z, Garnica S, Riess K, Martos F, Krause C, Oberwinkler F, Bauer R, Redecker D (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. Plos One 6:e16793

Yadav V, Kumar M, Kumar Deep D, Kumar H, Sharma R, Tripathi T, Tuteja N, Kumar Saxena A, Kumar Johri A (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in the phosphate transport to the host plant. J Biol Chem. doi:10.1074/jbc.M110.111021

Chapter 7
Effect of *Piriformospora indica* on Enhanced Biosynthesis of Anticancer Drug, Podophyllotoxin, in Plant Cell Cultures of *Linum album*

Vinod Kumar, Vikram Sahai, and V.S. Bisaria

7.1 Introduction

7.1.1 Piriformospora indica*: A Root Endophytic Fungus*

Arbuscular mycorrhizal fungi (AM fungi) represent the most ancient symbiosis with the plants. However, the AM fungi are non-cultivable outside the host plant. The absence of an authentic pure culture creates a problem in its production and, therefore, impedes the biotechnological applications of AM fungi. A root endophyte designated as *Piriformospora indica* was discovered from the desert soil of northwest India, which is similar to AM fungi. Like AM fungi, it has a broad and diverse spectrum and exerts plant growth-promoting effects on the host plants. But the most important advantage of *P. indica* over AM fungi is that it is a facultative symbiont and can be easily cultivated axenically on a variety of synthetic media (Varma et al. 2001). *P. indica* promotes the growth of plants and improves their productivity, enhances the uptake of phosphorus from the soil, increases the drought tolerance of host plants, delays the wilting of leaves, prolongs the aging of callus tissue, protects the plants from the attack of pathogens, and relieves the stress conditions caused by acidity, desiccation, and heavy metal toxicity (Podila and Varma 2004; Zuccaro et al. 2009; Yadav et al. 2010). A review of literature

V. Kumar
Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110 016, India

Department of Chemical and Biomolecular Engineering, Laboratory of Metabolic Engineering of Microorganisms, Pusan National University, Busan 609735, South Korea
e-mail: vinodiitd@yahoo.co.in

V. Sahai • V.S. Bisaria (✉)
Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110 016, India
e-mail: vikramsahai@hotmail.com; vbisaria@dbeb.iitd.ac.in; vsbisaria@yahoo.com

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_7,

substantiates that the fungus *P. indica* has extraordinary potential for growth promotion of plants by colonization of roots (Oelmüller et al. 2009; Varma et al. 2001). The fungus has exerted growth-promoting effects on a variety of mono- and dicotyledonous plants including medicinal and several economically important plants like *Bacopa monnieri* (Sahay and Varma 1999, 2000), *Azadirachta indica*, *Coffea arabica* (Singh et al. 2002, 2003), *Withania somnifera*, *Spilanthes calva* (Rai et al. 2001), *Tridex procumbens*, *Abrus precatorius* (Kumari et al. 2004), *Adhatoda vasica* (Rai and Varma 2005), and *Chlorophytum borivilianum* (Chauhan et al. 2006). In addition to fungal biomass, the growth-promoting effects have also been shown by the culture filtrate of *P. indica* (Varma et al. 1999, 2001).

7.1.2 Hairy Root Culture and Elicitation

Plants are inexhaustible source for a variety of chemicals such as flavors, fragrances, natural pigments, pesticides, and pharmaceuticals (Chattopadhyaya et al. 2004). Many of these valuable secondary metabolites are synthesized by plant kingdom only. Therefore we have to depend on plants for the production of these valuable secondary metabolites (Rao and Ravishankar 2002). The most important problem being faced today regarding these plant-derived chemicals is their affordability and availability. The conventional process of production of these secondary metabolites by the cultivation of the whole plant followed by the extraction of the desired product has a number of disadvantages such as low productivity and non-availability of these secondary metabolites from the plants throughout the year. Therefore there is a need to enhance their production so that their cost may come within the reach of common man. Plant cell culture provides an alternative to the whole plant cultivation for the production of these valuable secondary metabolites, which not only improves the productivity but is also independent of geographical and climatic factors and ensures the continuous supply of products, uniform quality, and yield (Georgiev et al. 2007). Furthermore, in a number of plant cell cultures, the amount of secondary metabolite produced is more than in the whole plant (Ravishankar and Rao 2000).

A route for improving secondary metabolite production is by transformation using the natural vector system of *Agrobacterium rhizogenes* (a gram-negative bacterium) which infects a variety of plants and causes the nepotistic plant disease known as "hairy root" disease. These transformed roots are genetically stable (Aird et al. 1988) that lead to stable and high-level production of secondary metabolites (Banerjee et al. 1995). They grow as fast as or even faster than the normal roots (Flores et al. 1999) and can be used as continuous source for the production of valuable secondary metabolites. Hairy roots do not require conditioning of the medium and can be grown in hormone- and vitamin-free culture medium. Moreover, the biosynthetic capacity of hairy roots is equivalent or sometimes more than the corresponding plant roots, and the hairy roots can be grown in bioreactors (Farkya and Bisaria 2008). Hairy roots also have been found to accumulate those

secondary metabolites which are found only in the aerial part of intact plant. For example, artemisinin which accumulates in the aerial part of *Artemisia annua* plant is also produced by hairy roots (Giri et al. 2000). There are a number of cases where secondary metabolites are synthesized at higher levels in hairy roots than in the corresponding untransformed roots (Bonhomme et al. 2000; Jacob and Malpathak 2004; Zhang et al. 2004).

One of the most effective strategies for enhancing secondary metabolite production in plant tissue and cell cultures is the treatment of plant cells with various elicitors (Qian et al. 2006). An elicitor may be defined as a substance which, when added in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds, and this improved biosynthesis of metabolites due to addition of trace amounts of elicitors is known as elicitation (Radman et al. 2003). Elicitors can be divided into two categories: biotic and abiotic. Biotic elicitors have a biological origin which include polysaccharides derived from plant cell walls (pectin or cellulose) and microorganisms (especially fungi and their extracts), while abiotic elicitors have a non-biological origin which include chemicals such as methyl jasmonate, arachidonic acid, copper sulfate, and silver nitrate.

7.1.3 *Podophyllotoxin (A Secondary Metabolite with Cytotoxic Activity) and* Linum album

Lignans are the dimerization products of phenylpropanoid pathway intermediates which are connected by the central carbons of their side chain. Podophyllotoxin (PT) is a naturally occurring lignan, which is a pharmacologically important compound for its cytotoxic properties. Though PT itself is too toxic for therapeutical use, but its three semisynthetic derivatives, etoposide, etopophos, and teniposide, are widely used as anticancer drugs and have shown good clinical effects against several types of neoplasms including testicular and small-cell lung cancers, lymphoma, leukemia, and Kaposi's sarcoma (Schacter 1996). Therefore PT is widely used for the commercial semisynthetic production of these derivatives.

Linum album is a perennial herbaceous plant which produces PT and its derivatives. The main lignan in *L. album* is PT (Smollny et al. 1998), and the suspension cultures of this plant are known to produce these lignans with highest productivity (Baldi et al. 2007). For the enhanced secondary metabolite production, transformed cell and organ cultures have been reported to be an alternative to suspension cultures (Giri et al. 2000; Baldi et al. 2008a). For example, hairy root cultures of *Linum flavum* have been reported to produce 6-methoxypodophyllotoxin (6-MPT) in 2–5 and 5–12 times higher concentration compared to non-transformed root and undifferentiated cell suspension cultures, respectively (Oostdam et al. 1993). Hairy root cultures of *L. album* have also been reported to produce lignans (PT and 6-MPT) (Farkya and Bisaria 2008). Baldi et al. (2010) studied the effect of

culture filtrates (autoclaved and membrane filtered) and live cells of *P. indica* on growth and lignan production by the cell suspension cultures of *L. album*. The maximum improvement in the growth (28.4 g/L DCW) and lignan content (PT: 20.1 mg/g DCW; 6-MPT: 2.3 mg/g DCW) was achieved on addition of live cells of *P. indica* at a concentration of 1.0 g/L for an exposure time of 24 h. A similar kind of study was carried out by Kumar et al. (2011a) who investigated the effect of addition of autoclaved and filter-sterilized culture filtrate of *P. indica* to the growing *L. album* hairy root cultures on growth and lignan production. They also found that the addition of culture filtrate resulted in a significant enhancement in growth and lignan content of *L. album* hairy root cells. The present article deals with the effect of culture filtrate, cell extract, and live cells of *P. indica* on growth and lignan production by *L. album* hairy root cultures.

7.2 Materials and Methods

7.2.1 Culture Maintenance and Growth of P. indica

The culture of *P. indica* was provided by Prof. Ajit Varma (Amity Institute of Herbal and Microbial Studies, Noida, India), and the stock culture was maintained on slants containing Kaefer-agar medium at pH 6.5 and 30 ± 1 °C. The seed culture was grown in a 500 mL Erlenmeyer flask containing 100 mL potato dextrose broth with an initial pH of 6.5 at 30 ± 1 °C on a rotary shaker at 200 rpm for 4 days. For elicitation and co-culture experiments, the fungus was grown on Kaefer medium for 8 days using the cultivation conditions mentioned above (Kumar et al. 2011b).

7.2.2 Development, Culture Maintenance, and Growth of L. album *Hairy Roots*

Agrobacterium rhizogenes LBA 9402-mediated genetically transformed high yielding hairy root line LYR2i of *L. album* was developed from cotyledon segments of aseptically germinated seeds (Farkya and Bisaria 2008). The root line was maintained by sub-culturing fresh root mass of 2.5 g/L, every 12th day in 250 mL Erlenmeyer flask containing 50 mL of Gamborg liquid medium (Gamborg et al. 1968) supplemented with 30 g/L sucrose on a gyratory shaker at 60 rpm and 25 ± 1 °C under 16/8 light/dark photoperiod. The pH of the media was adjusted to 5.8 before autoclaving. Hairy roots (12 days old) were used as inoculum to develop the suspension cultures. For elicitation and co-culture experiments, hairy roots were grown in Gamborg medium, containing 32.5 g/L sucrose and 0.75 g/L calcium chloride, with an inoculum level of 5.0 g/L on dry weight basis, incubated

on a gyratory shaker at 125 rpm and 25 ± 1 °C under 16/8 h light–dark regime. The intensity of the light used in all the experiments was 1,200 Lux. The fresh weight used for inoculation was calculated from the ratio of fresh weight to dry weight, which was equal to 12 (Farkya and Bisaria 2008; Kumar et al. 2011a).

7.2.3 Measurement of Hairy Root Growth

Hairy root growth was estimated by measuring dry cell weight (DCW) which was determined by drying hairy root cells on Whatman no. 1 filter paper at 25 ± 1 °C until a constant weight was achieved (Kumar et al. 2011a).

7.2.4 Estimation of Lignans and Phenylalanine Ammonia Lyase Activity

Lignans and phenylalanine ammonia lyase (PAL) activity in hairy root cells of *L. album* were measured by using the method followed by Kumar et al. (2011a). PAL enzyme activity was expressed as μkat (μmoles of cinnamic acid formed per sec) per kg protein.

7.2.5 Analysis of Protein Content in Hairy Root Cells

For the estimation of protein content in hairy root cells, the method of Bonjoch and Tamayo (2001) was followed. The method was based on the principle of protein-dye binding using bovine serum albumin as standard.

7.2.6 Preparation of Elicitors and Their Addition to L. album Hairy Root Cultures

The cultures of *P. indica* were grown as described above and were harvested in log and decline phase, i.e., on the 4th day and 8th day, respectively. The filter-sterilized and autoclaved culture filtrates of *P. indica* from log and decline phase, used as elicitors, were prepared according to the method followed by Kumar et al. (2011a). The two fungal elicitor preparations were added at six different levels (0.2, 0.5, 1, 2, 3, and 5 % v/v) on the 4th, 6th, 8th, 10th, and 12th day to growing *L. album* hairy root cultures.

7.2.7 *Preparation of Cell Extract and Its Addition to* L. album *Hairy Root Cultures*

For the preparation of cell extract, the submerged culture of *P. indica* was grown as described above. The culture broth was harvested after 5 days and the fresh mass of the *P. indica* was obtained by centrifugation at 4,000 × *g* for 10 min. This fresh mass was then dried at room temperature for 5–7 days till it became completely dry. After this it was grounded to a fine powder whose 10 % suspension was prepared in distilled water. Finally the pH was adjusted to 5.8 and autoclaved. The supernatant of this autoclaved suspension was used as an elicitor (Baldi 2008). Different concentrations of solution (0.2, 0.5, 1, 2, 3, and 5 % v/v) prepared from the cell extract of *P. indica* were aseptically added to *L. album* hairy root cultures on the 12th and 13th day of growth.

7.2.8 *Co-cultivation of* P. indica *with* L. album *Hairy Root Cultures*

The live, 5-day old, fungal cells of *P. indica* were inoculated at different concentrations (1, 2, 3, 4, and 5 g/L on DCW basis) into *L. album* hairy root cultures, and the co-culture of plant and fungus was established for different periods (24, 48, 72 and 96 h). For the co-cultivation, *L. album* hairy root culture was grown in Gamborg medium supplemented with 0.2 % peptone, 0.1 % yeast extract, and 0.1 % casamino acid hydrolysate using the culture conditions described above.

7.3 Results and Discussion

7.3.1 *Effect of Culture Filtrate, Cell Extract, and Live Cells of* P. indica *on Lignan Production by* L. album *Hairy Root Cultures*

Majority of the intermediates of phenylpropanoid pathway are produced by the plants in excess in the presence of signaling compounds by triggering defense/hypersensitive responses. Phenylpropanoids like PT and 6-MPT are either induced or produced constitutively at higher levels upon exposure to microbes or microbe-derived compounds. A variety of fungal elicitors which resulted in significant enhancement of phytochemicals in plant tissue cultures have been reported (Namdeo et al. 2002; Zhao et al. 2005). But there is still no way to predict a suitable fungal biotic elicitor for enhancement of a desired metabolite. A reduction in biomass upon treatment with biotic elicitors due to hypersensitivity responses is a

major cause of concern with respect to intracellular products as lowered biomass ultimately results in comparatively lower product concentration. However, *P. indica* being a known endophytic fungus with mutual synergism with plant roots was selected for the present study to see if its interaction with *L. album* increases the growth of lignan production in *L. album* hairy root cultures. For this, various concentrations of culture filtrate (autoclaved and filter sterilized), cell extract, and live cells of *P. indica* were added to growing *L. album* hairy root cultures during different phases of growth, and their effect on plant growth, PT, and 6-MPT contents was studied to determine the optimum concentration and time of addition.

7.3.1.1 Effect of Autoclaved Culture Filtrate

Effect of addition of autoclaved culture filtrate from log and decline phase of fungal growth on lignan (PT and 6-MPT) production is summarized in Tables 7.1 and 7.2. The level of enhancement in lignan content caused by the culture filtrate from log and decline phase was almost similar. The maximum increase in lignan content was obtained when 4-day old (log phase) autoclaved culture filtrate was added at a concentration of 3 % v/v on the 12th day (when the cells were actively synthesizing secondary metabolites), i.e., for an exposure time of 48 h. PT content and concentration were maximally improved by 2.1 times (8.6 mg/g) and 2.4 times (149.3 mg/L), respectively. On the other hand, maximum enhancement of 2.9-fold in 6-MPT content (4.5 mg/g) and 3.3-fold in 6-MPT concentration (77.5 mg/L) was obtained on addition of an 8-day old (decline phase) autoclaved culture filtrate at the same concentration and for the same duration (Table 7.2) (Kumar et al. 2011a).

7.3.1.2 Effect of Filter-Sterilized Culture Filtrate

From Tables 7.3 and 7.4, it is clear that the elicitation potential shown by filter-sterilized culture filtrate was greater than that of autoclaved one. *PT and 6-MPT contents were maximally enhanced by 3.3 times (13.0 mg/g) and 3.8 times (7.2 mg/g) in comparison to control culture on addition of culture filtrate from log and decline phase, respectively, at a level of 3 % v/v for exposure time of 48 h. PT and 6-MPT concentrations were also improved by 3.8 times (233.8 mg/L) and 4.4 times (131.9 mg/L), respectively, in comparison to control culture on addition of culture filtrate from decline phase for the same duration and concentration* (Table 7.4) (Kumar et al. 2011a).

The increase in the lignan content on addition of culture filtrate could be due to the elicitation caused by the presence of some signaling molecules such as jasmonic acid, methyl jasmonate, and salicylic acid and its analogues, which can induce plant defense responses and result in secondary metabolite accumulation (Rijhwani and Shanks 1998; Ketchum et al. 1999; Ge and Wu 2005). Similar results for addition of culture filtrates from log and decline phase indicated that compounds responsible for elicitation may have been released during log phase of fungal growth itself.

Table 7.1 Effect of addition of autoclaved culture filtrate of *P. indica* on day 10 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.1 ± 0.4	62.9 ± 2.5	23.4 ± 1.2	112.9 ± 1.6
4-day old culture filtrate of *P. indica*				
0.2	16.0 ± 0.4	50.3 ± 2.7	30.8 ± 1.6	100.1 ± 2.8
0.5	16.5 ± 0.4	41.0 ± 2.9	35.0 ± 1.2	84.7 ± 3.8
1.0	17.3 ± 0.1	72.8 ± 3.7	51.7 ± 2.9	165.3 ± 6.2
2.0	18.2 ± 0.6	79.0 ± 4.2	69.6 ± 3.1	189.5 ± 5.8
3.0	17.3 ± 0.4	83.1 ± 4.5	70.9 ± 3.5	196.7 ± 3.3
5.0	16.9 ± 0.5	60.1 ± 3.1	35.1 ± 1.2	118.0 ± 4.3
8-day old culture filtrate of *P. indica*				
0.2	15.4 ± 0.7	49.3 ± 1.8	27.3 ± 1.7	88.2 ± 4.0
0.5	16.6 ± 0.6	61.7 ± 3.7	37.1 ± 2.3	113.3 ± 3.9
1.0	17.3 ± 0.1	66.5 ± 4.1	43.3 ± 2.7	162.7 ± 4.7
2.0	18.6 ± 0.5	77.5 ± 3.9	54.8 ± 3.4	152.2 ± 2.1
3.0	18.2 ± 0.3	88.0 ± 3.4	63.4 ± 2.9	182.8 ± 7.9
5.0	16.9 ± 0.2	62.1 ± 2.8	47.3 ± 1.9	143.3 ± 1.6

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly the exposure time of *L. album* with the culture filtrate of *P. indica* was 4 days

Table 7.2 Effect of addition of autoclaved culture filtrate of *P. indica* on day 12 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.1 ± 0.4	62.9 ± 2.5	23.4 ± 1.2	112.9 ± 1.6
4-day old culture filtrate of *P. indica*				
0.2	14.6 ± 0.5	81.5 ± 2.4	27.8 ± 1.2	132.6 ± 3.1
0.5	15.9 ± 0.5	99.7 ± 4.3	39.1 ± 1.7	168.4 ± 7.4
1.0	16.8 ± 0.6	115.4 ± 6.2	48.7 ± 2.1	189.0 ± 4.2
2.0	16.9 ± 0.8	124.0 ± 4.6	62.1 ± 3.3	207.9 ± 9.4
3.0	17.5 ± 0.9	**149.3 ± 6.0**	74.7 ± 3.6	227.6 ± 2.1
5.0	16.7 ± 0.4	107.6 ± 5.1	43.2 ± 1.8	164.1 ± 7.3
8-day old culture filtrate of *P. indica*				
0.2	14.9 ± 0.7	75.9 ± 3.4	34.2 ± 1.4	141.1 ± 5.6
0.5	16.5 ± 0.7	96.1 ± 4.4	47.3 ± 2.5	162.4 ± 9.9
1.0	16.8 ± 0.9	102.9 ± 3.9	53.0 ± 2.0	185.6 ± 7.8
2.0	16.9 ± 0.7	110.5 ± 4.3	66.4 ± 3.1	202.4 ± 4.0
3.0	17.4 ± 0.9	138.8 ± 6.11	**77.5 ± 2.4**	237.3 ± 8.7
5.0	16.5 ± 1.0	98.1 ± 5.2	40.5 ± 1.4	175.6 ± 6.8

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly the exposure time of *L. album* with the culture filtrate of *P. indica* was 2 days

Table 7.3 Effect of addition of filter-sterilized culture filtrate of *P. indica* on day 10 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.6 ± 0.5	60.9 ± 3.1	29.7 ± 1.0	116.7 ± 2.1
4-day old culture filtrate of *P. indica*				
0.2	17.7 ± 0.5	114.4 ± 4.4	38.1 ± 1.4	189.8 ± 7.9
0.5	18.0 ± 0.7	130.6 ± 6.0	62.1 ± 1.2	240.0 ± 5.8
1.0	19.4 ± 0.5	158.4 ± 6.1	79.8 ± 1.9	284.5 ± 7.9
2.0	20.7 ± 0.3	208.7 ± 7.1	102.3 ± 2.7	317.4 ± 7.0
3.0	19.2 ± 0.7	213.1 ± 7.2	104.8 ± 2.8	360.6 ± 4.2
5.0	17.6 ± 0.5	171.8 ± 5.6	67.7 ± 2.4	312.6 ± 3.6
8-day old culture filtrate of *P. indica*				
0.2	17.3 ± 0.2	109.5 ± 4.7	31.3 ± 1.1	178.9 ± 3.2
0.5	18.8 ± 0.6	147.6 ± 3.9	42.1 ± 1.3	212.8 ± 3.3
1.0	20.3 ± 0.2	169.9 ± 5.7	55.8 ± 1.8	238.2 ± 5.5
2.0	21.8 ± 0.2	197.1 ± 8.6	67.4 ± 2.1	268.5 ± 5.2
3.0	20.9 ± 0.3	225.7 ± 5.6	94.5 ± 4.0	317.7 ± 8.8
5.0	18.4 ± 0.5	160.3 ± 4.4	63.3 ± 1.7	257.8 ± 5.1

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, PAL phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly the exposure time of *L. album* with the culture filtrate of *P. indica* was 4 days

Table 7.4 Effect of addition of filter-sterilized culture filtrate of *P. indica* on day 12 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.6 ± 0.5	60.9 ± 3.1	29.7 ± 1.0	116.7 ± 2.1
4-day old culture filtrate of *P. indica*				
0.2	15.6 ± 0.7	143.4 ± 07.0	56.0 ± 2.8	216.6 ± 9.6
0.5	16.1 ± 0.9	171.1 ± 08.4	68.6 ± 2.9	261.5 ± 3.1
1.0	16.7 ± 0.6	185.9 ± 09.3	91.8 ± 3.6	298.9 ± 2.3
2.0	16.9 ± 0.5	216.4 ± 10.2	102.3 ± 3.9	321.3 ± 8.5
3.0	17.8 ± 0.8	232.1 ± 12.2	124.3 ± 5.0	353.3 ± 6.5
5.0	16.1 ± 0.4	174.7 ± 08.2	75.5 ± 3.2	277.0 ± 3.4
8-day old culture filtrate of *P. indica*				
0.2	15.8 ± 0.6	148.3 ± 06.2	42.3 ± 2.0	236.9 ± 6.9
0.5	16.4 ± 0.3	159.8 ± 07.4	64.0 ± 2.7	288.4 ± 4.7
1.0	16.9 ± 0.5	177.8 ± 08.5	81.6 ± 3.6	315.5 ± 7.2
2.0	17.2 ± 0.4	191.0 ± 10.7	111.2 ± 4.59	339.5 ± 3.7
3.0	18.2 ± 0.4	**233.8 ± 12.6**	**131.9 ± 5.05**	371.4 ± 3.1
5.0	16.5 ± 0.3	171.6 ± 06.2	63.8 ± 2.3	308.5 ± 2.2

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly, the exposure time of *L. album* with the culture filtrate of *P. indica* was 2 days

The enhancement in case of culture filtrate from the decline phase could be also due to the presence of cellular components, released because of cell lysis. The greater enhancement in lignan accumulation with filter-sterilized culture filtrate indicates that the stimulatory components responsible for this enhancement may be heat labile and may have lost their elicitation activity on autoclaving (Kumar et al. 2011a). Baldi et al. (2010) also studied the effect of autoclaved and filtered culture filtrate of *P. indica* on growth and lignan production in suspension cultures of *L. album*. They reported maximum lignan content of 9.1 mg/g DCW (PT: 8.8; 6-MPT: 0.3) on addition of autoclaved culture filtrate (at a concentration of 2.5 % v/v) for an exposure time of 48 h. A similar study was done by Bais et al. (2000) who found that the addition of culture filtrate of *Phytophthora parasitica* at a level of 0.1 % v/v to the hairy root cultures of *Cichorium intybus* resulted in a 4.1- and 3.7-fold increase in the esculin and esculetin content, respectively.

7.3.1.3 Effect of Cell Extract

Different concentrations of the extract (0.2, 0.5, 1, 2, 3, and 5 % v/v) prepared from the cells of *P. indica* were aseptically added to *L. album* hairy root cultures on the 12th and 13th day of growth. The effects on plant growth and PT and 6-MPT contents were studied to determine the optimum concentration and time of addition of the elicitor. The addition of cell extract on the 13th day of growth did not result in any significant increase in PT and 6-MPT contents while there was significant increase in PT and 6-MPT contents on addition of cell extract on the 12th day of *L. album* growth. A maximum enhancement of 2.1-fold in PT concentration (138.6 mg/L) and 3.2-fold in 6-MPT concentration (71.4 mg/L) was achieved on addition of cell extract at a level of 1 % (v/v) for an exposure time of 48 h (Table 7.5).

Elicitation by fungal culture filtrates/cell extract might be due to combined/individual effects of many compounds of fungal origin like chitin, some disaccharides, and enzymes. There are many reports where polysaccharides and oligosaccharides extracted from the cell wall and dead cell extract of pathogenic fungi have been reported as an elicitor (Wang et al. 2001; Zhao et al. 2001; Yuan et al. 2002a). For example, *Fusarium* spp. have been reported to increase the flux towards phenylpropanoid pathway which caused an enhancement in the level of cinnamic acid, coumaric acid, and ferulic acid following infection of *Musa acuminate* roots (de Ascensao and Dubery 2003). *Fusarium solani* has also been reported to enhance the accumulation of 6-methoxymellein in cell suspension culture of carrot (Marinelli et al. 1990).

7.3.1.4 Effect of Live Cells of *P. indica* During Co-cultivation with *L. album*

Mutual interactions between plant and fungi do not imply absence of plant defense but require a sophisticated balance between the defense response of the plant and

Table 7.5 Effect of addition of cell extract of *P. indica* on day 12 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Culture filtrate conc. (v/v)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
0.0	15.4 ± 0.6	64.7 ± 2.5	22.5 ± 1.3	103.2 ± 2.1
0.2	15.8 ± 0.9	81.9 ± 4.2	29.0 ± 1.5	132.5 ± 8.0
0.5	16.3 ± 0.8	110.7 ± 5.4	38.8 ± 1.9	173.6 ± 6.9
1.0	18.1 ± 0.5	**138.6 ± 6.3**	**71.4 ± 3.7**	232.2 ± 7.5
2.0	16.8 ± 0.9	120.1 ± 4.8	58.5 ± 3.4	197.2 ± 3.4
3.0	16.3 ± 1.0	103.6 ± 3.4	46.7 ± 3.2	184.4 ± 6.6
5.0	15.5 ± 0.4	88.6 ± 2.1	32.5 ± 1.9	156.4 ± 2.6

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day. Accordingly, the exposure time of *L. album* with the cell extract of *P. indica* was 2 days

the nutrient demand of the endophyte. The interactions can be direct or indirect. Direct interactions may include physical or chemical antagonism or synergism between the fungi and plant cells. The indirect interactions may result in stimulation of production of antimicrobial compounds which are commonly known as phytoalexin. PT and related lignans are known to be produced as an arsenal of defense mechanism in plants. Hence the effect of co-cultivation of *P. indica* with *L. album* hairy root cultures was studied. The fungal cells were added to the late log phase of *L. album* hairy root growth, as suppression of cell growth in most of the elicitor-treated plant cell cultures has been a frequently observed phenomenon (Yuan et al. 2002b; Bennet et al. 1996). Hence, addition of the fungal cells in the late log phase will allow the plant cells to effectively utilize the available substrate to produce biomass without any inhibition, as lowered biomass will lead to the lower production of metabolites.

Co-cultivation of live *P. indica* cells with *L. album* hairy roots also resulted in enhancement of lignan concentration despite reduction in biomass. The hairy root cultures receiving 1–5 g/L fungal concentration on days 10, 11, 12, and 13 all achieved a higher PT and 6-MPT contents (mg/g) in the roots than the fungus-free control culture. The maximum increment of 2.1-fold in PT concentration (122.2 mg/L) and 2.5-fold in 6-MPT concentration (52.2 mg/L) was obtained when a fungal concentration of 2 g/L was added to a growing *L. album* hairy root cultures on the 12th day, i.e., for an exposure time of 48 h. Further increase in fungal cell concentration and exposure time resulted in significant decrease in lignan accumulation (Table 7.6). In the earlier reports based on co-cultivation, dose- and duration-dependent increase in production of secondary metabolites has been reported. For example, co-culturing of *Bacillus cereus* with hairy root cultures of *Salvia miltiorrhiza* caused a significant enhancement in tanshinone content of roots (Wu et al. 2007). Similarly, an increase in lignan content in *L. album* suspension cultures was reported upon co-cultivation with root endophytic fungi,

Table 7.6 Effect of addition of different concentrations of *P. indica* on days 10, 11, 12, and 13 to growing *L. album* hairy root cultures on growth and lignan production[a, b]

Day of addition (duration of co-culture)	Fungal conc. (g/L)	Hairy root DCW (g/L)	PT conc. (mg/L)	6-MPT conc. (mg/L)	PAL activity (μkat/kg protein)
13th day (24 h)	1.0	15.9 ± 0.9	81.5 ± 3.9	45.3 ± 2.4	158.6 ± 3.8
	2.0	15.4 ± 0.5	97.7 ± 6.0	49.7 ± 1.8	182.7 ± 1.6
	3.0	15.4 ± 0.7	77.46 ± 3.7	37.3 ± 1.9	138.8 ± 2.7
	4.0	15.1 ± 0.5	75.70 ± 4.9	35.9 ± 1.9	156.4 ± 0.6
	5.0	15.2 ± 1.2	73.83 ± 4.7	29.7 ± 1.5	126.6 ± 2.9
12th day (48 h)	1.0	14.7 ± 0.8	90.7 ± 4.5	42.3 ± 2.2	188.2 ± 5.4
	2.0	14.4 ± 0.9	**122.2 ± 7.3**	**52.2 ± 2.8**	224.3 ± 3.1
	3.0	13.3 ± 0.5	73.8 ± 4.5	36.0 ± 1.7	158.6 ± 3.9
	4.0	12.6 ± 0.4	70.2 ± 4.4	27.3 ± 1.4	154.4 ± 6.5
	5.0	12.2 ± 0.7	63.5 ± 3.5	22.0 ± 1.1	135.7 ± 2.8
11th day (72 h)	1.0	13.9 ± 0.8	65.7 ± 4.0	28.4 ± 1.7	115.8 ± 5.0
	2.0	13.4 ± 0.8	79.7 ± 4.3	39.0 ± 2.0	168.6 ± 3.3
	3.0	12.1 ± 0.8	55.6 ± 3.6	26.2 ± 1.5	142.7 ± 4.6
	4.0	11.9 ± 0.6	54.3 ± 3.3	21.3 ± 1.1	137.3 ± 5.7
	5.0	11.4 ± 0.9	50.8 ± 3.1	20.9 ± 1.1	131.4 ± 4.9
10th day (96 h)	1.0	13.1 ± 0.6	53.5 ± 3.2	22.0 ± 1.1	105.3 ± 5.4
	2.0	12.9 ± 0.6	63.7 ± 2.9	25.6 ± 1.3	117.3 ± 3.8
	3.0	11.5 ± 0.5	49.5 ± 3.1	18.6 ± 1.0	122.4 ± 6.0
	4.0	11.1 ± 0.7	43.2 ± 2.6	17.3 ± 0.9	95.5 ± 5.1
	5.0	10.9 ± 0.8	42.2 ± 2.6	15.2 ± 0.9	86.8 ± 6.1
Control	0.0	15.8 ± 1.0	59.1 ± 3.2	21.2 ± 1.1	104.8 ± 1.7

DCW dry cell weight, *PT* podophyllotoxin, *6-MPT* 6-methoxypodophyllotoxin, *PAL* phenylalanine ammonia lyase

[a]Values are mean ± standard deviations of three replicates

[b]The *L. album* culture was harvested on the 14th day

P. indica and *Sebacina vermifera (*Baldi et al. 2008b, 2010). Baldi et al. (2010) reported the maximum plant biomass concentration and PT and 6-MPT contents of 28.4 g/L, 20.1, and 2.3 mg/g DCW, respectively, when live cells of *P. indica* were added at a concentration of 1 g/L for an exposure time of 24 h.

7.3.2 *Effect of Culture Filtrate, Cell Extract, and Live Cells of* P. indica *on PAL Activity*

Phenylalanine ammonia lyase (PAL) catalyzes the first step of phenylpropanoid pathway which is deamination of phenylalanine to cinnamic acid. This is the rate-limiting step of lignan biosynthesis and also acts as the bridge between primary metabolism and natural product biosynthesis. *Lignan accumulation and PAL enzyme activity were found to be directly associated as maximum lignan*

accumulation (PT + 6-MPT) coincided with maximum increase of PAL enzyme activity. PAL activity was maximally enhanced by 3.1 times (371.4 μkat/kg protein) corresponding to 3.5-fold increase in lignan content (20.1 mg/g) upon addition of filter-sterilized culture filtrate from decline phase of P. indica *at a level of 3 % (v/v) to* L. album *hairy root cultures for an exposure time of 48 h* (Table 7.4). An increase of 2.1-fold in PAL activity (237.3 μkat/kg protein) was achieved on addition of autoclaved culture filtrate under similar conditions (Table 7.2) (Kumar et al. 2011a). Similarly in case of cell extract of *P. indica*, a maximum PAL activity of 232.2 μkat/kg protein, 2.3 times higher than the control, was found on addition of cell extract at a level of 1 % (v/v) on the 12th day of *L. album* growth (Table 7.5), which also resulted in maximum increase in lignan content. In case of interaction of live fungal and plant cells during co-culture studies, the maximum increment in PAL activity (224.3 μkat/kg) and protein also coincided with highest total lignan accumulation (Table 7.6).

A correlation between changes in the levels of phenylpropanoid biosynthetic enzymes and product accumulation has been demonstrated in other plant system as well. For example, a quantitative relationship between PAL levels and phenylpropanoid accumulation was established in transgenic tobacco (Nicholas et al. 1994). A similar effect was also observed in date palm roots, carrot cell suspension cultures, *Rubus fruticosus* cell suspension cultures, and *Taxus chinensis* on treatments with elicitor preparation from *Fusarium* spp. (Marinelli et al. 1990; Modafar et al. 2001; Yu et al. 2001; Yuan et al. 2001, 2002b; Nita-Lazar et al. 2004).

7.3.3 *Effect of Culture Filtrate, Cell Extract, and Live Cells of* P. indica *on Growth of* L. album *Hairy Root Cultures*

7.3.3.1 Effect of Culture Filtrate and Cell Extract

Although the addition of culture filtrate, both autoclaved and filter sterilized, resulted in growth-promoting effect on hairy roots in suspension cultures, the increase in hairy root dry cell weight was more significant in case of filter-sterilized culture filtrate. The maximum increase of 1.4-fold (21.8 g/L) in plant cell growth was achieved upon addition of filter-sterilized culture filtrate from decline phase of *P. indica* at a concentration of 2 % (v/v) for an exposure time of 96 h (Table 7.3). In case of autoclaved culture filtrate, the maximum enhancement of 1.2 times in plant cell growth (18.6 g/L) was obtained under similar conditions (Table 7.1) (Kumar et al. 2011a). In case of cell extract of *P. indica*, the maximum increase in hairy root DCW (18.1 g/L) was obtained on addition of cell extract on the 12th day of *L. album* growth at a level of 1 % v/v (Table 7.5). Therefore, it can be hypothesized that growth-promoting effect of *P. indica* might be due to some of the extracellular metabolites released in the medium during growth of fungal cells or could be due to

the presence of growth-stimulating factors present in cell extract of *P. indica*. Exposure time and concentration of culture filtrate to plant cells greatly affected this growth-promoting effect. An exposure time of 96 h and concentration of 2 % (v/v) were found to be optimum. Addition of culture filtrate for a shorter period, i.e., 48 h, resulted in comparatively lesser increment in growth of hairy roots. This might be due to insufficient time available for growth-promoting compounds to execute their effect.

P. indica has shown growth-promoting and yield enhancement activities on a variety of field-grown host plants (Sahay and Varma 1999, 2000; Rai et al. 2001; Singh et al. 2002, 2003; Kumari et al. 2004; Rai and Varma 2005; Chauhan et al. 2006; Oelmüller et al. 2009). In addition, its culture filtrate has also shown growth-promoting effects in field-grown plants (Varma et al. 1999, 2001).

7.3.3.2 Effect of Co-cultivation

Co-culture of live fungal cells with hairy roots lowered the growth of hairy roots (Table 7.6). This might be attributed to induction of strong hypersensitive reactions due to interaction between live fungal and plant cells. The reduction in growth was more significant upon increasing the concentration of fungal cells and duration of the co-culture. Although co-culture of *P. indica* with suspension culture of *L. album* had resulted in growth-promoting effect (Baldi et al. 2008b, 2010), the detrimental effect with hairy roots might be due to organized structure of hairy roots which may result in lesser interaction with fungal cells. Furthermore, the decrease in growth could also be due to competition of the hairy roots with fungal cells for the nutrients. This probably led to increased unavailability of nutrients for the hairy roots due to faster uptake of nutrients by the fungal cells; the reduction in hairy root growth was more severe with longer duration of exposure and higher concentration of the fungus.

The degree of enhancement was strongly dependent on the concentration of culture filtrate, cell extract, or live cells of *P. indica* and the time of their addition. The optimal time of addition of an elicitor depends on the right combination of the phase of the growth cycle during which it is added and the exposure time to result in maximal production of the desired metabolite, as the growth stage may influence not only the response to the elicitor treatment but also the pattern of production (Eilert 1987). *Addition of culture filtrate, cell extract, and live cells of* P. indica *resulted in an increase of the lignan (PT and 6-MPT) contents up to a concentration of 3 %, 1 %, and 2 % (v/v), respectively, after which the lignans decreased significantly on increasing the concentration of the elicitors*. An elicitor dose lower than the optimum suggests that the elicitor binding sites in cells were still not fully occupied for activating the biosynthesis of the metabolites, whereas an excessive dose caused a deleterious effect on the biosynthetic capacity of the cells. The deleterious effect of high concentrations of elicitors on biosynthesis of secondary metabolites has been observed in jasmonic acid-induced indole alkaloid biosynthesis (Rijhwani and Shanks 1998) and in methyl jasmonate-induced taxol

biosynthesis (Ketchum et al. 1999). Similar phenomenon has also been reported for ajmalicine accumulation in *Catharanthus roseus* suspension cultures when exposed to different concentrations of fungal elicitor extracts of *Trichoderma viride*, *Aspergillus niger*, and *Fusarium moniliforme* (Namdeo et al. 2002). High dosage of elicitor has been reported to induce hypersensitive response leading to cell death. Therefore, an optimum level was required for induction of secondary metabolite biosynthesis in plant cell cultures (Mukundan and Hjortsø 1990; Roewer et al. 1992). These results also indicate the importance of elicitor specificity in its selection for optimum induction of desired phytochemicals in plant cell cultures.

In addition to the concentration of an elicitor, the time of addition and hence the exposure time of elicitor to plant cells are also an important factor in maximizing its elicitation potential (Ketchum et al. 1999; Wang and Zhong 2002; Tabata 2004). An elicitor requires minimum time to execute its effect, but at the same time the longer incubation of elicitor may have deleterious effects on production. *The optimum time of the 12th day (an exposure time of 48 h) for culture filtrate, cell extract, and co-culture in present studies also confirmed this.* Longer exposure time may result in conversion or degradation of induced compounds. Therefore, a shorter exposure time is generally required to elicit phytochemical production in plant cell cultures. The decrease in lignan content beyond an exposure time of 48 h may be due to the conversion or degradation of PT to some other compounds. Such phenomenon was observed when the cells of *C. roseus* were exposed to fungal elicitor extracts for various time intervals. A three-fold higher ajmalicine production by *C. roseus* cells was elicited with extracts of *T. viride* and about two-fold increase in ajmalicine by the cells elicited with *A. niger* and *F. moniliforme* was also observed for an exposure time of 48 h. However, further increase of exposure time resulted in decrease of ajmalicine content (Namdeo et al. 2002). This pattern has also been reported by Rijhwani and Shanks (1998) for hairy root cultures of *C. roseus*, by Moreno et al. (1993) for cell suspension cultures of *C. roseus*, and by Negeral and Javelle (1995) for cell suspension cultures of tobacco.

7.4 Conclusions

The results of the present study clearly show that the addition of culture filtrate and cell extract of *P. indica* can improve the growth as well as lignan production in hairy roots of *L. album*. Among the various types of elicitors used, the maximum lignan (PT and 6-MPT) accumulation in *L. album* hairy root cultures was obtained with filter-sterilized culture filtrate of *P. indica*. The enhancement in lignan biosynthesis was found to be directly related to phenylalanine ammonia lyase activity. Although co-cultivation of live *P. indica* with *L. album* hairy roots caused a decrease in growth of *L. album*, the enhancement of lignan accumulation was substantial, leading to a significant net improvement in its production. *P. indica* seems to be promising for its applications to other plant cell tissue cultures to overproduce valuable secondary metabolites.

References

Aird ELH, Hamill JD, Robins RJ, Rhodes MJC (1988) Cytogenic analysis of hairy root cultures from a number of plant species transformed by *Agrobacterium rhizogenes*. Plant Cell Tiss Org Cult 15:47–57

Bais HP, Govindaswamy S, Ravishankar GA (2000) Enhancement of growth and coumarin production in hairy root cultures of Witloof chicory (*Cichorium intybus* L. cv. Lucknow local) under the influence of fungal elicitors. J Biosci Bioeng 90:648–653

Baldi A, Bisaria VS, Srivastava AK (2007) Biotechnological approaches for production of some plant based chemotherapeutics. In: Kayser O, Quax WJ (eds) Medicinal plant biotechnology—from basic research to industrial applications. Wiley-VCH Verlag, Weinheim, Germany, pp 117–156

Baldi A (2008) Production of anticancer drug podophyllotoxin by plant cell cultivation of *Linum album*. PhD thesis, Indian Institute of Technology Delhi, India

Baldi A, Bisaria VS, Srivastava AK (2008a) Improved podophyllotoxin production by transformed culture of *Linum album*. Biotechnol J 3:1256–1263

Baldi A, Jain A, Gupta N, Srivastava AK, Bisaria VS (2008b) Co-culture of mycorrhiza-like fungi (*Piriformospora indica* and *Sebacina vermifera*) with plant cells of *Linum album* for enhanced production of podophyllotoxins: a first report. Biotechnol Lett 30:1671–1677

Baldi A, Farkya S, Jain A, Gupta N, Mehra R, Datta V, Srivastava AK, Bisaria VS (2010) Enhanced production of podophyllotoxins by co-culture of transformed *Linum album* cells with plant growth-promoting fungi. Pure Appl Chem 82:227–241

Banerjee S, Zehra M, Kukreja AK, Kumar S (1995) Hairy roots in medicinal plants. Curr Res Med Arom Plants 17:348–378

Bennet M, Gallagher M, Fagg J, Bestwick C, Paul T, Beale M, Mansfield J (1996) The hypersensitive reaction, membrane damage and accumulation of autofluorescent phenolics on *Lettuu* cells challenged by *Bremia lactucae*. Plant J 9:851–885

Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux M, Jacquin-Dubreuil A (2000) Tropane alkaloid production by hairy roots of *Atropa belladonna* obtained after transformation with *Agrobacterium rhizogenes* 15834 and *Agrobacterium tumefaciens* containing *rol A, B, C* genes only. J Biotechnol 81:151–158

Bonjoch NP, Tamayo PR (2001) Protein content quantification by Bradford method. In: Reigosa RMJ (ed) Handbook of plant ecophysiology techniques. Kluwer Academic Publishers, Netherlands, pp 283–295

Chauhan AK, Das A, Kharkwal AC, Varma A (2006) Impact of micro-organism on environment and health. In: Chauhan AK, Varma A (eds) Microbiology series-microbes: health and environment. IK International Publishing House Pvt Ltd, New Delhi

Chattopadhyaya S, Srivastava AK, Bisaria VS (2004) Production of phytochemicals in plant cell bioreactors. In: Srivastava PS, Narula A, Srivastava S (eds) Plant biotechnology and molecular markers. Anamaya Publishers, New Delhi, pp 117–128

de Ascensao ARFDC, Dubery IA (2003) Soluble and wall-bound phenolics and phenolics polymers in *Musa acuminate* roots exposed to elicitors from *Fusarium oxysporum* f. sp. *Cubense*. Phytochemistry 63:679–686

Eilert U (1987) Elicitation: methodology and aspects of application. In: Constabel F, Vasil I (eds) Cell culture and somatic cell genetics of plants, vol 4. Academic, San Diego, CA, pp 153–196

Farkya S, Bisaria VS (2008) Exogenous hormones affecting morphology and biosynthetic potential of hairy root line (LYR2i) of *Linum album*. J Biosci Bioeng 102:140–146

Flores HE, Vivanco JM, Loyola-Vargas VM (1999) Radical biochemistry: the biology of root-specific metabolism. Trend Plant Sci 4:220–226

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Ge X, Wu J (2005) Induction and potentiation of diterpenoid tanshinone accumulation in *Salvia miltiorrhiza* hairy roots by β-aminobutyric acid. Appl Microbiol Biotechnol 68:183–188

Georgiev MI, Pavlov AI, Bley T (2007) Hairy root type plant in vitro systems as sources of bioactive substances. Appl Microbiol Biotechnol 74:1175–1185

Giri A, Ravindra ST, Dhingra V, Narasu ML (2000) Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua*. Curr Sci 81:378–382

Jacob A, Malpathak N (2004) Green hairy root cultures of *Solanum khasianum* Clarke-a new route to *in vitro* solasodine production. Curr Sci 87:1442–1447

Ketchum REB, Gibson DM, Croteau RB, Shuler ML (1999) The kinetics of taxoid accumulation in cell suspension culture of taxus following elicitation with methyl jasmonate. Biotechnol Bioeng 62:97–105

Kumar V, Rajauria G, Sahai V, Bisaria VS (2011a) Culture filtrate of root endophytic fungus *Piriformospora indica* promotes the growth and lignan production of *Linum album* hairy root cultures. Process Biochem 47:901–907

Kumar V, Sahai V, Bisaria VS (2011b) High-density spore production of *Piriformospora indica*, a plant growth promoting endophyte, by optimization of nutritional and cultural parameters. Bioresour Technol 102:3169–3175

Kumari R, Pham GH, Sachdev M, Garg AP, Varma A (2004) Symbiotic fungi for eco-friendly environment: a perspective. Nat Prod Rad 3:396–400

Marinelli F, Ronchi VN, Pini D, Salvadori P (1990) Induction of 6-methoxymellein and 6-hydroxymellein production in Carrot cells. Phytochemistry 29:849–851

Modafar CE, Tantaoui A, Saddik E, Boustani E (2001) Differential induction of phenylalanine ammonia-lyase activity in date palm roots in response to inoculation with *Fusarium oxysporum* f. sp. *albedinis* and to elicitation with fungal wall elicitor. J Plant Physiol 158:715–722

Moreno PRH, van der Heijden R, Verpoorte R (1993) Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 12:702–705

Mukundan U, Hjortsø MA (1990) Effect of fungal elicitor on thiophene production in hairy root cultures of *Tagetes patula*. Appl Microbiol Biotechnol 33:145–147

Namdeo A, Patil S, Fulzele DP (2002) Influence of fungal elicitors on production of ajmalicine by cell cultures of *Catharanthus roseus*. Biotechnol Prog 18:159–162

Negeral J, Javelle F (1995) Induction of phenylpropanoid and tyramine metabolism in pectinase- or pronase-elicited cell suspension cultures of tobacco. Physiol Plant 95:569–574

Nicholas JB, John OW, Meromit A, Hassar TN (1994) Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Plant Biol 91:7608–7612

Nita-Lazar M, Heyraud A, Gey C, Braccini I, Lienart Y (2004) Novel oligosaccharides isolated from *Fusarium oxysporum* L rapidly induce PAL activity in *Rubus* cells. Acta Biochem Pol 51:625–634

Oelmüller R, Sherameti I, Tripathi S, Varma A (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Oostdam A, Mol JNM, van der Plas LHW (1993) Establishment of hairy root cultures of *Linum flavum* producing the lignan 5- methoxypodophyllotoxin. Plant Cell Rep 12:474–477

Podila GK, Varma A (2004) Basic research and applications of mycorrhizae. Microbiology Series. IK International, India, New York

Qian Z-G, Zhao Z-J, Xu Y, Qian X, Zhong J-J (2006) Novel synthetic 2, 6 dichloro-isonicotinate derivatives as effective elicitors for inducing the biosynthesis of plant secondary metabolites. Appl Microbiol Biotechnol 71:164–167

Radman R, Saez S, Bucke C, Keshavarz T (2003) Elicitation of plants and microbial cell systems. Appl Biochem Biotech 37:91–102

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128

Rai M, Varma A (2005) Arbuscular mycorrhiza-like biotechnological potential of *Piriformospora indica*, which promotes the growth of *Adhatoda vasica* Nees. Elect J Biotechnol 8:107–112

Rao SR, Ravishankar GA (2002) Plant cell culture: chemical factories of secondary metabolites. Biotechnol Adv 20:101–153

Ravishankar GA, Ramachandra Rao S (2000) Biotechnological production of phyto-pharmaceuticals. J Biochem Mol Biol Biophys 4:73–102

Rijhwani S, Shanks JV (1998) Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol Prog 14:442–449

Roewer IA, Colutier N, Nessier CL, De luce V (1992) Transient induction of tryptophan decarboxylase and strictosidine synthase in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 11:86–89

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Sahay NS, Varma A (2000) Biological approach towards increasing the survival rates of micropropagated plants. Curr Sci 78:126–129

Schacter L (1996) Etoposide phosphate: what, why, where, and how? Semin Oncol 6:1–7

Singh AN, Singh AR, Varma A (2002) *Piriformospora indica*- in vitro raised leguminous plants: A new dimension in establishment and phyto-promotion. Ind J Biotechnol 1:372–376

Singh AN, Singh AR, Kumari M, Rai MK, Varma A (2003) Biotechnological importance of *Piriformospora indica*—A novel symbiotic mycorrhiza-like fungus: an overview. Ind J Biotechnol 2:65–75

Smollny T, Wichers HJ, Kalenberg S, Shahsavari A, Peterson AW (1998) Accumulation of podophyllotoxin and related lignans in cell suspension cultures of *Linum album*. Phytochemistry 48:975–979

Tabata H (2004) Paclitaxel production by plant cell culture technology. Adv Biochem Eng Biotechnol 87:1–23

Varma A, Verma S, Sudha, Sahay N, Bűtehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sudha, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: an axenically culturable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) Mycota IX. Springer, Berlin, Heidelberg, New York, pp 123–150

Wang C, Wu J, Mei X (2001) Enhancement of taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. Appl Microbiol Biotechnol 55:404–410

Wang W, Zhong JJ (2002) Manipulation of ginsenoside heterogeneity in cell culture of *Panax notoginseng* by addition of jasmonates. J Biosci Bioeng 93:48–53

Wu J-Y, Ng J, Shi M, Wu S-J (2007) Enhanced secondary metabolite (tanshinone) production of *Salvia miltiorrhiza* hairy roots in a novel root–bacteria coculture process. Appl Microbiol Biotechnol 77:543–550

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in the phosphate transport to the host plant. J Biol Chem 285:26532–26544

Yu LJ, Lan WZ, Qin WM, Xu HB (2001) Effects of salicylic acid on fungal elicitor-induced membrane-lipid peroxidation and taxol production in cell suspension cultures of *Taxus chinensis*. Process Biochem 37:477–482

Yuan YJ, Wei ZJ, Wu ZL, Wu JC (2001) Improved taxol production in suspension cultures of *Taxus chinensis* var. *mairei* by *in situ* extraction combined with precursor feeding and additional carbon source introduction in an airlift loop reactor. Biotechnol Lett 23:1659–1662

Yuan YJ, Li C, Hu ZD, Wu JC (2002a) A double oxidative burst for taxol production in suspension culture of *Taxus chinensis var. mairei* induced by oligosaccharide from *Fusarium oxysprum*. Enzyme Microb Technol 30:774–778

Yuan YJ, Li C, Hu ZD, Wu JC, Zeng AP (2002b) Fungal elicitor-induced cell apoptosis in suspension cultures of *Taxus chinensis* var. mairei for taxol production. Process Biochem 38:193–198

Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun X, Tang K (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci (USA) 101:6786–6791

Zhao J, Qiu H, Guo YQ, Zhu WH (2001) Elicitor-induced indole alkaloid biosynthesis in *Catharanthus roseus* cell culture is related to Ca^{2+} influx and the oxidative burst. Plant Sci 161:423–431

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333

Zuccaro A, Basiewicz M, Zurawska M, Biedenkopf D, Kogel K-H (2009) Karyotype analysis, genome organization, and stable genetic transformation of the root colonizing fungus *Piriformospora indica*. Fungal Genet Biol 46:543–550

Chapter 8
Piriformospora indica Promotes Growth of Chinese Cabbage by Manipulating Auxin Homeostasis: Role of Auxin in *P. indica* Symbioses

Joy Michal Johnson, Yin-Chen Lee, Iris Camehl, Chao Sun, Kai-Wun Yeh, and Ralf Oelmüller

8.1 Introduction: Auxin in Plant–Microbe Interactions

Auxin is the classical phytohormone involved in the development of new organs or promotion of growth. Nodule formation in response to infection with rhizobacteria (Perrine-Walker et al. 2010), the clubroot disease (Ando et al. 2008; Ishikawa et al. 2007; Devos and Prinsen 2006; Devos et al. 2005; Schuller and Ludwig-Müller 2006), or the transformation of root cells into galls/knots by endoparasitic nematodes (Lee et al. 2011a; Grunewald et al. 2009a, b; Wasson et al. 2009) provides examples for microbe-induced developmental reprogramming, in which auxin is involved. Mycorrhizal fungi (Fiorilli et al. 2009; Luo et al. 2009; Ludwig-Müller and Güther 2007; Amiour et al. 2006; Reboutier et al. 2002), plant-growth-promoting rhizobacteria (Contesto et al. 2010; Costacurta and Vanderleyden 1995), or beneficial root-colonizing endophytic fungi (Lee et al. 2011b; Contreras-Cornejo et al. 2009; Felten et al. 2009; Schäfer et al. 2009a, b; Vadassery et al. 2008; Sirrenberg et al. 2007) interfere with auxin metabolism or auxin functions in the plants. Phytohormones including auxins are also involved in plant defense and disease resistance (Kazan and Manners 2009; Bari and Jones 2009; Robert-Seilaniantz et al. 2007). Several microbes produce auxin and release it into the environment or directly into the root, thereby activating auxin signal transduction pathways in the plant cell (cf. Perrine-Walker et al. 2010; Spaepen et al. 2007; Reboutier et al. 2002). However, microbe-induced morphological changes in the roots cannot be caused exclusively by auxin released by the microbe, since

J.M. Johnson • I. Camehl • C. Sun • R. Oelmüller (✉)
Friedrich-Schiller-Universität Jena, Institute of General Botany and Plant Physiology, Dornburger Str.159, 07743 Jena, Germany
e-mail: joymkau@gmail.com; iris.camehl@gmx.de; csun@ice.mpg.de; b7oera@hotmail.de

Y.-C. Lee • K.-W. Yeh
Institute of Plant Biology, College of Life Science, National Taiwan University, Taipei 106, Taiwan
e-mail: ykwbppp@ntu.edu.tw

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_8,

exogenously applied auxin cannot replace the microbes (cf. Lee et al. 2011b; Felten et al. 2010). The complex developmental programs induced by the microbes require a highly coordinated plant response for which local changes in auxin homeostasis may be initiated by microbial signals.

We study the symbiotic interaction of an endophytic fungus *Piriformospora indica* with different plant species. The ubiquitous fungus promotes growth of all plant species which have been tested so far (Weiß et al. 2011), including the model plant *Arabidopsis thaliana* (Vadassery et al. 2008; 2009) and the agriculturally important crop Chinese cabbage (Sun et al. 2010, Lee et al. 2011b). Growth promotion of these two plant species by *P. indica* involves auxin; however, the role of this phytohormone in the two symbioses is quite different. The comparative analysis of these two symbioses allows interesting conclusions of how *P. indica* controls growth programs on the basis of different genetic backgrounds of individual plant species.

8.2 *P. indica* and Chinese Cabbage

Growth and development of shoots and roots of Chinese cabbage (*Brassica campestris* cv. Chinensis) seedlings is strongly promoted by *P. indica*, and the fresh weight of the seedlings increases approximately twofold (Lee et al. 2011b, Sun et al. 2010). The strong stimulation of root hair development resulted in a bushy root phenotype. Since the auxin level in the infected Chinese cabbage roots was twofold higher compared to the uncolonized controls, the growth-promoting effect in Chinese cabbage clearly involved auxin. We generated a double-subtractive expressed sequence tag library from Chinese cabbage roots grown in the presence or absence of the fungus and isolated genes/gene fragments which are upregulated by *P. indica* in the roots. Many of the identified cDNA fragments are related to auxin metabolism and function: genes for intercellular auxin transport carrier proteins such as AUX1 or PINs, for auxin perception and signal proteins, or for proteins related to cell wall acidification (Lee et al. 2011b). In an initial study, we expressed one of these genes, *BcAUX1,* in *Arabidopsis* and demonstrated that the transgenic lines show a strong promotion in growth and biomass production. This confirms that *BcAUX1* is a target of *P. indica* in Chinese cabbage. However, as expected, exogenous application of auxin could not replace *P. indica*. Thus, several questions arise: (a) What is the origin of auxin in this symbiosis? (b) How does the fungus manipulate the auxin homeostasis in Chinese cabbage roots to induce the growth response? (c) Since we did not observe such a dominant role of auxin in the *P. indica*/*Arabidopsis* symbiosis (Vadassery et al. 2008), what is the difference between the two hosts? (d) Since colonized roots of both plant species are bigger and contain longer roots and root hairs, is the beneficial effect of the plant simply caused by better excess of larger roots to nutrients in the medium/soil?

8.3 Origin of Auxin

P. indica-colonized Chinese cabbage roots contain approximately twice as much auxin as the uncolonized control roots, while the auxin level in the leaves is not affected by the fungus (Lee et al. 2011b). Several lines of evidence indicate that the higher auxin level in the roots is not of fungal origin. First, the bushy root hair phenotype and the longer primary and secondary roots could not be obtained when auxin was applied exogenously to the seedlings. Second, growth promotion of Chinese cabbage roots was also obtained by a preparation of a cell wall-derived fraction from the fungus, which does not contain auxin. This indicates that the fungus activates growth-promoting programs in the roots, which include auxin biosynthesis of plant origin. Third, we have previously demonstrated that *P. indica*-colonized *Arabidopsis thaliana* roots do not contain elevated auxin levels, although their growth is promoted by the fungus. We grew seedlings of both species together with the fungus in the same Petri dish and could confirm that only the roots of Chinese cabbage seedlings contain more auxin (Lee et al. 2011b). Based on these observations, we conclude that the higher auxin level in colonized Chinese cabbage roots is most likely of plant origin. Apparently, *P. indica*-induced growth promotion of Chinese cabbage seedlings is caused by fungal interference with the plant auxin biosynthesis and homeostasis. The difference in the regulation in Chinese cabbage to the regulation in *Arabidopsis* roots must be caused by differences in developmental programs of the two species: *P. indica*-induced growth promotion in *Arabidopsis* is not or less dependent on an overall increase in the auxin level in the root (cf. below).

8.4 Auxin Targets in Chinese Cabbage Roots

The identified auxin-related genes, which are upregulated in colonized Chinese cabbage roots (Lee et al. 2011b), allow conclusions of how *P. indica* induces growth in Chinese cabbage (Fig. 8.1). Upregulation of *TIR1*, the gene for the auxin receptor, is consistent with the idea that the roots are more sensitive to the phytohormone. Stimulation of auxin-induced genes requires the activation of the Aux/IAA degradation machinery, and many components involved in this process have been identified in the differential display analysis (e.g., UBC10, 26S proteasome subunit 4; Lee et al. 2011b). Furthermore, upregulation of the *AUX1* and *BIG* mRNA levels (2 genes for auxin transporters) implies that the fungal interaction results in a more efficient influx of auxin into the root cell. The *PIN3* mRNA level for an auxin efflux regulator is downregulated by the fungus, suggesting that auxin release from the cell is reduced. Auxin responses during plant/microbe interactions are dynamic; therefore, root-associated microbes can actively modify the host's auxin transport (cf. Grunewald et al. 2009a, b). Finally, several isolated cDNA fragments code for components involved in cell wall

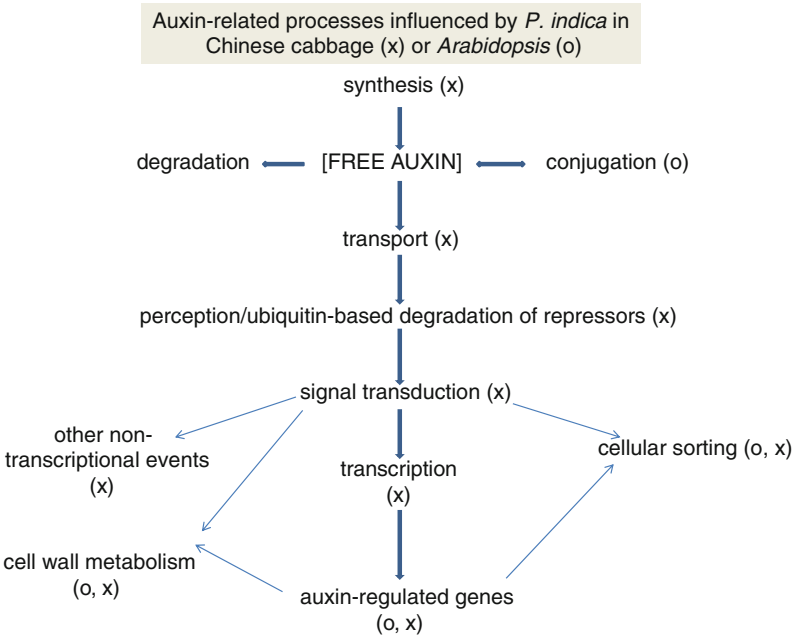

Fig. 8.1 Auxin targets of *P. indica*

acidification. This requires the stimulation of the export machinery for cell wall loosening proteins into the apoplast through the sorting processes in the endoplasmic reticulum. Exocytosis-related proteins in the endoplasmic reticulum are important targets of the fungus in *Arabidopsis* roots (Peškan-Berghöfer et al. 2004). Currently we are characterizing four *P. indica*-responsive genes in Chinese cabbage, which control exocytosis of proteins during endoplasmic reticulum/Golgi passage. Another complex aspect during auxin-controlled growth regulation is connected to the cell volume, which increases by promoting water and ion uptake and by rearranging the cytoskeleton. That both processes are targeted by *P. indica* is supported by the identified cDNA fragments in the screen (cf. Lee et al. 2011b, and unpublished data). In total, the differential expression of these auxin-related genes demonstrates that this phytohormone plays a crucial role in *P. indica*-mediated growth promotion and alteration of root morphology in Chinese cabbage. The bushy root hair phenotype of *P. indica*-colonized Chinese cabbage roots is important for improving the acquisition to water and minerals.

The identified auxin-related target genes/proteins of *P. indica* are upregulated locally, in an organ- or even cell-specific manner. It is important to understand how the fungus can manipulate auxin homeostasis and regulate auxin maxima in a controlled manner. The Chinese cabbage/*P. indica* symbiosis provides us a tool to study this scenario, to identify the underlying mechanism, and to use these tools to manipulate plants for biotechnological applications.

8.5 An Excursion to the *P. indica* Symbiosis with *Arabidopsis* Roots

Large-scale microarray analysis of *Arabidopsis* roots, colonized by *P. indica*, did not reveal many auxin-related genes as a target of the fungus. Expression of *DR5* promoter::ß-glucuronidase gene fusions, which are upregulated by auxin, is not significantly affected by the fungus (Vadassery et al. 2008). Mutants with reduced auxin levels (*ilr1-1*, *nit1-3*, *tfl2*, *cyp79 b2b3*) responded to *P. indica*, which indicated that severe alterations in auxin homeostasis in *Arabidopsis* do not prevent the growth response to *P. indica* (Vadassery et al. 2008). Auxin-related genes which are upregulated in *P. indica*-colonized Chinese cabbage roots are not upregulated in *P. indica*-colonized *Arabidopsis* roots, although growth of both species is promoted by the fungus. However, we obtained clear evidence of the role of auxin in the *P. indica*/*Arabidopsis* symbiosis, by analyzing the *sur1-1* mutant.

sur1-1 lacks a P450-dependent monooxygenase, which catalyzes the N-oxidation of indole-3-acetaldoxime and directs the pathway to indole glucosinolates. The mutant accumulates indole-3-acetaldoxime that is converted to auxin. Thus, *sur1-1* can be considered as an auxin overproducer (Boerjan et al. 1995; Mikkelsen et al. 2004); the seedlings are much smaller than the wild type, and their root length is approximately half the length of the wild type. Growth of *sur1-1* seedlings is strongly stimulated by *P. indica*, and colonized *sur1-1* seedlings are almost as big as wild-type seedlings cocultivated with the fungus. The free auxin level in *sur1-1* roots is reduced in *P. indica*-colonized seedlings, while the conjugated auxin level increases (Vadassery et al. 2008). The complete rescue of the dwarf phenotype of colonized *sur1* seedlings indicates that the fungus interferes with the auxin homeostasis by converting excess auxin into inactive conjugates. Thus, control of local-free auxin levels may be an important target of the fungus in *Arabidopsis*.

8.6 *P. indica*-Mediated Growth Promotion is Independent of the Root Architecture in *Arabidopsis*

Larger roots with numerous secondary roots and root hairs allow better access to water and nutrients in the medium/soil. In nature, it is likely that this contributes substantially to plant performance. However, the question arises whether this is the only reason for better performance of *P. indica*-colonized plants. Since the root architecture is strongly influenced by auxin and cytokinin, we analyzed the response of *Arabidopsis* mutants to *P. indica* with altered root architectures or root/shoot biomass ratios. Interestingly, we noted that the growth response to *P. indica* is not related of the architecture and size of the roots in *Arabidopsis*. For instance, the *ahk2 ahk3* seedlings have long roots relative to the aerial parts (Riefler et al. 2006), the *35S::CKX1* and *35S::CKX2* lines have stunted and bushy

roots (Werner et al. 2003), and the *sur1-1* roots are approximately half as long as the wild type. *tfl2*, in addition to its dwarf phenotype, has fewer lateral roots and shorter root hairs and a greatly reduced root surface (Bennett et al. 2005). All of these mutants respond to *P. indica* suggesting that the root size and architecture have little effect on the growth response induced by the fungus. Furthermore, root hair development is controlled by ROOT HAIR DEFECTIVE2 (RHD2), a plasma membrane localized NADP oxidase (Foreman et al. 2003). *rhd2* mutants have shorter root hairs, but their response to *P. indica* was comparable to the wild type. The *incomplete root hair elongation* (*ire*) mutant is defective in an AGC kinase and exhibits also a short root hair phenotype (Oyama et al. 2002); however, its response to the fungus is also comparable to the wild type (Camehl et al. 2011). Thus, also *Arabidopsis* mutants in which the root architecture is altered by lesions not directly related to phytohormones respond to the *P. indica*. Consequently, better performance of plants in the presence of the fungus is not exclusively caused by the stimulation of root growth.

8.7 Conclusions

We introduced Chinese cabbage as a novel host for *P. indica*. This agriculturally important crop shows a quite different developmental strategy in response to *P. indica* when compared to the model plant *Arabidopsis*. The breeding strategies for Chinese cabbage over the last 200 years are based on the generation of varieties with increased biomass. *Arabidopsis* plants were not exposed to such a selective pressure. Since growth stimulation is most efficiently mediated by manipulating auxin homeostasis, it is reasonable that this phytohormone plays a more dominant role in the symbiosis with Chinese cabbage than in *Arabidopsis*. A comparative analysis of the two symbiotic systems will help to understand how *P. indica* manipulates plant growth programs.

References

Amiour N, Recorbet G, Robert F, Gianinazzi S, Dumas-Gaudot E (2006) Mutations in DMI3 and SUNN modify the appressorium-responsive root proteome in arbuscular mycorrhiza. Mol Plant Microbe Interact 19:988–997

Ando S, Tsushima S, Kamachi S, Konagaya K, Tabei Y (2008) Alternative transcription initiation of the nitrilase gene (BrNIT2) caused by infection with *Plasmodiophora brassicae* Woron. in Chinese cabbage (*Brassica rapa* L.). Plant Mol Biol 68:557–569

Bari R, Jones JD (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69:473–488

Bennett RN, Wenke T, Freudenberg B, Mellon FA, Ludwig-Müller J (2005) The *tu8* mutation of *Arabidopsis thaliana* encoding a heterochromatin protein 1 homolog causes defects in the induction of secondary metabolite biosynthesis. Plant Biol 4:348–357

Boerjan W, Cervera MT, Delarue M, Beeckman T, Dewitte W, Bellini C, Caboche M, Onckelen HV, Montagú MV, Inzé D (1995) Superroot, a recessive mutation in *Arabidopsis,* confers auxin overproduction. Plant Cell 7:1405–1419

Camehl I, Drzewiecki C, Vadassery J, Shahollari B, Sherameti I, Forzani C, Munnik T, Hirt H, Oelmüller R (2011) The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in *Arabidopsis*. PLoS Pathog 7:e1002051

Contesto C, Milesi S, Mantelin S, Zancarini A, Desbrosses G, Varoquaux F, Bellini C, Kowalczyk M, Touraine B (2010) The auxin-signaling pathway is required for the lateral root response of *Arabidopsis* to the rhizobacterium *Phyllobacterium brassicacearum*. Planta 232:1455–1470

Contreras-Cornejo HA, Macías-Rodríguez L, Cortés-Penagos C, López-Bucio J (2009) *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. Plant Physiol 149:1579–1592

Costacurta A, Vanderleyden J (1995) Synthesis of phytohormones by plant-associated bacteria. Crit Rev Microbiol 21:1–18

Devos S, Prinsen E (2006) Plant hormones: a key in clubroot development. Commun Agric Appl Biol Sci 71:869–872

Devos S, Vissenberg K, Verbelen JP, Prinsen E (2005) Infection of Chinese cabbage by *Plasmodiophora brassicae* leads to a stimulation of plant growth: impacts on cell wall metabolism and hormone balance. New Phytol 166:241–250

Felten J, Kohler A, Morin E, Bhalerao RP, Palme K, Martin F, Ditengou FA, Legué V (2009) The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and *Arabidopsis* through auxin transport and signaling. Plant Physiol 151:1991–2005

Felten J, Legué V, Ditengou FA (2010) Lateral root stimulation in the early interaction between *Arabidopsis thaliana* and the ectomycorrhizal fungus *Laccaria bicolor*: Is fungal auxin the trigger? Plant Signal Behav 5:864–867

Fiorilli V, Catoni M, Miozzi L, Novero M, Accotto GP, Lanfranco L (2009) Global and cell-type gene expression profiles in tomato plants colonized by an arbuscular mycorrhizal fungus. New Phytol 184:975–987

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422:442–446

Grunewald W, Cannoot B, Friml J, Gheysen G (2009a) Parasitic nematodes modulate PIN-mediated auxin transport to facilitate infection. PLoS Pathog 5:e1000266

Grunewald W, van Noorden G, Van Isterdael G, Beeckman T, Gheysen G, Mathesius U (2009b) Manipulation of auxin transport in plant roots during Rhizobium symbiosis and nematode parasitism. Plant Cell 21:2553–2562

Ishikawa T, Okazaki K, Kuroda H, Itoh K, Mitsui T, Hori H (2007) Molecular cloning of *Brassica rapa* nitrilases and their expression during clubroot development. Mol Plant Pathol 8:623–637

Kazan K, Manners JM (2009) Linking development to defense: auxin in plant-pathogen interactions. Trends Plant Sci 14:373–382

Lee C, Chronis D, Kenning C, Peret B, Hewezi T, Davis EL, Baum TJ, Hussey R, Bennett M, Mitchum MG (2011a) The Novel Cyst Nematode Effector Protein 19C07 Interacts with the Arabidopsis Auxin Influx Transporter LAX3 to Control Feeding Site Development. Plant Physiol 155:866–880

Lee Y-C, Johnson J.M, Chien C-T, Sun C, Cai D, Lou B, Oelmüller R, Yeh K-W. (2011b) Growth promotion of Chinese cabbage and *Arabidopsis* by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin. Mol Plant-Microb Interact 24:421–431

Ludwig-Müller J, Güther M (2007) Auxins as signals in arbuscular mycorrhiza formation. Plant Signal Behav 2:194–196

Luo ZB, Janz D, Jiang X, Göbel C, Wildhagen H, Tan Y, Rennenberg H, Feussner I, Polle A (2009) Upgrading root physiology for stress tolerance by ectomycorrhizas: insights from

metabolite and transcriptional profiling into reprogramming for stress anticipation. Plant Physiol 151:1902–1917

Mikkelsen MD, Naur P, Halkier BA (2004) *Arabidopsis* mutants in the C-S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. Plant J 37:770–777

Oyama T, Shimura Y, Okada K (2002) The *IRE* gene encodes a protein kinase homologue and modulates root hair growth in *Arabidopsis*. Plant J 30:289–299

Perrine-Walker F, Doumas P, Lucas M, Vaissayre V, Beauchemin NJ, Band LR, Chopard J, Crabos A, Conejero G, Péret B, King JR, Verdeil JL, Hocher V, Franche C, Bennett MJ, Tisa LS, Laplaze L (2010) Auxin carriers localization drives auxin accumulation in plant cells infected by Frankia in *Casuarina glauca* actinorhizal nodules. Plant Physiol 154:1372–1380

Peškan-Berghöfer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V, Varma AK, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. Physiol Plant 122:465–477

Reboutier D, Bianchi M, Brault M, Roux C, Dauphin A, Rona JP, Legué V, Lapeyrie F, Bouteau F (2002) The indolic compound hypaphorine produced by ectomycorrhizal fungus interferes with auxin action and evokes early responses in nonhost *Arabidopsis thaliana*. Mol Plant Microbe Interact 15:932–938

Riefler M, Novak O, Strnad M, Schmülling T (2006) *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell 18:40–54

Robert-Seilaniantz A, Navarro L, Bari R, Jones JD (2007) Pathological hormone imbalances. Curr Opin Plant Biol 10:372–379

Schäfer P, Pfiffi S, Voll LM, Zajic D, Chandler PM, Waller F, Scholz U, Pons-Kühnemann J, Sonnewald S, Sonnewald U, Kogel KH (2009a) Phytohormones in plant root-*Piriformospora indica* mutualism. Plant Signal Behav 4:669–671

Schäfer P, Pfiffi S, Voll LM, Zajic D, Chandler PM, Waller F, Scholz U, Pons-Kühnemann J, Sonnewald S, Sonnewald U, Kogel KH (2009b) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. Plant J 59:461–474

Schuller A, Ludwig-Müller JA (2006) Family of auxin conjugate hydrolases from *Brassica rapa*: characterization and expression during clubroot disease. New Phytol 171:145–157

Sirrenberg A, Gobel C, Grond S, Czempinski N, Ratzinger A, Karlovski P, Santos P, Feussner I, Pawlowski K (2007) *Piriformospora indica* affects plant growth by auxin production. Physiol Plant 131:581–589

Spaepen S, Vanderleyden J, Remans R (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol Rev 31:425–448

Sun C, Johnson J.M, Cai D, Sherameti I, Oelmüller R, Lou B (2010) *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. J Plant Physiol 167:1009–1017

Vadassery J, Ranf S, Drzewiecki C, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R (2009) A cell wall extract from *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J 59:193–206

Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B, Novák O, Strnad M, Ludwig-Müller J, Oelmüller R (2008) *Piriformospora indica*-mediated growth promotion in *Arabidopsis* is sensitive to high auxin levels, requires trans-cytokinin biosynthesis and the cytokinin receptor combination CRE1/AHK2. Mol Plant Microbe Interact 21:1371–1383

Wasson AP, Ramsay K, Jones MG, Mathesius U (2009) Differing requirements for flavonoids during the formation of lateral roots, nodules and root knot nematode galls in *Medicago truncatula*. New Phytol 183:167–179

Weiß M, Sýkorová Z, Garnica S, Riess K, Martos F, Krause C, Oberwinkler F, Bauer R, Redecker D (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS One 6:e16793

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15:2532–2550

Chapter 9
Cocultivation of *Piriformospora indica* with Medicinal Plants: Case Studies

Aparajita Das, Ram Prasad, R.B. Srivastava, Shivaji Deshmukh, M.K. Rai, and Ajit Varma

9.1 Introduction

A thorough review of literature has shown that *P. indica* has enormous potential for growth promotion of diverse groups of plants by colonization of host roots (Singh et al. 2000; Rai et al. 2001; Malla et al. 2002, Rai and Varma 2005; Oelmuller et al. 2009; Achatz et al. 2010). The fungus is of utmost biotechnological importance and is multifunctional (Singh et al. 2003: Kumar et al. 2011). *P. indica* is similar to arbuscular mycorrhizal fungi in many respects (Varma and Schuepp 1994; Varma et al. 1999, 2001; Singh et al. 2000; Rai and Varma 2005). But, unlike arbuscular mycorrhizal fungi, it can be cultured on artificial medium (Varma et al. 1999, 2001; Pham et al. 2004). Thus, this revolutionary endophytic fungus functions as a plant growth promoter and biofertilizer in nutrient-depleted soils. *P. indica* promotes the growth of plants and improves their productivity, increases the drought tolerance, delays the wilting of leaves, prolongs the ageing of callus tissue, enhances the uptake of phosphate from the soil and relieves the stress conditions caused by acidity, desiccation and heavy metal toxicity (Oelmuller et al. 2009; Yadav et al. 2010; Camehl et al. 2010; Kumar et al. 2011). Plants treated with *P. indica* resulted in increase in overall growth, flowering, nutrient uptake and enhancement in secondary metabolites production in plants (Dolatabadi et al. 2011a, b; Das et al. 2012).

A. Das • R. Prasad • A. Varma (✉)
Amity Institute of Microbial Technology (AIMT), Amity University Uttar Pradesh (AUUP), Sec-125, Expressway, Noida, UP, India
e-mail: adas@amity.edu; rprasad@amity.edu; ajitvarma@amity.edu

R.B. Srivastava
Defence Institute of Higher Altitude Research, Defence Research Development Organisation, Leh-Ladakh, Jammu and Kashmir, India
e-mail: rbs1_cnb@rediffmail.com

S. Deshmukh • M.K. Rai
Department of Biotechnology, SGB Amravati University, Amravati 444 602, Maharashtra, India
e-mail: shivgajanan@rediffmail.com; mkrai123@rediffmail.com

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_9,

Pronounced growth promotional effect was recorded with terrestrial orchids also (Prasad et al. 2004). For more information readers may refer to Chap. 1.

Medicinal plants are of great importance because of their healing properties due to the presence of various active principles and different secondary metabolites (Rai 1994). A large number of remedies have been offered to mankind by the plant kingdom, of which many are provided by aromatic plants (Wyk and Wink 2004). Over three quarters of the world population rely mainly on medicinal plants and plant extracts for health care. More than 30 % of the entire plant species, at one time or other, was used for medicinal purposes. The rural folks and tribals in India as well as in other developing countries even now depend largely on the surrounding plants/forests for their day-to-day needs. Medicinal plants are being looked upon not only as a source of health care but also as a source of income. The value of medicinal plants related trade in India is of the order of 5.5 billion US dollar and is further increasing day by day. The international market of herbal products is estimated to be US $ 62 billion. India shares in the global market of medicinal plants trade is less than 0.5 % (http://www.agricultureinformation.com/forums/questions-answers/34618-cultivation-medicinal-plants-india-government-support.html, dated 17th April 2012). It has been estimated that in developed countries such as United States, plant drugs constitute as much as 25 % of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80 %. Thus, the economic importance of medicinal plants is much more to countries such as India than to rest of the world. These countries provide two-third of the plants used in modern system of medicine, and the health care system of rural population depends on indigenous systems of medicine (Chandel and Sharma 1997; Lambert 1998; Kavitha et al. 2010). Traditional Indian Ayurveda medicines have a 70 % share of the formal medicine market in India (Lambert 1998).

India is one of the world's 12 biodiversity centres with the presence of over 45,000 different plant species. India's diversity is unmatched due to the presence of 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces and 426 biomes (habitats of specific species). Of these, about 15,000–20,000 plants have remarkable medicinal value. However, only 7,000–7,500 species are used for their medicinal values by traditional communities (Kurian and Sankar 2007, http://megforest.gov.in dated 26th April 2012). There are about 45,000 medicinal plant species in India, with hot spots in the region of Eastern Himalayas, Western Ghats and Andaman and Nicobar Island. The officially documented plants with medicinal potential are 3,000, but traditional practitioners use more than 6,000. In rural India, 70 % of the population depends on the traditional type of medicine, the Ayurveda (http://medicinalpltsinindiamp, dated 17th April 2012). Hence, the medicinal and aromatic plants are of immense importance for welfare of the mankind. Taking into consideration, the above facts, it is the need of the hour to employ techniques which may enhance the growth and yield of economically important plants. In this context, arbuscular mycorrhizal fungi and other endophytes like *P. indica* play a crucial role (Oelmuller et al. 2009; Harman 2011).

It has been extensively studied that symbiotic fungus *P. indica* promoted growth and enhanced active ingredients of the medicinal as well as economically important

plants by forming association with their roots. This novel fungus has been also established as an agent for biological hardening of tissue culture-raised plantlets (Sahay and Varma 1999, 2000; Oelmuller et al. 2009; Gosal et al. 2010). So far, more than 148 plants have been interacted with the fungus and documented. This include plants such as *Centella asiatica*, *Coriandrum sativum*, *Artemisia annua*, *Spilanthes calva*, *Arabidopsis thaliana*, *Cajanus cajan*, *Arachis hypogaea*, *Mimosa pudica*, *Cicer arietinum*, *Allium cepa*, *Hordeum vulgare*, *Zea mays*, *Saccharum officinarum*, *Withania somnifera*, *Solanum lysopersicum*, etc. (Bagde et al. 2010). This chapter provides an overview of case studies of selected medicinal plants and their interaction with the wonder fungus *P. indica*.

9.2 Interaction of *P. indica* with Different Medicinal Plants

A large number of medicinal plants like *Spilanthes calva*, *Withania somnifera*, *Bacopa monnieri*, *Coleus forskohlii* and others were inoculated with the *P. indica* in pots as well as in fields to study its influence on the host plants. The symbiotic fungus promoted the overall growth and development of all the medicinal plants tested so far. It also enhanced percentage flowering and fruit development in medicinal plants like *Coleus forskohlii*, *Spilanthes calva* and *Withania somnifera*. It also promoted enhancement of secondary metabolites contents in medicinal plants inoculated with *P. indica*. Details of case studies are discussed from Sect. 9.2.1 to 9.2.13.

9.2.1 Interaction with Artemisia annua

Artemisia annua is also known by many names like sweet wormwood, sweet sagewort or annual wormwood. It is a common type of wormwood which is native to temperate Asia but now found throughout the world (Kapoor et al. 2007). The plant has traditionally been grown in China for medicine and more recently in Europe for its aromatic leaves which are used in flavouring beverages. It has fern-like leaves, bright yellow flowers and with camphor-like scent (Fig. 9.1a). Its height averages about 2 m tall, and the plant has a single stem, alternating branches and alternating leaves which range 2.5–5 cm in length. It secretes many medicinal compounds including "artemisinin" which is well antimalarial drug (Kapoor et al. 2007). *P. indica* showed growth promotion in *A. annua* when inoculated in seedlings stages under field condition (Fig. 9.1c,d; Tables 9.1a and 9.1b).

P. indica promoted the growth of the tissue culture-raised plantlets when interacted in vitro. It showed higher rate of shoot and root development (Fig. 9.2a, b). Similarly, it helped in biohardening of tissue culture-raised plantlets when transferred to the natural environmental conditions. Inoculated plants showed a higher survival rate than the uninoculated control (uninoculated) plants. It was also reported that interaction with fungus not only increased the biomass but also the Artemisinin (an antimalaria drug) content.

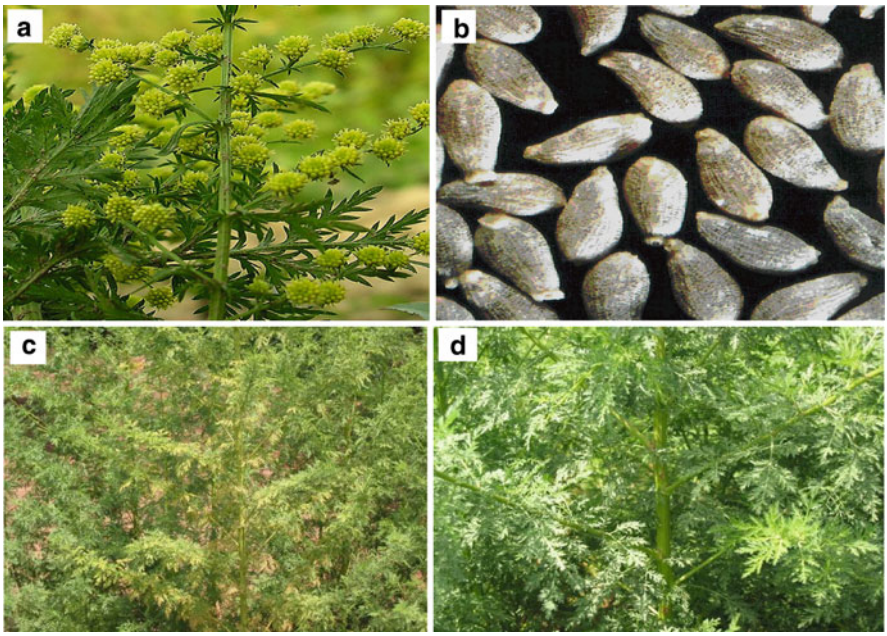

Fig. 9.1 Field grown plants of *Artemisia annua* (**a**) at flowering stage (**b**) Seeds; Five months old plants (**c**) Control plants without *P. indica* showing yellow-brown leaves, (**d**) Plants inoculated with *P. indica* showing dark green leaves

Table 9.1a Interaction of *Artemisia annua* seedlings with *P. indica* (field trial expt.)

Treatments	Height (in cm)	Mean
Control (Without *P. indica*)	152–165	158.5
Piriformospora indica	210–282	246.0

Data mean of 20 independent plants. Measured after 150 days. No chemical added. Irrigation by normal water

Table 9.1b Interaction of *Artemisia annua* seedling with *P. indica* (field trial expt.)

Treatment	Dry weight (in gm)/plant	Mean
Control (Without *P. indica*)	19–22	20.5
Piriformospora indica	64–82	73.0

Data mean of 20 independent plants. Measured after 150 days

9.2.2 Interaction with Tridax procumbens

Tridax procumbens Linn. belongs to family Asteraceae, found throughout India and is employed as indigenous medicine. It is reported for treatment of variety of ailments including jaundice (Kumari 2005; Bhagwat et al. 2008). It is commonly known as "Ghamra" and in English popularly called "coat buttons" because of the

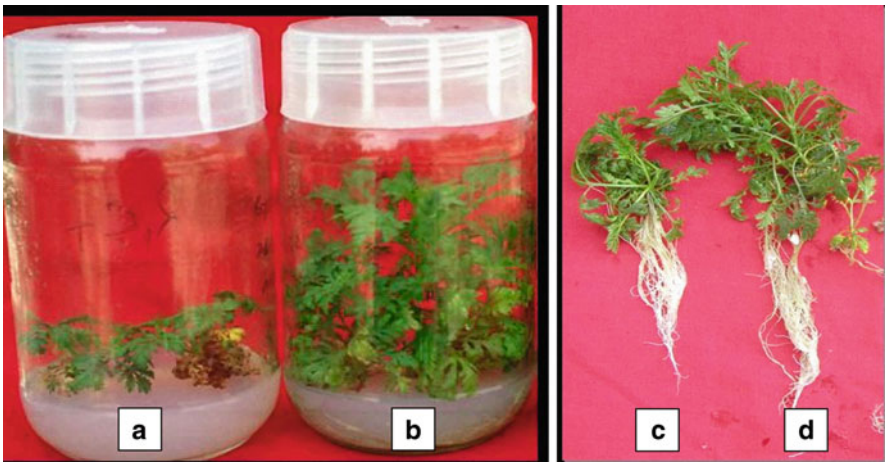

Fig. 9.2 Effect of *P. indica* on tissue culture-raised plantlets with high rate of shoots and root development. (**a**) Shoots without *P. indica*, (**b**) shoots with *P. indica*, (**c**) roots without *P. indica*, (**d**) roots with *P. indica*

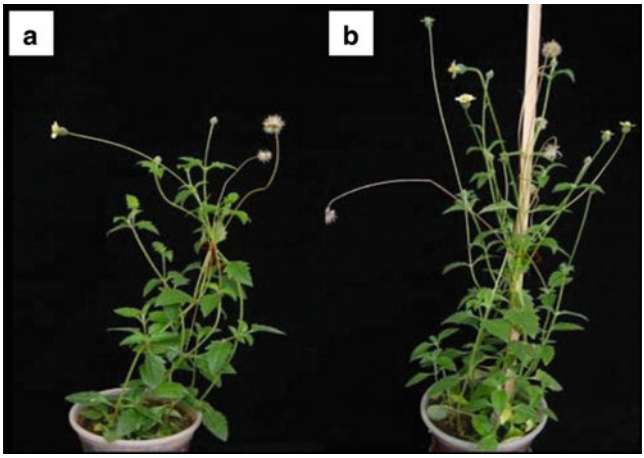

Fig. 9.3 Effect of *P. indica* on plant growth and biomass of *T. procumbens*. (**a**) Plants without fungus, (**b**) plants with fungus

appearance of its flowers. It has been extensively used in Indian traditional medicine as anticoagulant, antifungal and insect repellent, in bronchial catarrh, diarrhoea and dysentery. Moreover, it possesses wound healing activity and promotes hair growth. Antioxidant properties have also been demonstrated (Bhagwat et al. 2008). Interaction of *T. procumbens* with *P. indica* promoted the growth and biomass as depicted in Fig. 9.3 and Table 9.2 (Kumari 2005).

Table 9.2 Comparison of different growth parameters in control and *T. procumbens* plants inoculated with *P. indica*

Parameters	Control (without *P. indica*)	*P. indica* treated	Percent increase
Height	87.80 ± 1.58	108.44 ± 1.12	23
Shoot length	57.30 ± 0.99	73.04 ± 0.89	27
Root length	30.48 ± 0.89	35.44 ± 0.82	16
Fresh shoot weight	18.99 ± 0.35	23.55 ± 0.50	24
Dry shoot weight	06.80 ± 0.28	09.17 ± 0.29	35
Fresh root weight	06.87 ± 0.05	11.14 ± 0.46	62
Dry root weight	02.39 ± 0.27	04.65 ± 0.31	51
Percent colonization	nil	41	

Dead/sterile fungal biomass served as control. Height is given in centimetre, and weight is in gram. Each figure is an average of five independent replicates

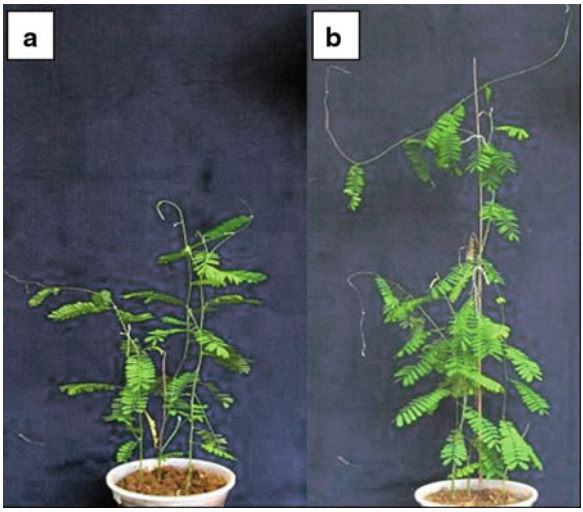

Fig. 9.4 Effect of fungi on plant growth and biomass of *A. precatorius* (**a**) without *P. indica*, (**b**) with *P. indica*

9.2.3 Interaction with Abrus precatorius

Abrus precatorius is a plant of the Fabaceae family. It is also known as Indian liquorice, jequirity, crab eye, *Glycyrrhiza glabra*, among others. The plant grows widely in fairly dry climates of tropical and subtropical regions, such as India, Sri Lanka, Nigeria and the West Indies. Plant pacifies vitiated "pitta", "vata", inflammation, vitiligo, skin disease, wounds, ulcer, alopecia, asthma, stomatitis and fever (Kumari 2005). The principal active ingredients present are abrin, abraline, choline, precatorine, abricin and abridin. Studies showed positive influence of the fungus *P. indica* on *Abrus precatorius* (Fig. 9.4 and Table 9.3).

Table 9.3 Comparison of different growth parameters in control and *A. precatorius* plants inoculated with *P. indica*

Parameters	Control (without *P. indica*)	*P. indica* treated
Height	102.23 ± 1.26	131.46 ± 0.80
Shoot length	66.96 ± 1.46	88.03 ± 1.58
Root length	35.26 ± 2.65	43.43 ± 0.75
Fresh weight	37.93 ± 1.02	43.95 ± 1.06
Fresh shoot weight	31.63 ± 1.40	35.66 ± 0.66
Dry shoot weight	14.00 ± 1.25	16.62 ± 0.50
Fresh root weight	6.30 ± 0.48	8.28 ± 0.56
Dry root weight	2.14 ± 0.12	3.06 ± 0.21

Dead/sterile fungal biomass served as control. Height is given in centimetre, and weight is in gram. Each figure is an average of five independent replicates

9.2.4 Interaction with Bacopa monnieri

Bacopa monnieri commonly known in India as Brahmi is an important Ayurvedic medicinal plant belonging to Scrophulariaceae family. In the traditional system of medicine, Brahmi is a reputed nervine tonic; it is also used to treat asthma, insanity, epilepsy, hoarseness, enlargement of the spleen, snake bite, rheumatism, leprosy, eczema and ringworm and as a diuretic, appetitive and cardiotonic. The main active ingredient of *B. monnieri* is believed to be the bacosides (Prasad et al. 2004).

Tissue culture technology could play an important role in the clonal propagation, germplasm conservation and improvement of *B. monnieri*. Shoot regeneration has been reported from the distal ends of 1–12 mm long internode segments of *B. monnieri* cultured on growth regulator-free medium, longer internode being more conducive to regeneration (Fig. 9.5). *P. indica* is documented to promote plant growth and protects the host against root pathogens and insects. *P. indica* promoted the plant growth and enhanced antioxidant activity as well as the active ingredient bacoside by several folds (Prasad et al. 2004). Plant treated with *P. indica* also showed significant increase in number of leaves, number of branches, root and shoot length as depicted in Fig. 9.5 and Table 9.4.

It was found that there was an inherent problem with the micropropagated plants during the time of transplantation from the laboratory to field. It was noticed that the rate of survival was very low, up to 40 % in the field conditions. The salient reason could be the "transient transplant shock" that resulted in the stunted growth. For employing the technique, inoculation of micropropagated plantlets with active cultures of AMF or mycorrhiza-like fungi appears to be critical for their survival and growth. These pre-acclimatized plantlets when transferred to field, overcome the transient transplant shock, and were able to cope up with changed environment of transplantation which also helps in their successful establishment. Pre-establishment of mycorrhiza in the host roots also helps in the development of the synergistic effect with other rhizosphere microflora that competes in the ecosystem for successful survival (Prasad et al. 2004).

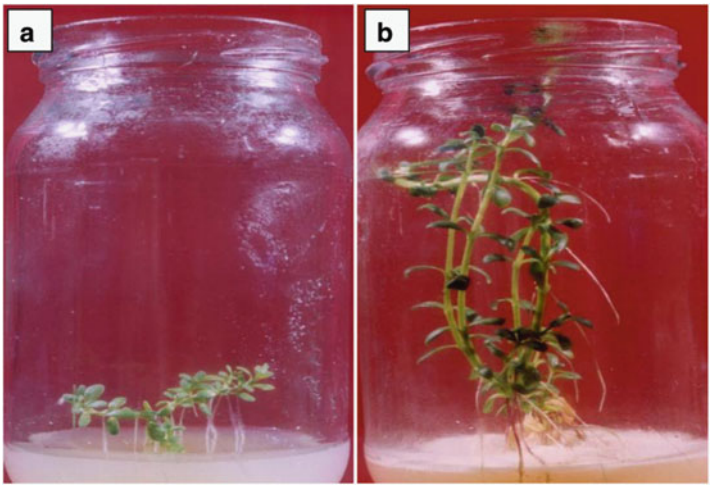

Fig. 9.5 In vitro grown plants of *B. monnieri*. (**a**) Plants without *P indica*, (**b**) plants with *P. indica*

Table 9.4 Effect of *P. indica* on growth of *B. monnieri* in 90 days time on Kaefer semisolid medium. Control: Without *P. indica*

Bio-inoculant	Number of leaves	Number of branches	Number of aerial roots	Root length (cm)	Shoot length (cm)	Total weight (f. w. in g)	
						Shoot	Root
Control	46.75	2	22.5	2.12	11.06	0.21	0.38
P. indica	76.5	6.6	38	6.5	23.12	1.26	0.96

9.2.5 *Interaction with* Coleus forskohlii

Coleus forskohlii (willd.) (Briq. syn. *C. Barbatus* (Andr.) Benth) is an aromatic herbaceous species belonging to the family Lamiaceae. It is native to India and is recorded in Ayurvedic *Materia Medica* under the Sanskrit name "Makandi" and "Mayani" (Shah 1996; Patil et al. 2001). It grows wild in the subtropical warm temperate climates of India, Nepal, Burma, Sri Lanka and Thailand. It is also found in Egypt, Arabia, Ethiopia, tropical East Africa and Brazil (Willemse 1985; Chandel and Sharma 1997; Kurian and Sankar 2007). *C. forskohlii* is a perennial plant that grows to about 45–60 cm tall. It has four angled stems that are branched, and nodes are often hairy. Leaves are 7.5–12.5 cm in length and 3–5 cm in width, usually pubescent, narrowed into petioles. Inflorescence is raceme, 15–30 cm in length; flowers are stout, 2–2.5 cm in size, usually perfect and calyx hairy inside. Upper lip of calyx is broadly ovate. The blue or lilac corolla is bilabiate. The root is typically golden brown, thick, fibrous and radially spreading. Roots are tuberous, fasciculated, 20 cm long and 0.5–2.5 cm in diameter, conical fusiform, straight, orangish within and strongly aromatic. *C. forskohlii* is the only species of the genus

to have fasciculated tuberous roots (Kavitha et al. 2010). The entire plant is aromatic, and whole plant *C. forskohlii* (roots, flowering shoots and leaves) have commercial importance. The plant contains 0.05–0.1 % forskolin/g fresh weight which is a diterpenoid and used as drug. Roots are the major source of forskolin (coleonol), although diterpenoids are found in almost all parts (Chandel and Sharma 1997). Forskolin from *C. forskohli*i is an activator of adenylate cyclase and thus leads to the production of second messenger cyclic AMP (cAMP) (Kavitha et al. 2010). Leaves also contain diterpenoid methylene quinine, coleonol, barbatusin and cyclobutatusin. Barbatusin is used against lung carcinoma and lymphatic leukaemia (Kurian and Sankar 2007). Other secondary compounds found in *C. forskohlii* are monoterpenes, monoterpene glycosides, sesquiterpenes and phenolic glycosides (Ahmed and Viswakarma 1988; Ahmed and Merotra 1991; Petersen 1994). In the traditional Ayurvedic medicine, *C. forskohlii* has been used for treating heart diseases, abdominal colic, respiratory disorder, insomnia, convulsions, asthma, bronchitis, intestinal disorders, burning sensation, constipation, epilepsy and angina (Ammon and Muller 1985). The plant is also used for veterinary purposes (De Souza and Shah 1988). Forskolin is also used in the preparation of medicines that suppresses hair greying and restoring grey hair to its normal colour (Keikichi et al. 1988). Furthermore, forskolin is valued for anti-allergic activity (Gupta et al. 1991). Roots are hypotensive and spasmolytic and are given to children in constipation. It is effective against thrombosis and is employed in glaucoma therapy, owning to its adenylated cyclase stimulant activity (Kurian and Sankar 2007; Chandel and Sharma 1997; Kavitha et al. 2010). This indigenous species, besides being used as a medicinal plant, is used as a potent source of essential oil (Patil et al. 2001). The essential oil present in tubers has an attractive and delicate odour with a spicy note (Misra et al. 1994). The essential oil has potential use in the food flavouring industry and can be used as an antimicrobial agent (Chowdhary and Sharma 1998). There is a wide variation in morphology, essential oil content and yield parameters among the genotypes of *C. forskohlii* (Patil et al. 2001). Vishwakarma et al. (1988) screened 38 genotypes collected from various locations to identify the potential genotypes for forskolin. The content of forskolin varied substantially with different genotypes, from 0.01 to 0.44 % on the fresh weight basis.

C. forskohlii was interacted with root endophytic fungus *P. indica* that mimics AMF under field condition. Growth of *C. forskohlii* in the presence of *P. indica* resulted in an enhancement in aerial growth and biomass production of medicinal plants. The height, number of branches, average length of the branches and number of leaves and leaf area of *P. indica*-treated 6-month-old plants was significantly increased compared to the untreated control plants (Table 9.5a and Fig. 9.6). Although the fungus also promoted the number (22 %) and lengths (32 %) of the roots, they looked fibrous and tough in texture (Table 9.5a). This demonstrates that the initial promotion of rooting shifted towards the development of fibrous root structures in the presence of the fungus. Consequently, after 6 month on the field, the overall weights of colonized roots were lower than the weight of the uncolonized controls (Table 9.5b). Also the thickness of colonized roots was dramatically reduced. As a result, shoot growth was promoted, and root growth

Table 9.5a Influence of *P. indica* on plant length (cm), number of branches, average length of branches (cm), number of leaves, leaf area (cm^2), number of roots and root thickness (cm) of field grown *C. forskohlii* plants (growth period: 6 months)

Parameters	Without *P. indica*	*P. indica*	Percent increase/ decrease over the control
Shoot length(cm)	43.94 ± 2.43a	55.07 ± 1.68b	+20.19
Number of branches	15.00 ± 1.24a	20.00 ± 1.32b	+24.21
Average length of branches (cm)	19.04 ± 2.35a	32.88 ± 2.05b	+42.14
Number of leaves	89.44 ± 6.46a	169.22 ± 13.91b	+46.05
Leaf area (cm^2)	8.79 ± 1.97a	12.50 ± 2.92b	+29.71
Number of roots	44.00 ± 3.08a	56.00 ± 2.87b	+22.81
Root length (cm)	16.44 ± 0.77a	24.06 ± 0.89b	+31.67
Root thickness (cm)	6.04 ± 4.61a	1.18 ± 2.29b	−80.46

Each data represents the mean of two independent replicates (Year 2008 and 2009), and each replicate represents 18 plants; control and *P. indica* represent non-treated and treated with symbiotic fungus *P. indica*, respectively; per cent increase represents per cent increase of treated over non-treated data; mean values within a same row followed by different letters differ significantly at $p \leq 0.05$ according to student's *t* test

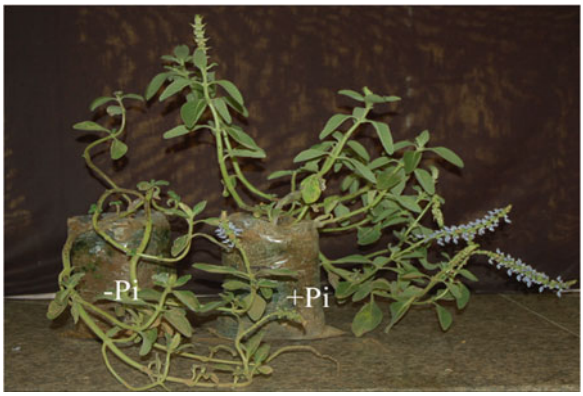

Fig 9.6 Influence of *P. indica* on 6-month-old *C. forskohlii* in field condition. Photographed after 6 months. Figure represents plant morphology as a result of interaction between *P. indica* on *C. forskohlii* under field condition. Each polythene bag contained 2.5 kg of unsterile sand, field soil and compost (1:1:0.25 w/w). The fungal inoculum was 2 % (w/v). Each bag contained 30-day-old rooted plant cuttings. Irrigation was done on alternate days using underground water. -Pi: Plants without inoculum of *P. indica*; +Pi: Plants treated with fungus

was retarded by *P. indica* when medicinal plants *C. forskohlii* when treated with *P. indica*. Flowering occurred earlier and more vigorously in *P. indica*-colonized plants. Colonized plants flowered at least 7 days earlier than the untreated controls. After 180 days, 31 % of non-treated but 81 % of *P. indica*-treated plants flowered. Moreover, the number and length of the inflorescence were significantly higher in colonized plants compared to their untreated counterparts (Das et al. 2012).

Table 9.5b Influence of *P. indica* on plant biomass (growth period 6 months)

Parameters	Without *P. indica*	*P. indica*	Percent increase/decrease over control
Dry shoot weight, g	16.63 ± 02.92a	30.82 ± 03.27b	+46.04
Dry root weight, g	08.54 ± 03.57a	04.34 ± 03.01b	−49.18
Percent root colonization	nil	25.55	

Each data represents the mean of two independent replicates (Year 2008 and 2009), and each replicate represents 18 plants; control and *P. indica* represent non-treated and treated with symbiotic fungus *P. indica*, respectively; per cent increase represents per cent increase of treated over non-treated data; mean values within a same row followed by different letters differ significantly at $p \leq 0.05$ according to student's *t* test; each figure in percent root colonization (Spores of *P. indica*) represents the mean of three independent replicates, and each replicate represents 90 root segments

9.2.6 *Interaction with* Adhatoda vasica

Adhatoda vasica Nees, commonly known as Malabar nut belongs to family Acanthaceae. It is an evergreen shrub. The plant is used for the preparation of medicine for asthma, bronchitis and other pulmonary disorders. It is also used as antiarthritis, antiseptic, antimicrobial, expectorant, sedative and antituberculosis (Dey 1980; Singh and Jain 1987). Glycodin®, a well-known product used for the cure of bronchitis, is extracted from the leaves of the plant. In Ayurveda, a number of medicines are manufactured by this plant. There is a pressing need of rapid multiplication of this plant due to increasing demand by pharma industries.

Rai and Varma (2005) studied the role of *P. indica* in growth promotion of *A. vasica*. The cuttings of *A. vasica* were inoculated with *P. indica* to assess the growth promoting property of *P. indica*. They reported that *P. indica* enhanced the growth of *A. vasica* (Fig. 9.7). The authors further observed profuse proliferation of roots of *A. vasica* after inoculation of *P. indica*. Root colonization of *A. vasica* by *P. indica* augmented with time from 53 % after 2 months to 95 % after 6 months. There was a significant enhancement in the growth rate of the plants inoculated with *P. indica*. The growth was very fast up to 2 months (Fig. 9.8) and slowed down thereafter. At each observation, growth was significantly higher for the plants inoculated with *P. indica* as compared to the control plants.

The fresh and dry weight of shoots and roots of *A. vasica*-inoculated plants was higher than that of the corresponding controls (Fig. 9.9). This suggests that *P. indica* is an appropriate endophyte for fast growth of the plants. After additional trials and evaluation of active principles or secondary metabolites production, *P. indica* may be recommended for growth enhancement of *A. vasica*.

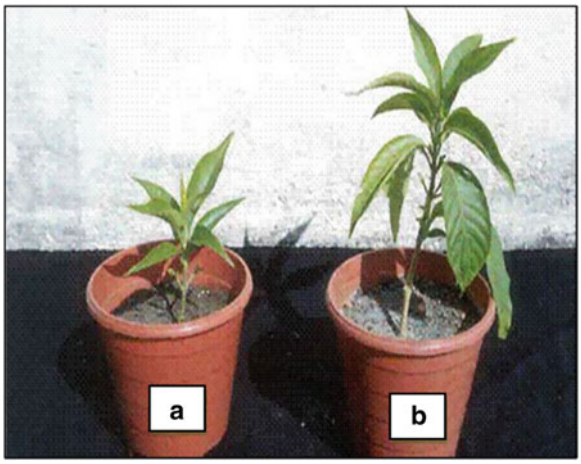

Fig. 9.7 Effect of *P. indica* on *A. vasica* (**a**) Plants without *P indica*, (**b**) Plants with *P. indica*

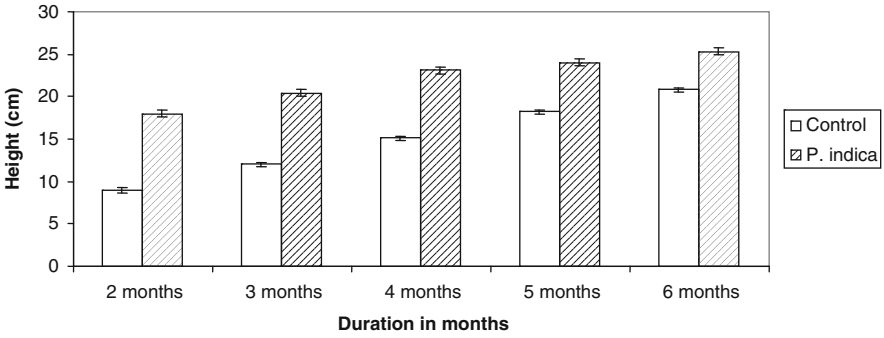

Fig. 9.8 Growth response of *A. vasica* (in cm) after inoculation with *P. indica*, control plants are without *P. indica*

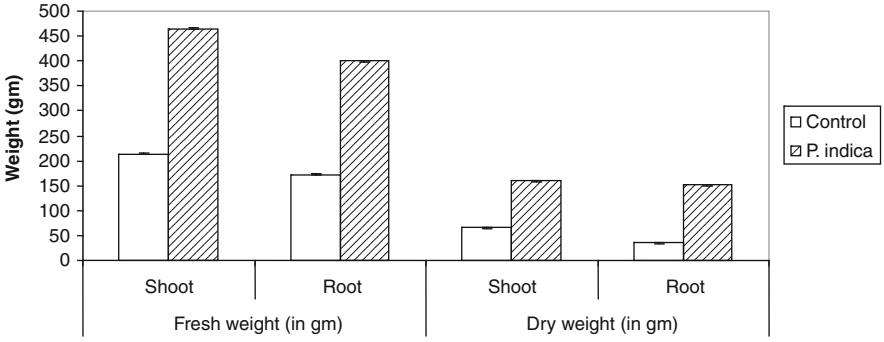

Fig. 9.9 Fresh and dry weight of *A. vasica* after 6 months after interaction with *P. indica*, control plants are without *P. indica*

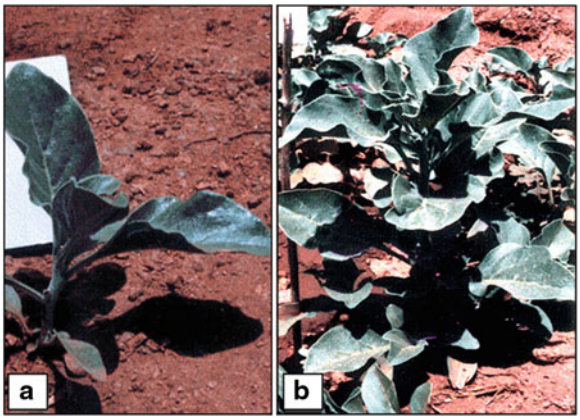

Fig. 9.10 Pronounced growth response in *W. somnifera* after inoculation with *P. indica*: (**a**) control (without *P. indica*), (**b**) inoculated with *P. indica*

9.2.7 *Interaction with* Withania somnifera *and* Spilanthes calva *in Field Trials*

W. somnifera is also known as Indian ginseng and belongs to the family Solanaceae. There are more than 91 pharmaceutical products manufactured from the roots of this plant and are potential sources of a promising drug for cancer (Devi 1996). *S. calva* is a member of Asteraceae and is called as "toothache plant" and is well known for enhancing immunity (Rai et al. 2001). The plant has anti-ageing property and cures diseases of gums like pyorrhoea and is also useful in toothache. The leaves of this plant stimulate salivation which is due to an active chemical known as "spilanthol". Because of high medicinal value, there is an increasing demand for these plants in national and international market (Rai et al. 2001). Therefore, there is a greater need to augment the growth and secondary metabolites of these plants. This inoculation of beneficial microorganisms like arbuscular mycorrhizal fungi or other growth promoting endophytes serves the purpose.

Pronounced growth response in *W. somnifera* and *S. calva* after inoculation with *P. indica* was noted (Figs. 9.10a, b and 9.11a, b). The data revealed that the plants treated with *P. indica* were superior in development compared to control plants (uninoculated with *P. indica*). A significant increase in the shoot length was observed in the inoculated plants. The microscopic examination of stained root samples revealed a high colonization of *S. calva* and *W. somnifera* by *P. indica* in 62 and 73 % root length, respectively (Fig. 9.12).

The basal and leaf area of treated plants was also increased (Table 9.6). Interestingly, in inoculated *S. calva* plants, some large, kidney-shaped heads were observed among the normal round heads. These kidney-shaped heads were never observed in control plants. The length of the inflorescence and the number of flowers in inoculated *S. calva* were also increased (Table 9.6; Fig 9.11a, b). Similarly, in the inoculated plants of *W. somnifera*, the number of flowers was higher (Table 9.6) as compared to controls. In both medicinal plants, seed count was higher as compared to controls.

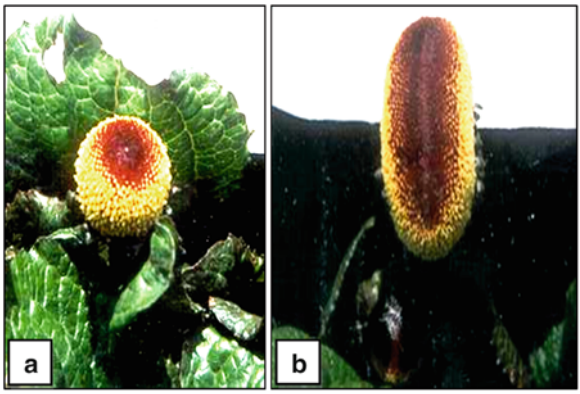

Fig. 9.11 Pronounced growth response and flowering in *S. calva* after inoculation with *P. indica*. (**a**) Control (without *P. indica*), (**b**) inoculated with *P. indica*

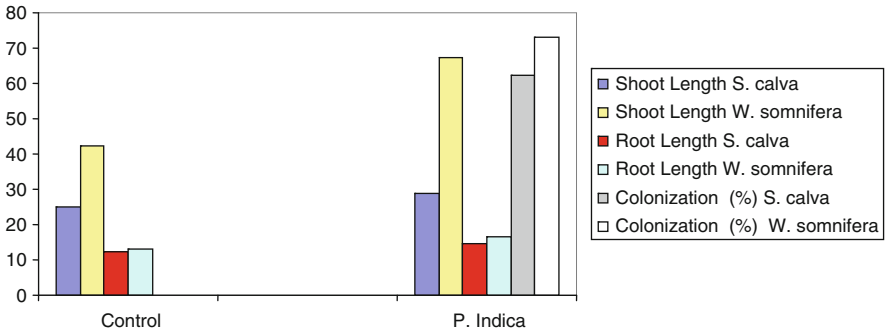

Fig. 9.12 Influence of *P. indica* on shoot and root length and on per cent root colonization of S. *calva* and *W. somnifera* in a field trial. The control plants were treated with an equal amount of autoclaved mycelium

*9.2.8 Interaction with Turmeric (*Curcuma longa *L) in a Field Trial*

Turmeric (*Curcuma longa* L.) is a perennial crop of Zingiberaceae family, widely cultivated in India and other parts of the world. In medieval Europe, turmeric became known as Indian saffron, since it was widely used as an alternative to the far more expensive saffron spice (Ruby et al. 1995). The plant has wide spectrum of medicinal properties and commercial applications due to presence of the well-known bioactive component curcumin. The microbial bioinoculants such as, phosphate solubilizing bacteria (PSB) and arbuscular mycorrhizal fungi (AM fungi) were used for the improvement of the crop, which are shown by the enhancement in the productivity (Eigner et al. 1999). It is a very important medicinal plant and extensively used in Ayurveda, Unani and Siddha medicine as the treatment for

Table 9.6 Influence of *P. indica* on morphology and growth of host plants (*S. calva* and *W. somnifera*) 90 days after inoculation in a field trial. The control plants were treated with equal amount of autoclaved mycelium; control: without *P. indica*

Hosts	Treatment	Basal area (cm^2)	Leaf area length of head (cm^2)	Basal area (cm^2)	Diameter of inflorescences (cm)	No. of flower inflorescences	No. seeds fruit
S. calva	Experimental	7.06 (±0.47)	37.67 (±2.28)	2.49 (±0.09)	5.06 (±0.32)	48.57 (±0.4)	1006 (±7.63)
	Control	4.11 (±0.57)	26.00 (±2.87)	1.48 (±0.03)	4.16 (±0.76)	11.50 (±3.6)	716 (±0.36)
W. somnifera	Experimental	11.40 (±0.61)	45.59 (±0.34)	Nil	Nil	307.40 (±0.53)	46.33 (±5.77)
	Control	5.57 (±0.49)	13.08 (±0.73)	Nil	Nil	81.80 (±1.57)	35.33 (±4.93)

All values are mean ± S.D.; differences between inoculated and control plants are significant at $P < 0.05$

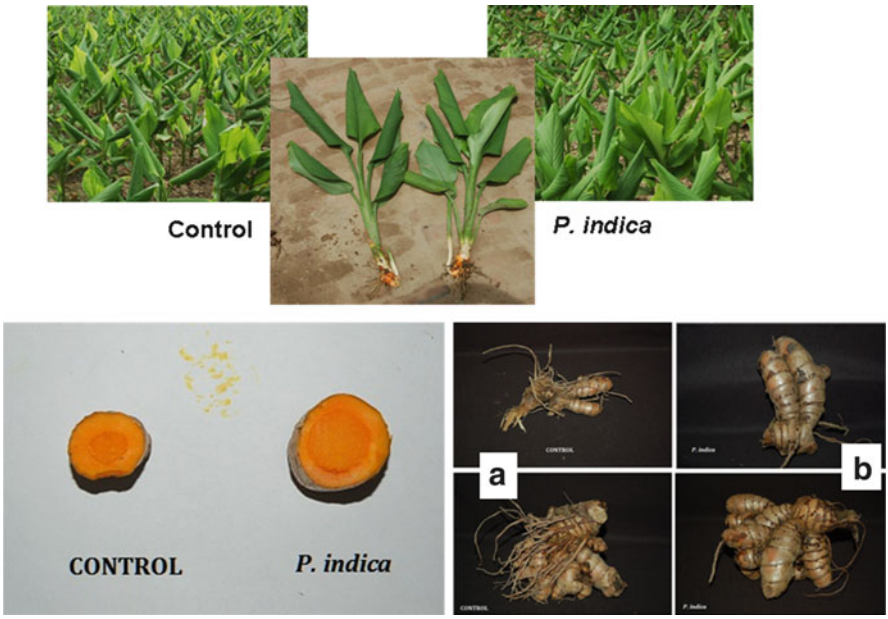

Fig. 9.13 Effect of the *P. indica* on the field grown plant and rhizomes of turmeric (*Curcuma longa* L.) (**a**) Plants without *P. indica*, (**b**) plants treated with *P. indica*. Control: plants without *P. indica*, *P. indica*: inoculated with *P. indica*

various diseases (Dasgupta et al. 1969). It is also used as a food additive (spice), preservative and colouring agent in many countries such as China and Southeast Asia. The turmeric is also used in Ayurveda for the treatment of sprains and swelling caused by injury (Ammon et al. 1992).

The present study focused to understand the interaction of the *P. indica with* turmeric plants. The impact of *P. indica* as bio-inoculant on growth and yield of turmeric was assessed by morphological parameters of the turmeric plant both in treated (with *P. indica*) and untreated (control) plants in field trials. It was observed that the plants of turmeric treated with *P. indica* demonstrated remarkable growth as compared to the control (Fig. 9.13). There was enhancement of 12 % in yield of treated turmeric plants. The rhizomes of the plant treated with *P. indica* were found to be healthier and thicker than the control (Fig. 9.13).

9.2.9 Estimation of Total Alkaloids and Withanolides in Inoculated and Control Plants

Quantitative determination of total alkaloids, withanolides and withaferin A was made by TLC densitometry. Ashwagandha roots were extracted with methanol (20 ml × 3), filtered and evaporated. The extract thus obtained was defatted with

Table 9.7 Percentage of alkaloid content in inoculated *Withania somnifera* (with *P. indica*) and uninoculated plants

Alkaloids	Inoculated with *Piriformospora indica (%)*	Uninoculated plants (without *P. indica*) (%)
Withanolides	0.52	0.60
Total alkaloids	0.34	0.38

n-hexane (10 ml × 3) and then extracted with 1 % sulphuric acid (5 ml × 3), basified with ammonia, extracted with chloroform (10 ml × 3), dried over anhydrous sodium sulphate, filtered, evaporated and weighed for total alkaloid content. The sulphuric acid insoluble was extracted with diethyl ether (10 ml × 3), dried over anhydrous sodium sulphate, filtered, evaporated and estimated as crude withanolide. The total withanolides and alkaloid contents increased after inoculation of *P. indica*. This suggests that *P. indica* can be used for enhancement of active principles (Table 9.7).

9.2.10 *Enhancement of Antimycotic Activity in* Spilanthes calva *due to Increase in Active Principles after Inoculation of* P. indica

Plants of *Spilanthes calva* inoculated with *P. indica* and uninoculated (control) exhibited antifungal activity against *Fusarium oxysporum* (opportunistic human pathogen) and *Trichophyton mentagrophytes* (potential human pathogen). A significant antimycotic activity in aqueous and petroleum ether extracts of *S. calva* was recorded. Petroleum ether extract of *S. calva was* more effective than aqueous extract in inoculated as well as uninoculated plants. The minimum inhibitory concentration of *F. oxysporum* was recorded as 125 µl/ml when treated with aqueous extract of inoculated plants, whereas 250 µl/ml aqueous extract of inoculated plant was needed to inhibit the growth of *T. mentagrophytes*. *F. oxysporum was* found to be more sensitive than *T. mentagrophytes*. The value of MIC for *F. oxysporum* was very high (1,000 µl/ml) when aqueous extract of uninoculated plant was tested. In addition, aqueous extract of uninoculated plant of *S. calva* did not inhibit the growth of *T. mentagrophytes* which is a potential fungal pathogen causing skin and nail infections in human beings (Rai et al. 2004).

Petroleum ether extract of inoculated plants of *S. calva* showed a remarkable antifungal activity. Extract (62.5 µl/ml) was sufficient to inhibit the growth of 1 ml spore suspension of *F. oxysporum*, whereas in extract of uninoculated plants the MIC value reached to 500 µl/ml of spores. Similarly, for *T. mentagrophytes*, 125 µl extract of inoculated plants was enough to arrest the growth, while MIC value was quite high (1,000 µl/ml) in case of petroleum ether extract of uninoculated plants.

The extract of inoculated plants of *S. calva* showed significantly higher activity compared to uninoculated plants. Additionally, extract of petroleum ether was

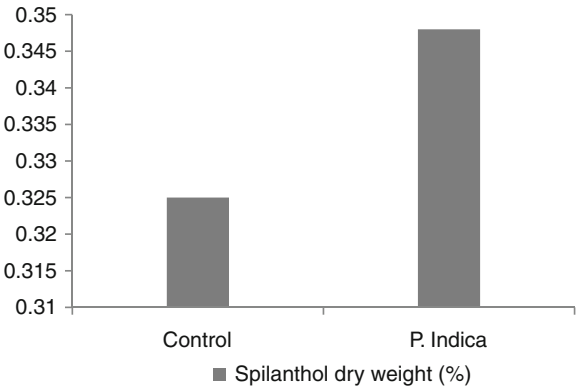

Fig. 9.14 Estimation of spilanthol in roots of *Spilanthes calva*. Control: plants without *P. indica*, *P. indica*: plants treated with *P. indica*

found to be more active than aqueous extract which shows synthesis of higher quantity of spilanthol in inoculated plants. The enhancement of spilanthol was revealed by the chemical analysis of the roots of the plant (Fig. 9.14).

*9.2.11 Interaction with Safed Musli (*Chlorophytum sp.*)*

Chlorophytum sp., which is commonly called as "Safed Musli", is an important medicinal herb belonging to family Liliaceae, having fleshy roots of medicinal importance for the cure of physical weakness, diabetes and arthritis. The dried fasciculed storage roots of this herb, popularly known as "Musli", have strong aphrodisiac, antistress and immuno-modulatory properties due to the presence of steroidal saponins and polysaccharides. The continued collection of these medicinal plants has resulted in the fast depletion of its population, may be due to low rate of multiplication through vegetative means and shy flowering behaviours (Mathur et al. 2008; Gosal et al. 2010).

Chlorophytum plants when inoculated with the fungus *P. indica*, after 30 days of growth in green house, significant increase in root length and the number of lateral roots were observed over uninoculated control (Gosal et al. 2010). Survival rate of musli plants as recorded after transplantation in soil in the greenhouse was improved with microbial *P. indica* biotization (Mathur et al. 2008; Gosal et al. 2010). In vitro plantlets with roots when interacted with P. indica, there was 86 % of plantlets survival in green house, at biohardening stage. An important observation was noted that even the micropropagated un-rooted shoots could form roots under in vivo conditions when treated with *P. indica* with more than 43 % plantlet establishment (Mathur et al. 2008). Biotization with *P. indica* alone or in combination with *P. fluorescens* has led to increase in saponin content (Gosal et al. 2010).

9.2.12 *Interaction of* P. indica *with Fennel (*Foeniculum vulgare*)*

Fennel (*Foeniculum vulgare*) a member from the family Apiaceae is one of the most important aromatic plants widely applied in culinary and medicinal preparations. It is generally considered indigenous to the Mediterranean area, but it is also cultivated elsewhere (Russia, India, China and Japan). Fennel is used against digestive disorders such as spasmodic gastrointestinal complaints and bloating. It may be an effective diuretic and a potential drug for the treatment of hypertension, nervous disturbances, paediatric colic and some respiratory disorders due to its antispasmodic effects. Essential oils are mainly concentrated in the fruits and provide their unique aroma and taste. Anethole and fenchone are the most important volatile components of *F. vulgare* essential oil (Dolatabadi et al. 2011a).

P. indica significantly increased growth of the inoculated of fennel plants in comparison to uninoculated control plants. As well as it significantly increased dry weight of 1,000 fruits in comparison to controls. Not only biomass but the concentration of essential oil increased in pot cultures inoculated with *P. indica* in comparison to controls (Dolatabadi et al. 2011a). Their work revealed through GC and GC-MS studies that the level of anethole was also enhanced with *P. indica* inoculation.

9.2.13 *Interaction with* Linum album

Lignans constitute a large group of secondary metabolites synthesized by many plants. These compounds are usually formed from two phenylpropanoid units and manifest considerable biological activity. Podophyllotoxin, a lignan with antiviral and antineoplastic activities, is used today primarily as a precursor for the semi-synthesis of established cancer therapeutics such as etoposide, teniposide and Etopophos. *Linum album* is an herbaceous and medicinal plant that has important lignan such as podophyllotoxin. Podophyllotoxin and 6-methoxy podophyllotoxin have antiviral and anticancer properties (Chashmi et al. 2011). Podophyllotoxin has been obtained by solvent extraction from the rhizomes of plants *Podophyllum peltatum* and *P. hexandrum* that belong to the family Berberidaceae. Due to endangered status of its natural source and economically unfeasible chemical synthesis, there is an imperative need to search for alternate ways to produce these lignans by cell cultures. Cell cultures of *Linum album* are known to produce these lignans with highest productivity (Baldi et al. 2008).

Baldi et al. (2008) developed cell suspension cultures of *Linum album* from internode portions of in vitro-germinated plant in Gamborg's B5 medium supplemented with 0.4 mg naphthalene acetic acid/l. The highest biomass was 8.5 g/l with podophyllotoxin and 6-methoxypodophyllotoxin at 29 and 1.9 mg/l, respectively, after 12-day cultivation. They were able to successfully coculture *L. album* cells with axenically cultivable arbuscular mycorrhiza-like fungi,

P. indica and *Sebacina vermifera*, for the first time. These enhanced podophyllotoxin and 6-methoxypodophyllotoxin production by about four- and eight-fold, respectively, along with a 20 % increase in biomass compared to the control cultures.

9.3 Conclusions

From the studies of the interaction of *P. indica* with selected medicinal plants, it can be concluded that *P. indica* acts as a potential colonizer and the plant growth promoter fungus which induced a faster development of the aerial part by promoting early maturation with respect to flowering and biomass. Besides this, it also enhances the absorption of the nutrients by underground roots. The increased growth of *P. indica*-colonized plants is due to enhanced nutrient uptake such as phosphorus and nitrogen from the soil. *P. indica* is also involved in the transportation of the phosphate to the host plant. The fungus colonizes both monocots and dicots. The wonder fungus also provided protection when inoculated into the tissue culture-raised plants by overcoming the "transient transplant shock" on transfer to the field or green house and rendered very high survival percentage on transplanted in vitro plants. Finally, the fungus is multifunctional and thus should be used for the growth promotion of medicinally important plants.

Acknowledgements Authors are thankful to Dr. Amit C. Kharkwal for providing turmeric (*Curcuma longa* L) data and figures. Ajit Varma is thankful to ICAR, DBT and DRDO for partial financial support.

References

Achatz B, Kogel KH, Franken P, Waller F (2010) Piriformospora indica mycorrhization increases grain yield by accelerating early development of barley plants. Plant Signal Behav 5:1685–87

Ahmed B, Merotra R (1991) Coleoside-B: a new phenolic glycoside from *Coleus forskohlii*. Pharmazie 46:157–158

Ahmed B, Viswakarma RA (1988) Coleoside, a monoterpene glycoside from *Coleus forskohlii*. Phytochemistry 27:3309–3310

Ammon HPT, Anazodo MI, Safayhi H, Dhawan BN, Srimal RC (1992) Curcumin: a potent inhibitor of leukotriene B4 formation in rat peritoneal polymorphonuclear neutrophils (PMNL). Planta Med 58:26–28

Ammon HPT, Muller AB (1985) Forskolin: from an ayurvedic remedy to a modern agent. Planta Med 46:473–477

Bagde US, Prasad R, Varma A (2010) Interaction of mycobiont: *Piriformospora indica* with medicinal plants and plants of economic importance. Afr J Biotechnol 9:9214–9226

Baldi A, Jain A, Gupta N, Srivastava AK, Bisaria VS (2008) Co-culture of arbuscular mycorrhiza-like fungi (Piriformospora indica and Sebacina vermifera) with plant cells of Linumalbum for enhanced production of podophyllotoxins: a first report. Biotechnol Lett 30:1671–1677

Bhagwat DA, Killedar SG, Adnaik RS (2008) Antidiabetic activity of leaf extract of *Tridax procumbens*. Int J Green Pharm 2:126–128

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Ralf O (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and non beneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol 185:1062–1073

Chandel KPS, Sharma N (1997) Micropropagation of *Coleus forskohlii* (Willd.) Briq. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. Hi Tech and micropropagation. Springer, Berlin, pp 74–84

Chashmi NA, Sharifi M, Yousefzadi M, Behmanesh M, Palazon J (2011) The production of cytotoxic lignans by hairy root cultures of *Linum Album*. World Acad Sci, Engineer Technol 80:401–402

Chowdhary AR, Sharma ML (1998) GC-MS investigations on the essential oil from *Coleus forskohlii* Briq. Indian Perfumer 42:15–16

Das A, Kamal S, Shakil NA, Sherameti I, Oelmüller R, Dua M, Tuteja N, Johri AK, Varma A (2012) The root endophyte fungus *Piriformospora indica* leads to early flowering, higher biomass and altered secondary metabolites of the medicinal plant. Coleus forskohlii. Plant Signal Behav 7:103–112

Dasgupta SR, Sinha M, Sahana CC, Mukherjee BP (1969) A study of the effect of an extract of Curcuma longa Linn. on experimental gastric ulcers in animals. Ind J Pharmacol 1:49–54

De Souza NJ, Shah V (1988) Forskolin—an adenylate cyclase activating drug from an Indian herb. Econ Med Plant Res 2:1–16

Devi PU (1996) *Withania somnifera* Dunal (Ashwgandha): potential plant source of a promising drug for cancer chemotherapy and radiosensitization. Ind J Exp Biol 34:927–932

Dey AC (1980) Indian medicinal plants used in ayurvedic preparations. In: Bishen Singh, Mahendra Pal Singh (eds), Dehra Dun, pp 202

Dolatabadi HK, Goltapeh EM, Jaimand K, Rohani N, Varma A (2011a) Effects of *Piriformospora indica* and *Sebacina vermifera* on growth and yield of essential oil in fennel (*Foeniculum vulgare*) under green house conditions. J Basic Microbiol 51:33–39

Dolatabadi HK, Goltapeh EM, Moieni A, Jaimand K, Pakdaman B, Sardrood P (2011b) Effect of *Piriformospora indica* and *Sebacina vermifera* on plant growth and essential oil yield in *Thymus vulgaris* in vitro and in vivo experiments. Symbiosis 53:29–35

Eigner D, Scolz D (1999) *Ferula asa-foetida* and *Curcuma longa* in traditional medicinal treatment and diet in Nepal. J Ethnopharmacol 67:1–6

Gosal SK, Karlupia A, Gosal SS, Chhibba IM, Varma A (2010) Biotization with *Piriformospora indica* and *Pseudomonas fluorescens* improves survival rate, nutrient acquisition, field performance and saponin content of micropropogated Chlorophytum sp. Ind J Biotechnol 9:289–297

Gupta PP, Srimal RC, Tandon JS (1991) Antiallergic activity of coleonol, a diterpene from *Coleus forskohlii*, In: Proc 25th Indian Pharmacol Soc Conf. Dec 29–31, Ahmedabad, Gujrat, India, pp 142

Harman GE (2011) Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. New Phytol 189:647–649

Kapoor R, Chaudhary V, Bhatnagar AK (2007) Effects of arbuscular mycorrhiza and phosphorus application on artemisinin concentration in *Artemisia annua* L. Mycorrhiza 17:581–587

Kavitha C, Rajamani K, Vadivel E (2010) *Coleus forskohlii*: a comprehensive review on morphology, phytochemistry and pharmacological aspects. J Med Plant Res 4:278–285

Keikichi S, Koji T, Akira F, Makota E (1988) Forskolin containing composing for hair graying suppression. Eur Pat Appl EP 295(903):21

Kumar K, Sarma MVRK, Saharan K, Srivastava S, Kumar L, Sahai V, Bisaria VS, Sharma AK (2011) Effect of formulated root endophytic fungus *Piriformospora indica* and plant growth promoting rhizobacteria fluorescent pseudomonads R62 and R81 on *Vigna mungo*. World J Microbiol Biotechnol. doi:10.1007/s11274-011-0852-x

Kumari R (2005) Plant–Microbe Interaction in in vitro and in vivo condition. Ph D Thesis, Ch. Charan Singh University, Meerut, India

Kurian A, Sankar A (2007) Important medicinal plants in trade. In: Peter KV (ed) Medicinal plants, vol 2. New India Publishing Agency, New Delhi, pp 294–295

Lambert JDH (1998) Medicinal plants: Their importance to National Economics. In: Medicinal plants: a global heritage. In: Proceedings of the International Conference on Medicinal Plants for survival. 16–19 Feb, Bangalore, India, pp 122–127

Malla R, Singh A, Eyaullah MD, Yadav V, Varma A, Rai M (2002) *Piriformospora indica* and plant growth promoting rhizobacteria: an appraisal. In: Rao GP, Manoharachari C, Bhat DJ, Rajak RC, Lakhanpal TN (eds) Frontiers of fungal diversity in India (Prof Kamal Festscrift). International Book Distributing Co, Lucknow, India, pp 401–419

Mathur A, Mathur AK, Verma P, Yadav S, Gupta ML, Darokar MP (2008) Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum* Sant. Et Fernand. Afr J Biotechnol 7:1046–1053

Misra LN, Tyagi BR, Ahmad A, Bahl JR (1994) Variablity in the chemical composition of the essential oil of *Coleus forskohlii* genotypes. J Essent Oil Res 6:243–247

Oelmuller R, Sherameti I, Tripathi S, Varma A (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Patil S, Hulamani NC, Rokhade AK (2001) Performance of genotype of *Coleus forskohlii* Briq. for growth, yield and essential oil content. Indian Perfumer 45:17–21

Petersen M (1994) Coleus spp In vitro culture and the production of forskolin and Rosmarinic acid. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, medicinal and aromatic plants VI. Springer, Berlin, pp 69–85

Pham HG, Kumari R, Singh A, Malla R, Prasad R, Sachdev M, Kaldorf M, Buscot F, Oelmüller R, Hampp R, Saxena AK, Rexer KH, Kost G, Varma A (2004) Axenic culture of symbiotic fungus *Piriformospora indica*. In: Varma A, Abbott LK, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Germany, pp 593–613

Prasad R, Garg AP, Varma A (2004) Interaction of medicinal plant with plant growth promoting rhizobacteria and symbiotic fungi. In: Podila G, Varma A (eds) Basic research and applications: mycorrhizae. Microbiology series. IK International, India, pp 363–407

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128

Rai M, Varma A (2005) Arbuscular mycorrhiza-like biotechnological potential of *Piriformospora indica*, which promotes the growth of *Adhatoda vasica* Nees. Electron J Biotechnol 8:107–112

Rai M, Varma A, Pandey AK (2004) Antifungal potential of *Spilanthes calva* after inoculation of *Piriformospora indica*. Mycosis 47:479–481

Rai MK (1994) Herbal medicines in India: retrospects' and prospectus. Fetoterapia LXV 6:483–491

Ruby AJ, Kuttan G, Dinesh Babu K, Rajasekharan KN, Kuttan R (1995) Antitumor and antioxidant activity of natural curcuminoids. Cancer Lett 94:79–83

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Sahay NS, Varma A (2000) Biological approach toward increasing the survival rates of micropropagated plants. Curr Sci 78:126–129

Shah V (1996) Cultivation and utilization of medicinal plants (supplement). RRL and CSIR, Jammu—Tawai, pp 385–411

Singh A, Sharma J, Rexer KH, Varma A (2000) Plant productivity determinants beyond minerals, water and light. Piriformospora indica: a revolutionary plant growth promoting fungus. Curr Sci 79:101–106

Singh AN, Singh AR, Kumari M, Rai MK, Varma A (2003) Biotechnological importance of Piriformospora indica. A novel symbiotic mycorrhiza- like fungus: an overview. Ind J Biotechnol 2:65–75

Singh V, Jain DK (1987) Taxonomy of angiosperms. Rastogi, Meerut, India
Varma A, Schuepp H (1994) Positive influence of arbuscular mycorrhizal fungus on in vitro raised hortensia plantlets. Angew Bot 68:108–113
Varma A, Singh A, Sudha Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: an axenically culturable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) The Mycota IX, Fungal associations. Springer, Berlin, pp 123–150
Varma A, Verma S, Sudha SN, Britta B, Franken P (1999) *Piriformospora indica*—a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Appl Environ Microbiol USA 65:2741–2744
Vishwakarma RA, Tyagi BR, Ahmed B, Hussain A (1988) Variation in forskolin content in the roots of *Coleus forskohlii*. Planta Med 54:471–472
Willemse RH (1985) Notes on East African *Plectranthus* species (Labiatae). Kew Bull 40:93–96
Wyk BEV, Wink M (2004) Medicinal plants of the world. Times Edition, Singapore
Yadav V, Kumar M, Kumar DD, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena A, Johri A (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in the phosphate transport to the host plant. J Biol Chem 285:26532–2654

Chapter 10
Biophysical Phenomics: Evaluation of the Impact of Mycorrhization with *Piriformospora indica*

Merope Tsimilli-Michael and Reto J. Strasser

10.1 Introduction

Mycorrhizae (ecto- and endo-) are mutualistic microsymbionts of about 90 % of higher plants in natural, semi-natural and agricultural environments, with a well-documented beneficial role concerning plant growth and crop yield, especially when the soil conditions are suboptimal (degraded habitats and nutrient-deficient or polluted soil) or during stress periods (see e.g., Varma 1995, 1998; Varma and Schuepp 1996; Biro et al. 2006). They are therefore a main parameter in ecosystem functions and highly advantageous in sustainable agriculture.

Piriformospora indica, which belongs to basidiomycota, is also a root endophyte that can colonise all higher plants tested so far (including Arabidopsis and conifers), with arbuscular mycorrhizal fungi (AMF)-like characteristics and the added important advantage that, contrary to AMF that are obligate endosymbionts, it can grow axenically (it is cultivable in vitro, on agar plates or in liquid media; see e.g., Verma et al. 1998; Varma et al. 1999, 2001). Shoot and root length, biomass, basal stem, leaf area, overall size, number of inflorescences and flowers and seed production, as well as tolerance to temperature, drought and heavy metals, are all enhanced

M. Tsimilli-Michael (✉)
Ath. Phylactou 3, CY 1100 Nicosia, Cyprus
e-mail: tsimicha@spidernet.com.cy

R.J. Strasser
Weed Research Laboratory, Nanjing Agricultural University, Nanjing, China

Research Unit Environmental Science and Management, North-West University, Potchefstroom, Republic of South Africa

Laboratory of Bioenergetics, University of Geneva, Geneva, Switzerland
e-mail: Reto.Strasser@unige.ch

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_10,

in a broad range of plants, including medicinal plants, when colonised by *P. indica* (Sahay and Varma 1999; Varma et al. 2001, 2002; Rai et al. 2000, 2001, 2004; Singh and Varma 2001; Singh et al. 2000, 2002, 2003; Kumari et al. 2004; Rai and Varma 2005). Hence, *P. indica* can well be considered as a powerful new candidate symbiont for improving plant production and crop yield in sustainable agriculture, flori–horticulture and agroforestry. However, the success of any microbial inoculation in practice has to be tested for each case, since the effectiveness of symbiosis depends on complex interactions between plant, symbionts and environment.

Most of the tests focus on the estimation of root colonisation parameters (e.g., frequency, intensity) and/or the evaluation of physiological/morphological parameters (e.g., shoots and roots length and dry weight). Since mycorrhizal activity has multiple effects on the physiology and vitality of the host plant at different levels, it was reasonable to expect that it would affect, as well, the performance of the photosynthetic apparatus, which is highly sensitive to any environmental change.

Hence, we have extended our general theoretical approach of stress (Strasser 1985, 1988; Tsimilli-Michael et al. 1995, 1996) to address the establishment of symbiosis as an adaptation of plants in general, and of the photosynthetic apparatus in particular, to a changing environment (Tsimilli-Michael and Strasser 2002). Accordingly, we have applied as a testing method for recognising and, moreover evaluating, the impact of symbiosis in several cases (Tsimilli-Michael et al. 2000; Biro et al. 2006; Strasser et al. 2007; Tsimilli-Michael and Strasser 2008; Zubek et al. 2009; Jurkiewicz et al. 2010) the same experimental approach we use when addressing the impact of any other biotic or abiotic stress. This method provides a description of a biophysical phenotype in respect to the photosynthetic apparatus, which we hence termed as *Biophysical Phenomics* (Strasser and Tsimilli-Michael 2005; Strasser et al. 2007; Tsimilli-Michael and Strasser 2008). This biophysical phenotyping is a description of the behaviour/performance of photosystem (PS) II and PSI, hence an in vivo vitality analysis, in terms of different structural and functional parameters. The parameters are deduced from the JIP-test, which is an analysis of the fast chlorophyll (Chl) *a* fluorescence transient OJIP exhibited by all oxygenic photosynthetic organisms upon illumination. The method provides early diagnosis and the experimentation is simple, fast and non-invasive.

We will here review this method with representative examples of JIP-test application that reveal the beneficial impact of symbiosis with *P. indica* on the photosynthetic performance of the host plants. We will also demonstrate that the behaviour patterns of the photosynthetic machinery are similar upon colonisation either with *P. indica* or with AMF.

10.2 Biophysical Phenomics: In Vivo Analysis of Photosynthetic Behaviour/Performance

10.2.1 The Fast Chlorophyll a Fluorescence Transient OJIP

Chlorophyll (Chl) *a* fluorescence, emitted by all oxygenic photosynthetic organisms when illuminated, originates at ambient temperature basically from PSII. The analysis of the Chl *a* fluorescence kinetics and spectra has been proven to be a very useful, non-invasive tool for the investigation of stress effects on the structure and function of the photosynthetic machinery.

The fluorescence kinetics exhibited by dark-adapted photosynthetic samples upon illumination comprises a fast rise followed by a slow decline (Kautsky curve). Our method exploits the fast fluorescence rise (from 10 μs to 1 s), measured in dark-adapted leaves with a high time resolution fluorimeter (Handy-Pea fluorimeter, Plant Efficiency Analyser, Hansatech Instruments Ltd., King's Lynn Norfolk, PE 30 4NE, UK). The transients were induced by red light (peak at 650 nm) of 3,500 μmol photons m^{-2} s^{-1} intensity provided by an array of three light-emitting diodes and recorded for 1 s with 12 bit resolution. The data acquisition was every 10 μs from 10 μs to 0.3 ms, every 0.1 ms (0.3–3 ms), every 1 ms (3–30 ms), every 10 ms (30–300 ms) and every 100 ms (300 ms to 1 s).

The fast fluorescence rise is generally accepted to reflect the accumulation of the reduced form (Q_A^-) of the PSII primary electron quinone acceptor Q_A, equivalent to the closure of PSII reaction centres (RCs), which is the net result of Q_A reduction due to PSII activity and Q_A^- reoxidation due to PSI activity. When the photosynthetic sample is kept in the dark long enough to allow the full reoxidation of Q_A^-, hence the reopening of all RCs, the fluorescence intensity at the onset of illumination is denoted as F_0 (minimal fluorescence). The maximum intensity F_P at the end of the fast rise, depending on the achieved redox state of Q_A acquires its maximum possible value (denoted then as F_M) if the illumination is strong enough (usually above 500 μmol photons m^{-2} s^{-1} red light) to ensure the reduction of all Q_A (equivalently, closure of all RCs). Transients recorded with high-time-resolution fluorimeters (as e.g. the Plant Efficiency Analyzers PEA, Handy-PEA (hPEA), Pocket PEA (pPEA), Senior PEA (sPEA) and Multi-Functional PEA (mPEA), or the FluorPen and FIM instruments) provide additional and/or more accurate information concerning the processes leading to Q_A^- accumulation. It was shown that the fluorescence rise kinetics is polyphasic, exhibiting clearly, when plotted on logarithmic time scale, the steps J (at 2 ms) and I (30 ms) between the initial (F_0) and the maximum (F_P or F_M) fluorescence level (hence denoted as OJIP transient); moreover, a precise detection of F_0 (taken at 20 μs), as well of the initial slope that offers a link to the maximum rate of PSII photochemical reaction, is provided (Strasser and Govindjee 1991; Strasser et al. 1995; for reviews see Strasser et al. 2000, 2004).

10.2.2 The JIP-Test

The shape of the OJIP transient is very sensitive to stress. Strasser and co-workers have developed the JIP-test, used today world wide, which is an analysis of the OJIP fluorescence transient, by which the changes in the shape of OJIP are utilised for the detection and evaluation of the impact of several types of stress at different sites in the photosynthetic process; all three (interrelated) components of plants' vitality, namely photosynthetic activity, adaptability and stability, are hence assessed (for a review, see Strasser et al. 2004 and references therein; for the recent extension of the JIP-test that includes electron transfer through PSI, see Tsimilli-Michael and Strasser 2008; Strasser et al. 2010).

The JIP-test employs two types of data processing:

(a) Utilisation of the whole transient. Using the differences of suitably normalised transients exhibited by stressed and non-stressed plants, more bands can be detected, denoted by the series O–L–K–J–I–H–G–P, which are usually hidden among the O–J–I–P steps of the original transients (Strasser et al. 2004); the bands between the O- and P-step are labelled in alphabetic order, from slower to faster events. These phases provide a wealth of information, as they allow the recognition and, moreover, a semi-quantitative evaluation of the impact of stress on different sites of the photosynthetic machinery.

(b) Utilisation of selected original fluorescence data. On the basis of the *Energy Flux Theory in Biomembranes* (Strasser 1978, 1981), a theoretical model was developed, by which structural and functional parameters of the whole sequence of events from exciton trapping to the reduction of the PSI electron acceptor side are derived. Thus, changes in the OJIP shape are translated to changes of the structural and functional parameters.

The following original data extracted from the recorded OJIP are used (see also Table 10.1): the maximal measured fluorescence intensity, F_P, equal here to F_M since the excitation intensity is high enough to ensure the closure of all active (Q_A reducing) RCs; the fluorescence intensity at 20 μs, considered as F_0 (all RCs open); the fluorescence intensities at 50 and 300 μs ($F_{50\ \mu s}$ and $F_{300\ \mu s}$) required for the calculation of the initial slope, taken as $(dF/dt)_0 \cong (F_{300\ \mu s} - F_{50\ \mu s})/(250\ \mu s)$; the fluorescence intensities at 2 ms (J step; F_J) and at 30 ms (I-step; F_I); the complementary area (Area) above the fluorescence curve, i.e., the area between the curve, the horizontal line $F = F_M$ and the vertical lines at $t = 20$ μs and at $t = t_{F_M}$ (the time at which F_M is reached).

A schematic summary of the JIP-test concept, based on the energy fluxes and their bifurcations from PSII to the end electron acceptors of PSI, is shown in Fig. 10.1 (grey or white arrows for fluxes utilised or not for electron transfer, respectively). The figure includes definitions and equations, demonstrating also how the parameters are linked with the fluorescence signals selected from the OJIP fluorescence transient. Subscript "0" indicates that a parameter refers to the starting conditions (onset of illumination; all RCs open) of the photosynthetic sample. For more details, see Table 10.1.

Table 10.1 Glossary, definition of terms and formulae of the JIP-test parameters (see also Fig. 10.1) used for the analysis of the Chl *a* fluorescence transient OJIP emitted by dark-adapted photosynthetic samples (after Strasser et al. 2010)

Data extracted from the recorded fluorescence transient OJIP	
F_t (or, simply F)	Fluorescence at time t after onset of actinic illumination
$F_{20\ \mu s}$	First reliable recorded fluorescence at 20 μs
$F_{300\ \mu s}$	Fluorescence at 300 μs
$F_J \equiv F_{2\ ms}$	Fluorescence at the J-step (2 ms) of OJIP
$F_I \equiv F_{30\ ms}$	Fluorescence at the I-step (30 ms) of OJIP
F_P	Maximal recorded fluorescence, at the peak P of OJIP
t_{F_M}	Time (in ms) to reach the maximal possible fluorescence F_M
Area	Total complementary area between the fluorescence transient and $F = F_M$
Fluorescence parameters derived from the extracted data	
$F_0 \cong F_{20\ \mu s}$	Minimal fluorescence, when all RCs are open
F_M (=F_P)	Maximal fluorescence, when all RCs are closed ($F_M = F_P$ when the actinic light intensity is above 500 μmol photons $m^{-2}\ s^{-1}$ and provided that all RCs are active as Q_A reducing)
$F_\upsilon \equiv F_t - F_0$	Variable fluorescence at time t
$F_V \equiv F_M - F_0$	Maximal variable fluorescence
$S_m \equiv \text{Area}/(F_M - F_0) = \text{Area}/F_V$	Normalised Area
$V_t \equiv F_\upsilon/F_V \equiv (F_t - F_0)/(F_M - F_0)$	Relative variable fluorescence at time t
$M_0 \equiv [(\Delta F/\Delta t)_0]/(F_M - F_{50\ \mu s})$ $\equiv 4(F_{300\ \mu s} - F_{50\ \mu s})/(F_M - F_{50\ \mu s})$	Approximated initial slope (in ms^{-1}) of the fluorescence transient normalised on the maximal variable fluorescence F_V
Biophysical parameters derived from the fluorescence parameters	
$EC_0/RC = S_m = \text{Area}/(F_M - F_0)$	A measure of total electron carriers per RC
De-excitation rate constants of PSII antenna	
$k_N = (ABS)\ k_F\ (1/F_M)$	Nonphotochemical de-excitation rate constant (ABS: absorbed energy flux; k_F: rate constant for fluorescence emission)
$k_P = (ABS)\ k_F\ (1/F_0 - 1/F_M) = k_N\ (F_V/F_0)$	Photochemical de-excitation rate constant
Specific energy fluxes (per Q_A-reducing PSII reaction centre—RC)	
$ABS/RC = M_0\ (1/V_J)\ (1/\varphi_{Po})$	Absorption flux (of antenna Chls) per RC (also a measure of PSII apparent antenna size)
$TR_0/RC = M_0\ (1/V_J)$	Trapped energy flux (leading to Q_A reduction) per RC
$ET_0/RC = M_0\ (1/V_J)\ (1 - V_J)$	Electron transport flux (further than Q_A^-) per RC
$RE_0/RC = M_0\ (1/V_J)\ (1 - V_I)$	Electron flux reducing end electron acceptors at the PSI acceptor side, per RC

(continued)

Table 10.1 (continued)

Quantum yields and efficiencies	
$\varphi_{Pt} \equiv TR_t/ABS = [1 - (F_t/F_M)] = \Delta F_t/F_M$	Quantum yield for PSII primary photochemistry at any time t, according to the general equation of Paillotin (1976)
$\varphi_{Po} \equiv TR_0/ABS = [1 - (F_0/F_M)]$	Maximum quantum yield for PSII primary photochemistry
$\psi_{Eo} \equiv ET_0/TR_0 = (1 - V_J)$	Efficiency/probability that an electron moves further than Q_A^-
$\varphi_{Eo} \equiv ET_0/ABS = [1 - (F_0/F_M)]\,(1 - V_J)$	Quantum yield for electron transport (ET)
$\delta_{Ro} \equiv RE_0/ET_0 = (1 - V_I)/(1 - V_J)$	Efficiency/probability with which an electron from the intersystem electron carriers is transferred to reduce end electron acceptors at the PSI acceptor side (RE)
$\varphi_{Ro} \equiv RE_0/ABS = [1 - (F_0/F_M)]\,(1 - V_I)$	Quantum yield for reduction of end electron acceptors at the PSI acceptor side (RE)
$\gamma_{RC} = Chl_{RC}/Chl_{total} = RC/(ABS + RC)$	Probability that a PSII Chl molecule functions as RC
$RC/ABS = \gamma_{RC}/(1 - \gamma_{RC}) = \varphi_{Po}\,(V_J/M_0) = (ABS/RC)^{-1}$	Q_A-reducing RCs per PSII antenna Chl (reciprocal of ABS/RC)
Performance indexes (*products of terms expressing partial potentials at steps of energy bifurcations*)	
$PI_{ABS} \equiv \frac{\gamma_{RC}}{1-\gamma_{RC}} \cdot \frac{\varphi_{Po}}{1-\varphi_{Po}} \cdot \frac{\psi_o}{1-\psi_o}$	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors
$PI_{total} \equiv PI_{ABS} \cdot \frac{\delta_{Ro}}{1-\delta_{Ro}}$	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors

"Fluorescence" stands as a shortening of "fluorescence intensity"
Subscript "0" (or, "o" when written after another subscript) indicates that the parameter refers to the onset of illumination, when all RCs are assumed to be open
RC refers to the active (Q_A-reducing) PSII reaction centres

The energy fluxes are: for PSII absorption (ABS); trapping in PSII (TR_0), i.e. reduction of Pheo (pheophytin) and Q_A; electron transport (ET_0) from Q_A^- to the intersystem electron acceptors, i.e., Q_B (secondary electron quinone acceptor), PQ (plastoquinone), Cyt (cytochrome b_6/f) and PC (plastocyanin), or to any acceptor X (e.g., O_2) before PSI; reduction of end acceptors at the PSI electron acceptor side (RE_0), i.e., NADP (nicotinamide adenine dinucleotide phosphate) and Fd (ferredoxin).

The efficiencies/yields, defined as ratios of energy fluxes (and indicated by line arrows), are: the maximum quantum yield of primary photochemistry, $TR_0/ABS = \boldsymbol{\varphi}_{Po} = 1 - (F_0/F_M)$; the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^-, $ET_0/TR_0 = \boldsymbol{\psi}_{Eo} = 1 - V_J$; the quantum yield of electron transport further than Q_A^-,

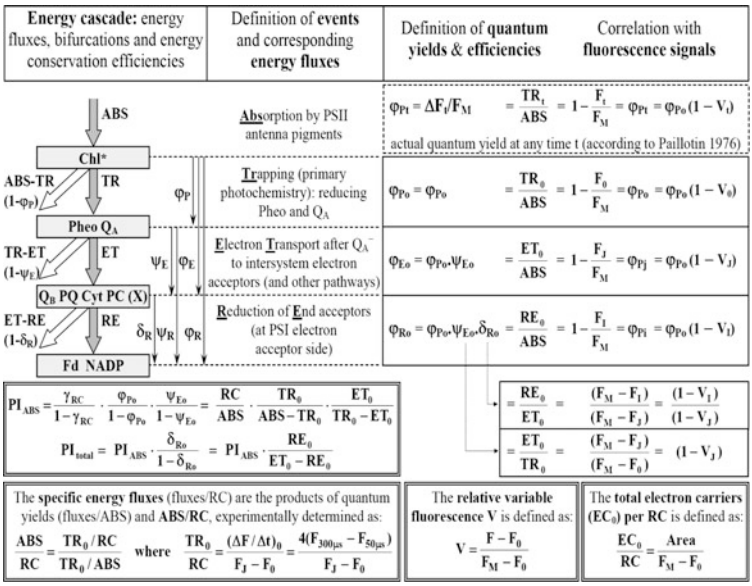

Fig. 10.1 A schematic summary of the JIP-test concept with definitions and equations, based on the energy fluxes (*wide arrows*) and their bifurcations from PSII to the end electron acceptors of PSI. Efficiencies/yields (*line arrows*) are defined as ratios of fluxes and further linked with fluorescence signals selected from the OJIP fluorescence transient. Adopted from Strasser et al. 2010. For details, see text and Table 10.1

$ET_0/ABS = \boldsymbol{\varphi}_{Eo} = \boldsymbol{\varphi}_{Po}.\ \boldsymbol{\psi}_{Eo}$; the efficiency with which an electron can move from the reduced intersystem electron carriers to the PSI end electron acceptors, $RE_0/ET_0 = \boldsymbol{\delta}_{Ro} = (1 - V_I)/(1 - V_J)$; the quantum yield for reduction of PSI end electron acceptors, $RE_0/ABS = \boldsymbol{\varphi}_{Ro} = \boldsymbol{\varphi}_{Po}.\ \boldsymbol{\psi}_{Eo}.\ \boldsymbol{\delta}_{Ro}$; the efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the PSI end electron acceptors, $RE_0/TR_0 = \boldsymbol{\psi}_{Ro} = \boldsymbol{\psi}_{Eo}.\ \boldsymbol{\delta}_{Ro}$. The definition of the relative variable fluorescence V is also given.

The derivation of the specific fluxes (fluxes per active, i.e., Q_A reducing, reaction centre-RC; arbitrary units) from the quantum yields (which are efficiencies on absorption basis; i.e., fluxes per ABS) is also depicted, as well as the derivation of the total electron carriers per reaction centre (EC_0/RC).

The figure includes also the definition of the performance indexes PI_{ABS} and PI_{total} (or PI_{tot}) as products of terms expressing energy bifurcations from PSII to the intersystem electron transport chain or to PSI end electron acceptors, respectively (where $\gamma_{RC} = Chl_{RC}/Chl_{total}$ is the fraction of PSII reaction centre Chl molecules relative to the total PSII Chl content; since $Chl_{total} = Chl_{antenna} + Chl_{RC}$, then $\gamma_{RC}/(1 - \gamma_{RC}) = Chl_{RC}/Chl_{antenna} = RC/ABS$).

10.3 Case Studies

We here chose a case study to demonstrate in detail how we apply biophysical phenomics to assess the impact of symbiosis on the photosynthetic mechanism. We started from the original fluorescence transients exhibited by dark-adapted leaves of onion plants (*Allium cepa*) that were previously obtained and partly used in an earlier publication of ours (Tsimilli-Michael and Strasser 2008), in which further details can be found. The plants were inoculated with *P. indica* or commercially available endomycorrhizae (AEGIS Endo Gel—SYTEN Company; composed of *Glomus intaradices* and *Glomus masseuse*) and grown in a greenhouse; non-inoculated plants of the same age were used as control. Figure 10.2 depicts the average Chl *a* fluorescence transient (±SD, from 20–25 replicates) for each case, expressed as F/F_0 (where F_0 is the initial fluorescence, at 20 μs), so that differences concerning the F_0 values would not interfere with the other differences. Further than demonstrating that inoculation results in bigger maximum variable fluorescence ($F_M - F_0$; normalised here on F_0), Fig. 10.2 reveals also that it causes a decrease of the heterogeneity among replicates, which is an indeed very interesting finding; moreover, heterogeneity decrease more when *P. indica* instead of AMF was used. Taken together, these two observations indicate that symbiosis has a stronger beneficial effect on the less favoured plants among the tested population.

Figure 10.3 presents the average Chl *a* fluorescence transients for the three cases (also expressed as F/F_0) to facilitate their comparison. Moreover, the figure depicts the main steps O, J, I and P and demonstrates the approximation used for calculating the initial slope. We observe that the transients exhibit differences concerning their shape. We note that the increase of the maximum variable fluorescence is equivalent to an increase of the maximum quantum yield of primary photochemistry φ_{Po} (see text; also Fig. 10.1 and Table 10.1), the parameter commonly used as the (only) criterion for comparing dark-adapted photosynthetic samples in respect to their activity.

In order to obtain a semi-quantitative evaluation of the observed differences and of other hidden differences, the three average fluorescence transients, denoted as C (non-inoculated), M (inoculated with AMF) and P (inoculated with *P. indica*), were expressed and plotted in Fig. 10.4 (left vertical axis) as kinetics of different expressions of relative variable fluorescence (see legend for details). In each of the plots (a), (b) and (c), the difference kinetics of the respective relative variable fluorescence is also presented (right vertical axis). This way of data processing permits us to get several interesting information.

The difference kinetics of $\Delta V \equiv \Delta[(F - F_0)/(F_M - F_0)]$, depicted in panel (a) together with the V kinetics, exhibit bands of negative sign in the O–I part of the OJIP transient, which indicates that processes from exciton trapping to PQ reduction (reflected in O–I; Strasser et al. 2004, 2007, 2010) are faster in inoculated

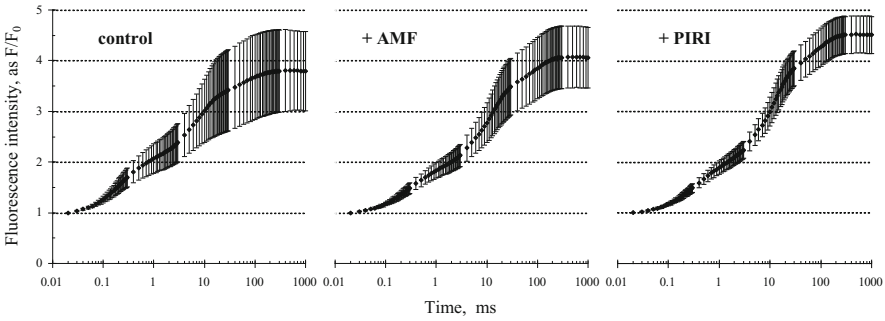

Fig. 10.2 Chl *a* fluorescence transients exhibited, upon illumination, by dark-adapted leaves of onion (*Allium cepa*) plants, which were grown without inoculation (control; *left panel*) or were inoculated with a commercial mixture of arbuscular mycorrhiza fungi (+AMF; *middle panel*) or with *P. indica* (+PIRI; *right panel*). The transients, expressed as F/F_0 (F_0 denoting the initial fluorescence intensity, taken at 20 μs, where all reaction centres (RCs) of photosystem (PS) II are considered to be open), are plotted on logarithmic time scale from 20 μs to 1 s. For each case, the average transient ± SD from 20 to 25 replicates is presented. Data were obtained from the study published by Tsimilli-Michael and Strasser (2008)

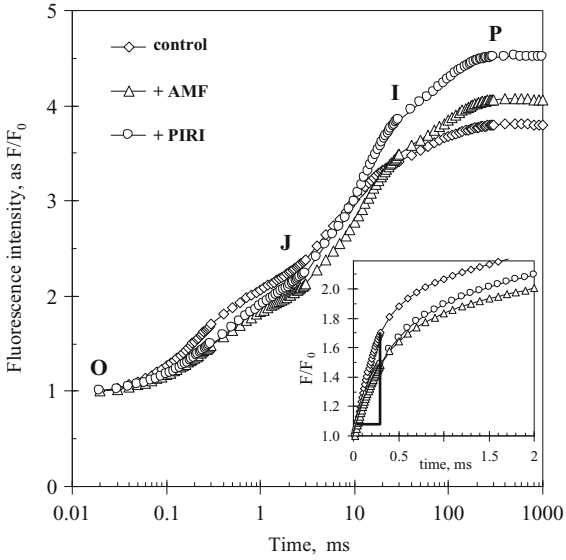

Fig. 10.3 The average Chl *a* fluorescence transients OJIP (from Fig. 10.2) are depicted, referring to onion plants that were grown without inoculation (control) or were inoculated with a mixture of arbuscular mycorrhiza fungi (+AMF) or with *P. indica* (+PIRI). The steps O (at 20 μs), J (at 2 ms), I (at 30 ms) and P (*peak*) are marked. The corresponding fluorescence intensities F_0, F_J, F_I and F_P (F_P is equal here to F_M—maximal possible intensity—due to the high excitation intensity), along with the initial slope $(dF/dt)_0$, are the selected fluorescence data used by the JIP-test for the translation of a transient to structural and functional parameters of the photosynthetic machinery (see Fig. 10.1). The approximation of the initial slope by $(\Delta F/\Delta t)_0$, taken between 50 and 300 μs, is graphically demonstrated in the *insert*, where the initial parts of the transients (up to 2 ms) are plotted on a linear time scale. For other details, see legend of Fig. 10.2

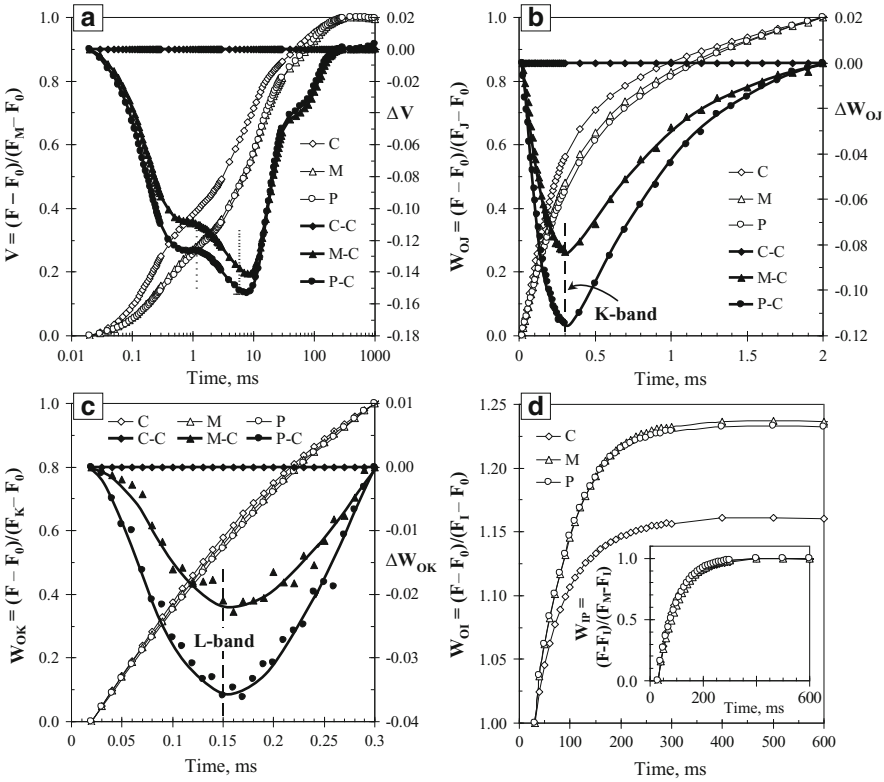

Fig. 10.4 The average Chl *a* fluorescence kinetics obtained from non-inoculated plants (C, control) and plants inoculated with arbuscular mycorrhiza fungi (M) or *P. indica* (P), and depicted as F/F_0 vs. t in Fig. 10.3, are presented (*open symbols*; *left vertical axis*) as kinetics of different expressions of relative variable fluorescence: (**a**) between F_0 and F_M, as: $V = (F - F_0)/(F_M - F_0)$; (**b**) between F_0 and F_J, as $W_{OJ} = (F - F_0)/(F_J - F_0)$; (**c**) between F_0 and $F_{300\ \mu s}$ $(= F_K)$, as $W_{OK} = (F - F_0)/(F_K - F_0)$; (**d**) between F_0 and F_I (for $F \geq F_I$), as $W_{OI} = (F - F_0)/(F_I - F_0)$ and, in the insert, between F_I and F_M (for $F \geq F_I$), as $W_{IP} = (F - F_I)/(F_M - F_I)$. In each of the plots (**a**), (**b**) and (**c**), the difference kinetics, ΔV, ΔW_{OJ} and ΔW_{OK} respectively, are also presented (*closed symbols*; *right vertical axis*). The difference kinetics were calculated by subtracting the kinetics of the control from all three kinetics and they are hence denoted as C–C, M–C and P–C. Plots (**b**) and (**c**) clearly reveal the K- and L-bands, respectively. For other details, see legend of Fig. 10.2

than in non-inoculated plants. Actually, in this time range each band is double, distinguished in a band for the O–J part (photochemical phase) and another for the J–I part (reflecting electron transfer from Q_A^- to PQ). The former indicates a higher probability of energy conservation and the latter a more efficient electron transfer to PQ. It is worth pointing out that, by this way of transient processing, the cases of AMF- and *P. indica*-inoculation cases are better resolved than in the original transients, revealing also that the effect is more pronounced when *P. indica* instead

of AMF was used. The third negative band in the I–P part of the OJIP transient will be discussed in respect to panel (d).

If we now focus on the differences concerning the O–J phase of the transients, which are revealed by the $\Delta W_{OJ} \equiv \Delta[(F - F_0)/(F_J - F_0)]$ kinetics, presented together with the W_{OJ} kinetics in panel (b), we observe bands at 300 μs, denoted in the JIP-test as K-bands. According to the interpretation of the K-band (analytically presented in Strasser et al. 2004), the negative sign of the K-bands in Fig. 10.4b indicates that the inoculated compared to the control samples have either a more active oxygen evolving system or a smaller PSII antenna size (i.e., smaller number of antenna molecules supplying excitation energy to the RC)—in other words a bigger number of active RCs per Chl. Both effects should be considered as beneficial for the photosynthetic mechanism, hence for the plant. It is worth noting that, as Fig. 10.4b reveals, the beneficial effect of *P. indica* is bigger than that of AMF.

In the plot of Fig. 10.4c, which presents the difference kinetics $\Delta W_{OK} \equiv \Delta[(F - F_0)/(F_K - F_0)]$ of the O–K phase, i.e., in the 20–300 μs time range, together with the W_{OK} kinetics, we observe bands at 150 μs, denoted in the JIP-test as L-bands. The negative sign of the L-band indicates that the extent of energetic connectivity among PSII units in the inoculated is bigger than in the non-inoculated samples, which reveals again a beneficial role of symbiosis, since energetic connectivity increases the utilisation of excitation energy and is also a factor of stability of a photosynthetic system (Strasser et al. 2004, 2007). Notably, the extent of connectivity appears to be bigger when *P. indica* instead of AMF was used.

Figure 10.4d exploits, with two different normalisations, the I–P part of the transients, which reflects processes related with the electron flow from reduced PQ (PQH_2) to PSI end electron acceptors (Schansker et al. 2005; Tsimilli-Michael and Strasser 2008; Strasser et al. 2010). With the normalisation employed for the main figure, the differences among the three cases concerning the relative amplitude of the I–P phase are depicted. We observe that this amplitude, being the same when the plants were inoculated with AMF or *P. indica*, is bigger than in non-inoculated plants. However, as revealed in the insert, where the normalisation was done between F_I and F_M, the kinetics of "filling up" the differing amplitudes are identical for all three cases. This means that symbiosis results in a bigger pool of end electron acceptors to be filled with electrons coming from PQH_2, but it does not affect the rate constants of the electron transfer pathway.

In conclusion, Fig. 10.4 demonstrates, though semi-quantitatively, that symbiosis has beneficial effects at different sites in the photosynthetic process, concerning both activity and stability, and that the beneficial effect of *P. indica* is bigger than that of AMF.

For a quantitative comparison we further analysed the fluorescence transients with the JIP-test equations and calculated a constellation of structural and functional parameters (see Fig. 10.1 and Table 10.1); each raw transient (20–25 replicates) was processed and the calculated parameters were averaged for each of the three cases of onion plants (non-inoculated or inoculated with AMF or *P. indica*). Among all the

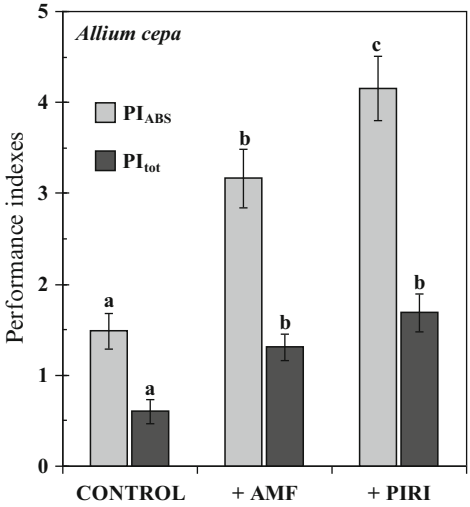

Fig. 10.5 Performance indexes PI_{ABS} and PI_{tot} (average values ± SE, from 20 to 25 replicates), calculated by analysing with the JIP-test the OJIP fluorescence transients depicted in Fig. 10.3. The plants (*Allium cepa*) were grown without inoculation (CONTROL) or were inoculated with a mixture of arbuscular mycorrhiza fungi (+AMF) or with *P. indica* (+PIRI). For each performance index, different letters above the columns indicate statistically significant differences ($P < 0.025$). For other details, see legend of Fig. 10.2

parameters determined by this analysis, the performance index PI_{tot} is the most sensitive as it evaluates the overall photosynthetic performance/behaviour; we here remind that PI_{tot} has been defined as the product of terms expressing potentials for photosynthetic performance (partial performances) at the sequential energy bifurcations from exciton up to the reduction of PSI end acceptors. Figure 10.5 presents the average PI_{tot} (± SE) for the three cases of onion plants. The other performance index, PI_{ABS}, which refers to the sequential energy bifurcations from exciton up to PQ reduction only, is also included. Figure 10.5 clearly demonstrates, in agreement with the semi-quantitative results from Fig. 10.4, that the positive effects of symbiosis are pronounced and that the benefit from inoculation with *P. indica* is bigger compared to that from inoculation with AMF.

What is more important is that the performance indexes PI_{tot} and PI_{ABS} correlate well with physiological parameters. Figure 10.6 demonstrates, as an example, the very good correlation of PI_{tot} and PI_{ABS} with the height of plants; the figure was constructed after processing fluorescence transients obtained in an earlier investigation of ours (partly used in Strasser et al. 2007), in which we applied the here presented approach for a comparative study of the beneficial role of the typical arbuscular mycorrhiza fungi *Glomus mosseae* and *Glomus caledonium* and of *P. indica* on chick pea (*Cicer arietinum* L. Chafa variety) plants exposed to cadmium (Cd) stress.

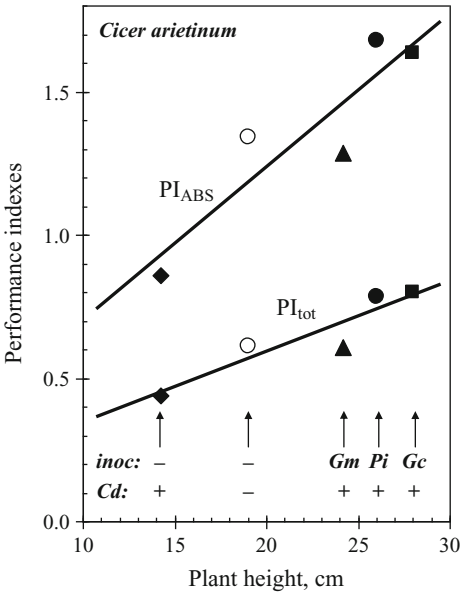

Fig. 10.6 Correlation of the performance indexes PI_{ABS} and PI_{tot} (biophysical parameters), derived by analysing with the JIP-test the OJIP fluorescence transients exhibited by dark-adapted leaves of chick pea (*Cicer arietinum* L. Chafa variety) plants, with the height of the plants (morphological parameter). The data refer to non-inoculated plants in the absence (*open circles*) or presence (*closed diamonds*) of cadmium (*Cd*) and to plants exposed to Cd stress after being inoculated with *G. mosseae* (*Gm*, *closed triangles*), *G. caledonium* (*Gc*, *closed squares*) or *P. indica* (*Pi*, *closed circles*), as indicated. Data were obtained from the study published by Strasser et al. (2007)

The performance indexes can be well used for routine screening of plants and evaluation of the overall impact of symbiosis on photosynthetic performance/behaviour. However, for a more detailed assessment of the impact at different sites the individual structural and functional parameters need to be compared. Figure 10.7 presents a collection of the so-called photosynthetic *behaviour patterns* for the analytically presented study on onion plants, the case of Cd stressed chick peas and also for four other cases where only AMF (or together with bacteria in 10.7c) was used for colonisation. Without entering in details that the reader can find in the cited references (see legend), we can clearly deduce from Fig. 10.7 that the impact of symbiosis with AMF or with *P. indica* is basically the same, both concerning the performance indexes and the individual parameters, despite differences of the extent of the impact among the six cases. It is also worth pointing out that similar behaviour patterns were found when *P. indica* or ectomycorrhiza (AEGIS Ecto Gel—SYTEN Company; composed of a spore mixture of *Rhizogon* sp., *Pisolithus* sp. and *Schleroderma* sp.) were used to colonise pine trees (*Pinus halepensis*), and that *P. indica* was found to be even more beneficial than ectomycorrhiza (Tsimilli-Michael and Strasser 2008).

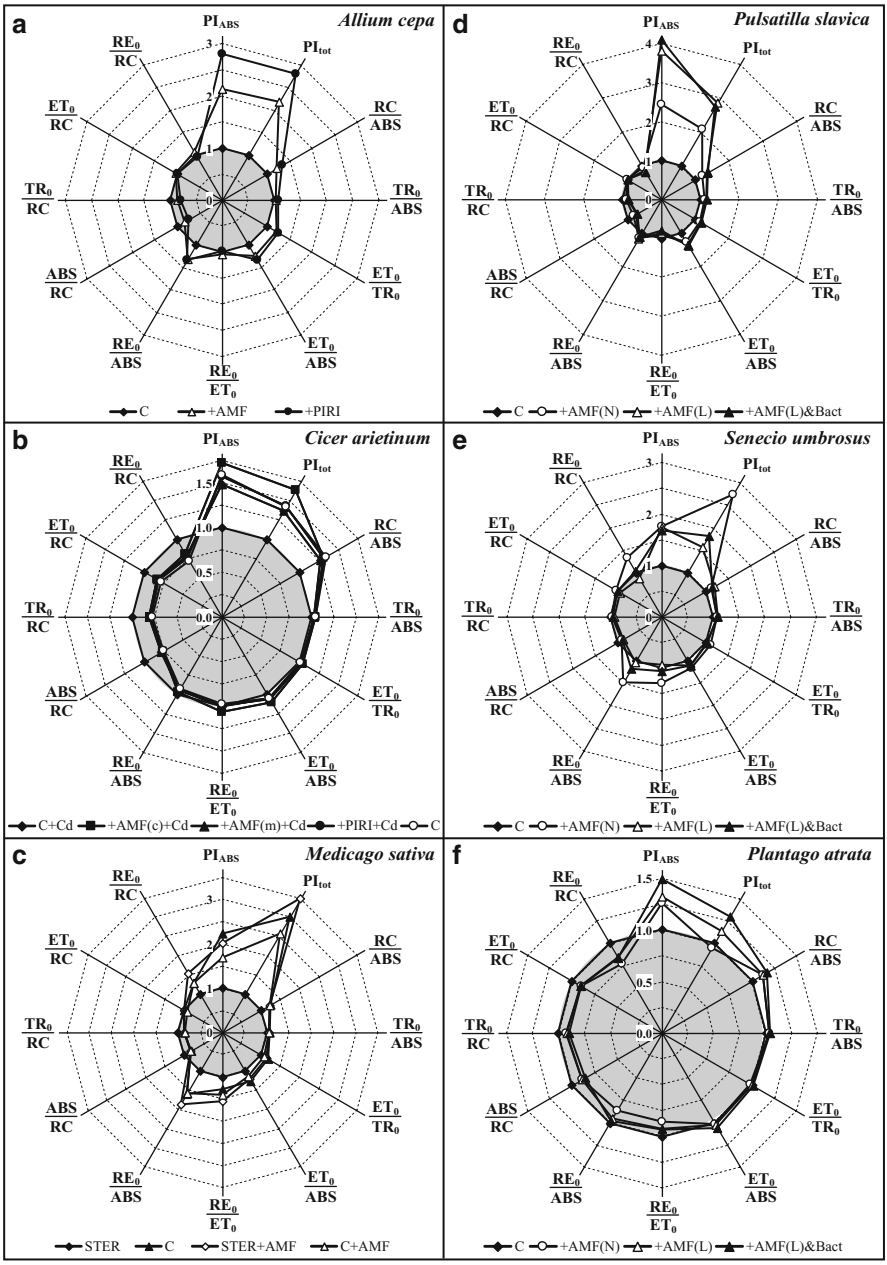

Fig. 10.7 A collection of photosynthetic behaviour patterns demonstrating the impact of symbiosis with arbuscular mycorrhiza fungi (AMF) and/or *P. indica* on six different plants grown under the same or different conditions. Each pattern comprises the same 12 structural and functional parameters and is thus presented by a dodecagon. For each plant, the parameters were derived by analysing with the JIP-test the fluorescence transients OJIP exhibited by dark-adapted leaves upon

10.4 Concluding Remarks

We have here presented a comprehensive review for the application of *Biophysical Phenomics*—a description of a biophysical phenotype in respect to the photosynthetic apparatus provided by the JIP-test—in the evaluation of the impact of mycorrhization with *P. indica* on the photosynthetic performance/behaviour, in comparison also with the impact of typical arbuscular mycorrhizal fungi. The results demonstrate that *P. indica* is equally, or even more, beneficial than AMF and that it affects in an AMF-like way the different components of the photosynthetic process. The additional take-home message of this review is that the JIP-test is a powerful tool for the in vivo and in situ recognition and evaluation of the effectiveness of symbiosis, which cannot be foreseen or taken for granted as it depends on complex interactions between plants, symbionts and environment. It should be also emphasised that the method provides early diagnosis and the experimentation is simple, fast and non-invasive. From the experimental point of view, the analytical presentation and application of our method, the JIP-test, for one

Fig. 10.7 (continued) illumination and are presented after normalisation on the corresponding values of the chosen reference case (non-inoculated plant), which is thus depicted by a regular dodecagon (values of all parameters equal to unity). Hence the deviations of the behaviour patterns of the inoculated samples from that of the reference sample demonstrate the fractional impacts of symbiosis. The photosynthetic parameters are: the quantum yields TR_0/ABS ($= \varphi_{Po}$), ET_0/ABS ($= \varphi_{Eo}$) and RE_0/ABS ($= \varphi_{Ro}$); the probabilities/efficiencies ET_0/TR_0 ($= \psi_{Eo}$) and RE_0/ET_0 ($= \delta_{Ro}$); the specific energy fluxes ABS/RC, TR_0/RC, ET_0/RC and RE_0/RC; the reaction centres per absorption (or per antenna Chl *a*), RC/ABS; the performance indexes PI_{ABS} and PI_{tot}. For the links of the parameters with fluorescence signals, see Fig. 10.1 and Table 10.1. The six panels were constructed from data obtained in previous studies of ours; for further details, see the corresponding publications. *Panel* (**a**) refers to onion (*Allium cepa*) plants grown without inoculation (C-control; reference case) or inoculated with a commercial mixture of arbuscular mycorrhiza fungi (+AMF) or *P. indica* (+PIRI); data from Tsimilli-Michael and Strasser, 2008. *Panel* (**b**) refers to chick peas (*Cicer arietinum* L. Chafa variety) that were exposed to cadmium (+Cd) stress, being either non-inoculated (C + Cd,; reference case) or inoculated with *G. caledonium* (+AMF (c) + Cd), *G. mosseae* (+AMF(m) + Cd) or *P. indica* (+PIRI + Cd); the case of non-inoculated and non-exposed to Cd stress is also depicted for comparison (C); data from Strasser et al. 2007. *Panel* (**c**) refers to alfalfa (*Medicago sativa* L.) plants grown without or with (+AMF) inoculation with *G. fasciculatum* M107, in untreated loamy chernozem soil (C), where the usual rhizosphere microflora was present, or in gamma-irradiated sterile soil (STER); the case of plants grown without inoculation in sterile soil (STER, *closed diamonds*) was used as the reference case; data from Tsimilli-Michael et al. 2000. *Panels* (**d**), (**e**) and (**f**) refer, respectively, to three endangered plant species (native in Tatra mountains, Poland), namely *Pulsatilla slavica*, *Senecio umbrosus* and *Plantago atrata*, that were either non-inoculated (C; reference case) or inoculated with native AMF isolated from their natural habitats (+AMF(N)), or a mixture of AMF strains available in the laboratory of Prof. Katarzyna Turnau in the Institute of Environmental Sciences, Jagiellonian University, Krakow, Poland (+AMF(L)) or a combination of AMF(L) with rhizobacteria (+AMF(L)&Bact); data from Zubek et al. 2009

case study (that of onion plants), can be followed easily by the reader, who can further consult, for a deeper understanding, the cited references.

References

Biro B, Köves-Péchy K, Tsimilli-Michael M, Strasser RJ (2006) Role of beneficial microsymbionts on the plant performance and plant fitness, Chap. 14. In: Mukerji KG, Manoharachary C, Singh J (eds) Microbial activity in the rhizosphere, soil biology (Varma A, Series Editor), vol 7. Springer, Berlin, pp 265–296

Jurkiewicz A, Ryszka P, Anielska T, Waligórski P, Bialońska D, Góralska K, Tsimilli-Michael M, Turnau K (2010) Optimization of culture conditions of *Arnica montana* L.: effects of mycorrhizal fungi and competing plants. Mycorrhiza 20:293–306

Kumari R, Pham GH, Prasad R, Sachdev M, Srivastava A, Yadav V, Verma PK, Sharma S, Malla R, Singh A, Maurya AK, Prakash S, Pareek A, Rexer K-H, Kost G, Garg AP, Oelmueller R, Sharma MC, Varma A (2004) *Piriformospora indica*: fungus of the millenium. In: Podila G, Varma A (eds) Basic research and applications: mycorrhizae. IK International/Kluwer Academic Press, India and New York/Holland, pp 259–295

Paillotin G (1976) Movement of excitations in the photosynthetic domains of photosystem II. J Theor Biol 58:237–252

Rai M, Varma A (2005) Arbuscular mycorrhiza-like biotechnological potential of *Piriformospora indica*, which promotes the growth of *Adhatpda vasica* Nees. Electron J Biotechnol 8:107–112

Rai M, Sahay N, Varma A (2000) In vitro growth promotion of maize and soybean by *Piriformospora indica*, an emerging endophyte. J Environ Biol 9:1–5

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthus calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in fields trial. Mycorrhiza 11:123–128

Rai M, Varma A, Pandey AK (2004) Antifungal potential of *Spilanthes calva* after inoculation of *Piriformospora indica*. Mycoses 47:479–481

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Schansker G, Tóth SZ, Strasser RJ (2005) Methylviologen and dibromothymoquinone treatments of pea leaves reveal the role of photosystem I in the Chl *a* fluorescence rise OJIP. Biochim Biophys Acta 1706:250–261

Singh A, Varma A (2001) AMF-like fungus, *Piriformospora indica*, shows promising applications. Int Symbiosis 1:1–4

Singh A, Sharma J, Rexer K-H, Varma A (2000) Plant productivity determinants beyond minerals, water and light. *Piriformospora indica*: a revolutionary plant growth promoting fungus. Curr Sci 79:101–106

Singh A, Singh A, Varma A (2002) *Piriformospora indica* - in vitro raised leguminous plants: a new dimension in establishment and phyto-promotion. Ind J Biotechnol 1:372–376

Singh A, Singh A, Kumari M, Rai MK, Varma A (2003) Biotechnology importance of *Piriformospora indica*—a novel symbiotic mycorrhiza-like fungus: an overview. Ind J Biotechnol 2:65–75

Strasser RJ (1978) The grouping model of plant photosynthesis. In: Akoyunoglou G (ed) Chloroplast development. Elsevier, Dordrecht, pp 513–524

Strasser RJ (1981) The grouping model of plant photosynthesis: heterogeneity of photosynthetic units in thylakoids. In: Akoyunoglou G (ed) Photosynthesis III. Structure and molecular organisation of the photosynthetic apparatus. Balaban International Science Services, Philadelphia, PA, pp 727–737

Strasser RJ (1985) Dissipative Strukturen als thermodynamischer Regelkreis des Photosyntheseapparates. Ber Deutsche Bot Ges Bd 98:53–72

Strasser RJ (1988) A concept for stress and its application in remote sensing. In: Lichtenthaler HK (ed) Applications of chlorophyll fluorescence. Kluwer Academic Publishers, The Netherlands, pp 333–337

Strasser RJ, Govindjee (1991) The F_0 and the O-J-I-P fluorescence rise in higher plants and algae. In: Argyroudi-Akoyunoglou JH (ed) Regulation of chloroplast biogenesis. Plenum, New York, pp 423–426

Strasser RJ, Tsimilli-Michael M (2005) State changes realizing adaptation to stress as the result of an optimized redistribution of functional microstates. In: van der Est, Bruce D (eds) Photosynthesis: fundamental aspects to global perspectives. Allen, Canada, pp 537–540

Strasser RJ, Srivastava A, Govindjee (1995) Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria. Photochem Photobiol 61:32–42

Strasser RJ, Srivastava A, Tsimilli-Michael M (2000) The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Yunus M, Pathre U, Mohanty P (eds) Probing photosynthesis: mechanism, regulation and adaptation, Chap. 25. Taylor and Francis, London, UK, pp 443–480

Strasser RJ, Tsimilli-Michael M, Srivastava A (2004) Analysis of the Chlorophyll *a* Fluorescence Transient. In: Papageorgiou GC, Govindjee (eds) Chlorophyll fluorescence: a signature of photosynthesis, advances in photosynthesis and respiration series (Govindjee—Series Editor) vol 19, chapter 12. Kluwer Academic Publishers, The Netherlands, pp 321–362

Strasser RJ, Tsimilli-Michael M, Dangre D, Rai M (2007) Biophysical phenomics reveals functional building blocks of plants systems biology: a case study for the evaluation of the impact of mycorrhization with *Piriformospora indica,* Chap. 21. In: Varma A, Oelmüller R (eds) Advanced techniques in soil microbiology, soil biology, vol 11. Springer, Berlin, Heidelberg, pp 319–342

Strasser JR, Tsimilli-Michael M, Qiang S, Goltsev V (2010) Simultaneous in vivo recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*. Biochim Biophys Acta 1797:1313–1326

Tsimilli-Michael M, Strasser RJ (2002) Mycorrhization as a stress adaptation procedure. In: Gianinazzi S, Haselwandter K, Schüepp H, Barea JM (eds) Mycorrhiza technology in agriculture: from genes to bioproducts. Birkhauser/Verlag, Basel/Boston/Berlin, pp 199–209

Tsimilli-Michael M, Strasser RJ (2008) In vivo assessment of stress impact on plants' vitality: applications in detecting and evaluating the beneficial role of mycorrhization on host plants. In: Varma A (ed) Mycorrhiza: state of the art, genetics and molecular biology, eco-function, biotechnology, eco-physiology, structure and systematics, 3rd edn. Springer, Berlin, Heidelberg, pp 679–703

Tsimilli-Michael M, Krüger GHJ, Strasser RJ (1995) Suboptimality as driving force for adaptation: a study about the correlation of excitation light intensity and the dynamics of fluorescence emission in plants. In: Mathis P (ed) Photosynthesis: from light to biosphere, vol V. Kluwer Academic Publishers, The Netherlands, pp 981–984

Tsimilli-Michael M, Krüger GHJ, Strasser RJ (1996) About the perpetual state changes in plants approaching harmony with their environment. Archs Sci Genève 49:173–203

Tsimilli-Michael M, Eggenberg P, Biro B, Köves-Pechy K, Vörös I, Strasser RJ (2000) Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and *Azospirillum* and *Rhizobium* nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the chlorophyll *a* polyphasic fluorescence transient O-J-I-P. Appl Soil Ecol 15:169–182

Varma A (1995) Mycorrhizae: their application in micropropagated plantlets. Crit Rev Biotechnol 15:179–199

Varma A (1998) Mycorrhizae, the friendly fungi: what we know, what we should know and how do we know? In: Varma A (ed) Mycorrhiza manual. Springer, Germany, pp 1–24

Varma A, Schuepp H (1996) Influence of mycorrhization on the growth of micropropagated plants. In: Mukerjee KG (ed) Concepts in mycorrhizal research. Kluwer Academic Publishers, Netherlands, pp 113–132

Varma A, Verma S, Sudha SN, Bütehorn B, Franken P (1999) *Piriformospora indica*—a cultivable plant growth promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sudha, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Varma A, Hock B (eds) Mycota IX. Springer, Berlin, Heidelberg, New York, pp 123–150

Varma A, Singh A, Sudha, Sahay NS, Kumari M, Bharati K, Sarbhoy AK, Maier W, Walter MH, Strack D, Franken P, Singh A, Malla R, Hurek T (2002) *Piriformospora indica*: a plant stimulator and pathogen inhibitor arbuscular mycorrhiza-like fungus. In: Markandey DK, Markandey NR (eds) Microorganisms in bioremediation. Capital Book Company, New Delhi, pp 71–89

Verma S, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn P, Franken P (1998) *Piriformospora indica* gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:895–909

Zubek S, Turnau K, Tsimilli-Michael M, Strasser RJ (2009) Response of endangered plant species to inoculation with arbuscular mycorrhizal fungi and soil bacteria. Mycorrhiza 19:113–123

Chapter 11
Enhanced Productivity Associated with Tripartite Symbiosis Between *Phaseolus*, Rhizobia, and *Piriformospora indica*: In Presence of Vermicompost

Reshma Tuladhar, Jeny Shrestha, Anjana Singh, and Ajit Varma

11.1 Introduction

Piriformospora indica, a fungus belonging to the member of Basidiomyocotina, is a plant growth promoter discovered by Verma et al. (1998). *P. indica* is structurally and functionally similar to arbuscular mycorrhizal fungi (AMF) in several aspects (Oelmüller et al. 2009; Singh 2004; Varma et al. 1999). However, unlike AMF, *P. indica* can be grown in artificial medium (Singh 2004).

This symbiotic fungus mainly associates with plant roots but does not invade the aerial part of the plant. Within the root cortex, they form inter- and intracellular hyphae, often differentiating into arbuscule-like structure and vesicle-like structures. The fungal hyphae multiply within the host cortical tissues without traversing through the endodermis.

P. indica has the ability to colonize the roots of wide host range. Initially, the effect on the growth was studied on *Zea mays*, *Pisum sativum*, *Nicotiana tabacum*, *Glycine max*, etc. (Rai and Varma 2005). Subsequently, it was discovered that the fungus has the ability to stimulate the growth of wide range of plants ranging from medicinal to timber-yielding plants (Rai et al. 2001; Singh 2004). Growth-promoting ability of *P. indica* has been observed in terrestrial orchids such as *Dactylorhiza purpurella*, *D. incarnate*, *D. majalis*, and *D. fuchsii* (Singh 2004). This endophyte has been proven as an effective growth promoter of hydroponic cultures (Fakhro et al. 2010). Plants associated with vesicular–arbuscular (VA) mycorrhiza are benefited compared to nonmycorrhizal plants. The mycorrhizal

R. Tuladhar • J. Shrestha • A. Singh (✉)
Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
e-mail: anjanas67@yahoo.com

A. Varma
Amity Institute of Microbial Technology, Amity University Uttar Pradesh, E-3 Block, IV Floor, Amity University Campus, Sector -125, Noida 201303, U.P, India
e-mail: ajitvarma@amity.edu

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_11,

associations have owed plants with resistance to stress such as toxicity, high salinity, and adverse soil pH (Atlas and Bartha 2000; Bagyaraj and Varma 1995).

More importantly the fungi promote the plant growth by facilitating in the uptake of the frequently limiting macronutrients such as nitrogen and phosphorous (Schachtman et al. 1998). Nitrogen gas though abundant in the atmosphere is not readily available for the plant. It is converted into the plant utilizable form by nitrogen-fixing bacteria. The symbiotic fixation by rhizobia in association with leguminous plants contributes for the largest part of nitrogen in terrestrial habitats (Atlas and Bartha 2000).

Larger part of phosphorous is often present in unavailable form in the soil as a result of adsorption, precipitation, or conversion to the organic form (Holford 1997). The rate of diffusion of phosphate is slow in soil. If the plant uptake rate is high, it creates a zone around the root that is depleted of phosphorous (Schachtman et al. 1998). Increased P absorption in plant has been associated with the presence of fungal symbiont (Harrison et al. 1995). The plant-associated mycorrhiza extends extrametrical hyphae in soil facilitating the adsorption of P followed by its translocation through hyphae and mycelia development within root tissue and eventually from fungus to root cells (Joner and Johansen 2000).

Both the rhizobial and mycorrhizal symbioses can act synergistically on promoting plant growth (Jia et al. 2004). Improving the growth of plant leading to the greater yield in the product has been a strategy in agriculture to meet the ever-increasing demand for food in the world. The use of vermicompost for culturing the bean plant inoculated with both rhizobia and mycorrhizal fungi was aimed to enhance the growth, productivity, and nutritional content of the plant.

11.2 Tripartite Symbiosis Within Mycorrhizal Fungi, Rhizobia, and Host

The synergistic interaction within *Rhizobium*-AMF and legume was found to increase the rate of P uptake and N-fixation resulting in an increased crop biomass (Azcon et al. 1991; Xavier and Germida 2002). In *Rhizobium*-AMF–legume tripartite symbiosis relationships, nodulation of rhizobia and establishment of AMF often occur simultaneously and synergistically. Rhizobia provide fixed nitrogen not only to the plant but also to the fungus. Besides, rhizobia can also assist in mobilizing nutrients from the soil and improving the growth of infected plants. AMF on other hand enhance plant growth by absorbing P from soil and transporting it to the roots (Jakobsen et al. 1992). The increment in the P supply as a result of AMF colonization was consistently associated with increase in N accumulation and N productivity (Jia et al. 2004). Coinoculation of soybean with rhizobia and AMF increased the growth under the condition of low P and N content in soil (Wang et al. 2011).

The interaction between rhizobia, AMF, and legume shares parts of the signaling pathways mediated by specific flavonoids (Antunes et al. 2006; Tajini et al. 2009).

The demand for P is found to be greater especially when the N supply in legumes depends upon the rhizobial symbiosis as up to 20 % of total plant P is allocated to nodules (Tajini et al. 2009). The nodule biomass is correlated with the availability of P in plant (Hellsten and Huss-Danell 2001) as the deficiency of P resulted in reduced nodule size (Gunawardena et al. 1992).

11.3 Tripartite Symbiosis Between *P. indica*, *Rhizobium leguminosarum*, and *Phaseolus vulgaris* in Presence of Vermicompost

Vermicompost is a product of biodegradation of organic materials through interactions between earthworms and microorganisms (Sallaku et al. 2009). It is produced by earthworms in the form of worm cast upon feeding on biodegradable materials. Being rich in nitrogen, phosphorus, and potassium (NPK) and important plant growth hormones, vermicompost has been popular organic compost known to enhance the biomass production of numbers of crops (Hidalgo 1999; Pashanasi et al. 1996). Vermicompost has been known to improve the texture and properties of soil (Edwards and Burrows 1988). Besides, it has been found to increase the soil microflora. There has been a significant increase in the colonization of the mycorrhiza in the plant in presence of vermicompost (Kale et al. 1992).

Dual inoculation of legume with mycorrhizal fungi and *Rhizobium* improved nodulation, mycorrhizal colonization, dry weight, and nitrogen and phosphorus content (Manjunath et al. 1984). The *Phaseolus* bean inoculated with *P. indica* and *R. leguminosarum* in presence of vermicompost showed increased length and dry weight of both root and shoot during harvesting stage of plant compared to treatment lacking vermicompost or single inoculation (Fig. 11.1).

The experiment was carried out in soil-filled earthen pot, and vermicompost was supplemented where it is required. Inoculation of *P. indica* and *R. leguminosarum* were done where necessary following the germination of seed. The length and weight of the root and shoot were measured 30 days after the sowing of the seeds for flowering stage. Similarly harvesting was done after 50 days.

The growth of plant in terms of shoot and root parameters was relatively highest in dual inoculation during the flowering stage (Figs. 11.1 and 11.2), however, not significantly different from the dual treatment supplemented with vermicompost. The growth of plant was found to be highest in the dual treatment supplemented with vermicompost during the harvesting stage (Figs. 11.1 and 11.3).

Similarly the yield was highest in the plant treated with *P. indica* and *R. leguminosarum* in presence of vermicompost (Fig. 11.4) followed by *P. indica*- and *R. leguminosarum*-treated plant without vermicompost. The yield was estimated from the number of bean pods in each plant.

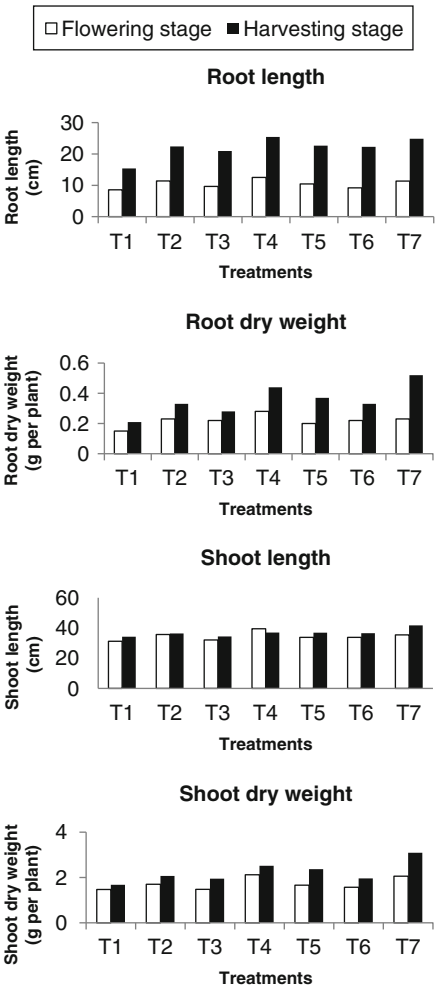

Fig. 11.1 Root and shoot length and dry weight production during flowering stage and harvesting stage in different plant treatments T1 (control), T2 (*P. indica*), T3 (*Rhizobium*), T4 (*P. indica* and *Rhizobium*), T5 (*P. indica* and vermicompost), T6 (*Rhizobium* and vermicompost), and T7 (*P. indica*, *Rhizobium*, and vermicompost)

11.4 Mycorrhizal Colonization Associated with Vermicompost

Measurements of the extent to which roots are mycorrhizal have been used to indicate the abundance of mycorrhizal fungi in soil (Hayman and Stovold 1979; Sparling and Tinker 1978). The active symbiotic phase is reflected from the mycorrhizal root colonization (Singh 2004). The mycorrhizal root colonization was highest in the plant inoculated with *P. indica* and Rhizobium with vermicompost supplement incorporated (Fig. 11.5). There was an increased colonization in the harvesting stage compared to the flowering stage (Fig. 11.5). The root colonization of *P. indica* in *Adhatoda vasica* was estimated to 95 % after 6 months (Rai and Varma 2005).

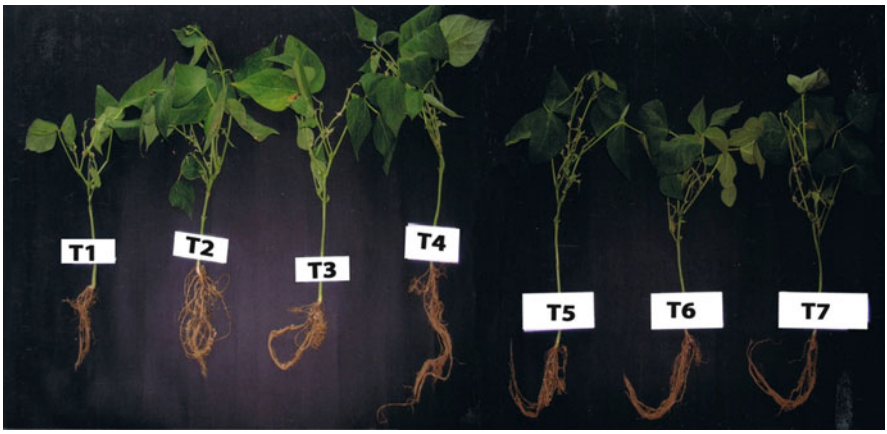

Fig. 11.2 *Phaseolus* plants of different treatment at flowering stage. T1 (control), T2 (*P. indica*), T3 (*Rhizobium*), T4 (*P. indica* and *Rhizobium*), T5 (*P. indica* and vermicompost), T6 (*Rhizobium* and vermicompost), and T7 (*P. indica*, *Rhizobium*, and vermicompost)

Fig. 11.3 *Phaseolus* plants of different treatment at harvesting stage. T1 (control), T2 (P. *indica*), T3 (*Rhizobium*), T4 (*P. indica* and *Rhizobium*), T5 (*P. indica* and vermicompost), T6 (*Rhizobium* and vermicompost), and T7 (*P. indica*, *Rhizobium*, and vermicompost)

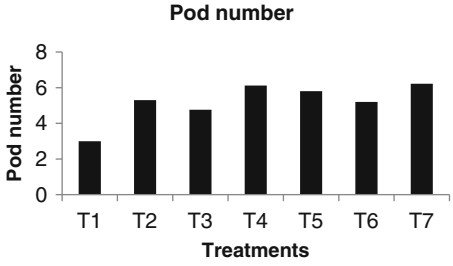

Fig. 11.4 Yield in *Phaseolus* pod followed by different treatments during the harvesting stage. The seven different treatments as mentioned in Fig. 11.1

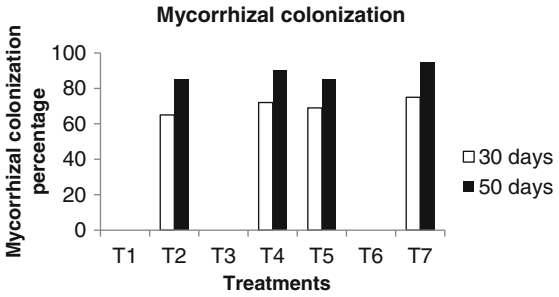

Fig. 11.5 Percentage of mycorrhizal colonization of root in *Phaseolus* in different treatments during flowering and harvesting stage. The seven different treatments as mentioned in Fig. 11.1

11.5 Nitrogen, Phosphorus, and Potassium Content in the Plant

Nitrogen, phosphorus, and potassium are most essential nutrients required for the growth of the plant. Nitrogen being the key building block of the protein and present in the nucleic acid is indispensable component of the cell. Phosphorous is present in biomolecules such as nucleic acid, phospholipids, and ATP making it important for plant. Potassium promotes the root growth in plants and assists the absorption of minerals.

Organic fertilizers are known to contain NPK essential for plant. Vermicompost has been superior organic manure enhancing biomass production of number of crops (Hidalgo 1999). It has high percentage of NPK and water retention ability which help increase the soil fertility (Acharya 1997; Edwards and Burrows 1988). The efficient uptake of these elements by plant is equally important for the growth of the plant, and this ensures the maximum utilization of the vermicompost.

The presence of rhizobia has been reported to significantly increase the uptake of N, P, and K by rice plants (Biswas et al. 2000). Nitrogen uptake has been increased in dual inoculation of *Bradyrhizobium japonicum* with endomycorrhizal fungi compared to the single (Shalaby and Hanna 1998). The importance of phosphorus in the rhizobia-AMF–legume tripartite symbiosis is reflected from the fact that for one molecule of nitrogen to be fixed to ammonia, 16 ATP is required (Theodorou and Plaxton 1993). Presence of vermicompost will enhance in the content of the essential nutrient in the soil as it led to significant increase in soil enzyme activities such as urease, phosphomonoesterase, and phosphodiesterase (Albiach et al. 2000). Besides plant growth-promoting bacteria stimulate solubilization of nutrients (Rodriguez and Fraga 1999) and production of growth hormones (Correa et al. 2004).

This will directly affect the nutrient content in the plant. In this experiment, the dual inoculation with *P. indica* and *Rhizobium* in presence of vermicompost led to highest percentage of NPK in the shoot compared to the rest of the treatment (Fig. 11.6). Similar result was observed in the percentage of NPK in the root (Fig. 11.7). The control plant without any treatment showed the lowest percentage of NPK content.

The significant increase in the shoot and root potassium and nitrogen content in the dual inoculation as compared with the single inoculation is consistent with the fact that the symbiosis, rather than compete with each other for the nutrient, complement each other by enhancing the plant's nutrient acquisition strategies.

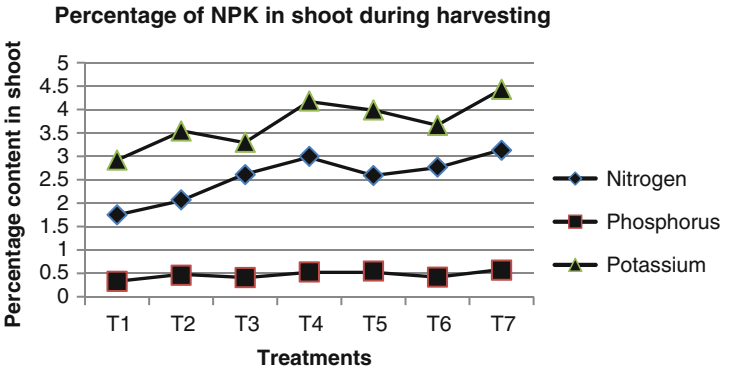

Fig. 11.6 Percentage of nitrogen (*diamond marked*), phosphorus (*square marked*), and potassium (*triangular marked*) present in the shoots of plants under different treatments during the harvesting stage

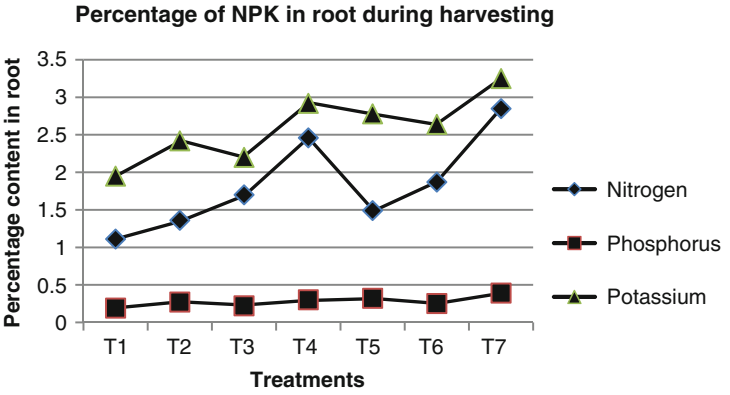

Fig. 11.7 Percentage of nitrogen (*diamond marked*), phosphorus (*square marked*), and potassium (*triangular marked*) present in the roots of plants under different treatments during the harvesting stage

11.6 Conclusion

The soil rich in NPK and other nutrients increases the rhizobial and mycorrhizal symbiotic relationship with plant which in turn have benefit on the growth and nutritional content of the plant. Vermicompost serves as the superior grade of compost for enriching the soil. This study opens possibility for further assessing the tripartite symbiotic relationship in presence of nutritional rich soil for the resistance of plant against potential plant diseases.

References

Acharya MS (1997) Integrated vermiculture for rural development. Int J Rural Stud 4:8–10

Albiach R, Canet R, Pomares F, Ingelmo F (2000) Microbial biomass content and enzymatic activities after application of organic amendments to a horticultural soil. Bioresour Technol 75:43–48

Antunes PM, Rajcan I, Goss MJ (2006) Specific flavonoids as interconnecting signals in the tripartite symbiosis formed by arbuscular mycorrhizal fungi, *Bradyrhizobium japonicum* (Kirchner) Jordan and soybean (*Glycine max* L. *Merrill*.). Soil Biol Biochem 38:533–543

Atlas RM, Bartha R (2000) Biogeochemical cycling. In: Earl W, Fogel L, Wong G (eds) Microbial ecology: fundamentals and applications. 4th edn. Pearson Education Asia, pp 417

Azcon R, Rubio R, Barea JM (1991) Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N_2-fixation (^{15} N) and nutrition of *Medicago sativa* L. New Phytol 117:339–404

Bagyaraj DJ, Varma A (1995) Interaction between arbuscular mycorrhizal fungi and plants and their importance in sustainable agriculture in arid and semi-arid tropics. Adv Microbial Ecol 14:119–142

Biswas JC, Ladha JK, Dazzo FB (2000) Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Sci Soc Am J 64:1644–1650

Correa JD, Barrios ML, Galdona RP (2004) Screening for plant growth-promoting rhizobacteria in *Chamaecytisus proliferus* (tagasaste), a forage tree-shrub legume endemic to the Canary Islands. Plant Soil 266:75–84

Edwards CA, Burrows I (1988) The potential of earthworm compost as plant growth media. In: Neuhauser E, Edwards CA (eds) Earthworms in waste and environmental management. SPB Academic, The Hague, Netherlands, pp 21–32

Fakhro A, Andrade-Linares DR, von Bargen S, Bandte M, Buttner C, Grosch R, Schwarz D, Franken P (2010) Impact of Piriformospora indica on tomato growth and on interaction with fungal and viral pathogens. Mycorrhiza 20:191–200

Gunawardena SFBN, Danso SKA, Zapata F (1992) Phosphorus requirement and nitrogen accumulation by three mungbean (*Vigna radiata* (L.) Wilczek cultivars. Plant Soil 147:267–274

Harrison MJ, Buuren L, Van M (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature 378:26–29

Hayman DS, Stovold GE (1979) Plant growth response to vesicular arbuscular mycorrhiza. New Phytol 71:41–47

Hellsten A, Huss-Danell K (2001) Interaction effects of nitrogen and phosphorus on nodulation in red clover (*Trifolium pratense* L.) Acta Agriculturae Scandinavica Section. Soil Plant Sci 80:135–142

Hidalgo P (1999) Earthworm castings increase germination rate and seedling development of cucumber. Mississippi Agricultural and Forestry Experiment Station, Research report 22:141–150

Holford ICR (1997) Soil phosphorus: its measurement and its uptake by plants. Aust J Soil Res 35:227–239

Jakobsen I, Abbot LK, Robson AP (1992) External hyphae of vesicular arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L.I. spread of hyphae and phosphorous inflow in roots. New Phytol 120:371–380

Jia Y, Gray VM, Straker CJ (2004) The influence of rhizobium and arbuscular mycorrhizal fungi on nitrogen and phosphorus accumulation by *Vicia faba*. Ann Bot 94:251–258

Joner EJ, Johansen A (2000) Phosphatase activity of external hyphae of two arbuscular mycorrhizal fungi. Mycol Res 104:81–86

Kale RD, Mallesh BC, Bano K, Bagyaraj DJ (1992) Influence of vermicompost application on the available macronutrients and selected microbial populations in a paddy field. Soil Biol Biochem 24:700–702

Manjunath A, Bagyaraj DJ, Gopala Gowda HS (1984) Dual inoculation with VA mycorrhiza and *Rhizobium* is beneficial to *Leucaena*. Plant Soil 78:445–448

Oelmüller R, Sherameti I, Tripathi S, Varma A (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Pashanasi B, Lavelle P, Alegre J, Charpentier F (1996) Effect of the endogeic earthworm, *Pontoscolex corethrurus* on soil chemical characteristics and plant growth in a low-input tropical agro-ecosystem. Soil Biol Biochem 28:801–808

Rai M, Varma A (2005) Arbuscular mycorrhiza-like biotechnological potential of *Piriformospora indica*, which promotes the growth of *Adhatoda vasica* Nees. Electronic J Biotech 8:107–110

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128

Rodriguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319–339

Sallaku G, Babaj I, Kaciu S, Balliu A (2009) The influence of vermicompost on plant growth characteristics of cucumber (*Cucumis sativus* L.) seedlings under saline conditions. J Food Agric Environ 7:869–872

Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant Physiol 116:447–453

Shalaby AM, Hanna MM (1998) Preliminary study in interaction between VA mycorrhizal fungus *Glomus mosseae*, *Bradyrhizobium japonicum* and *Pseudomonas syringae* in soybean plants. Aeca Microbiologica Polonica 47:385–391

Singh A (2004) Immunocharacterization of *Piriformospora indica* and other identical root endophytes. Ph.D. thesis. Jawaharla Nehru University, New Delhi

Sparling GP, Tinker PB (1978) Mycorrhizal in pennine grassland. J Appl Ecol 15:943–950

Tajini F, Suriyakup P, Vailhe H, Jansa J, Drevon JJ (2009) Assess suitability of hydroaeroponic culture to establish tripartite symbiosis between different AMF species, beans, and rhizobia. BMC Plant Biol 9:73

Theodorou ME, Plaxton WC (1993) Metabolic adaptations of plant respiration to nutritional phosphate deprivation. Plant Physiol 101:339–344

Varma A, Sudha S, Franken P (1999) *Piriformospora indica*- a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:2741–2744

Verma S, Varma A, Rexer KH, Kost G, Sarbhoy A, Bisen P, Butehorn B, Franken P (1998) *Piriformospora indica,* gen et sp nov, a new root colonizing fungus. Mycologia 95:896–903

Wang X, Pan Q, Chen F, Yan X, Liao H (2011) Effect of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. Mycorrhiza 21:173–181

Xavier LJC, Germida JJ (2002) Response of lentil under controlled conditions to co-inoculation with arbuscular mycorrhizal fungi and rhizobia varying in efficacy. Soil Biol Biochem 34:181–188

Chapter 12
Inhibitory Interactions of Rhizobacteria with the Symbiotic Fungus *Piriformospora indica*

Ajit Varma, Agnes Fekete, Abhishek Srivastava, Anil K Saxena, Moritz Frommberger, Dan Li, Silvia Gschwendter, Irena Sherameti, Ralf Oelmueller, Philippe Schmitt-Kopplin, Swati Tripathi, and Anton Hartmann

A. Varma • S. Tripathi
Amity Institute of Microbial Technology, Amity University, Block E-3, Sector 125, Noida, UP, India
e-mail: ajitvarma@amity.edu; swati.tripathi@rediffmail.com

A. Fekete • M. Frommberger • P. Schmitt-Kopplin
Helmholtz Zentrum München, German Research Center for Environmental Health, Research Unit Analytical Biogeochemistry, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany
e-mail: agnes.fekete@uni-wuerzburg.de; frommberger@helmholtz-muenchen.de; schmitt-kopplin@helmholtz-muenchen.de

A. Srivastava
School of Engineering and Sciences, International University of Bremen, Campus Ring 6, 28759 Bremen, Germany
e-mail: abhishek_srivastava@hotmail.com

A.K. Saxena
Divisions of Microbiology, Indian Agricultural Research Institute, New Delhi 110012, India
e-mail: saxena461@yahoo.com

D. Li • S. Gschwendter • A. Hartmann (✉)
Research Unit Microbe–Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany
e-mail: dan.li@helmholtz-muenchen.de; silvia.gschwendtner@helmholtz-muenchen.de; anton.hartmann@helmholtz-muenchen.de

I. Sherameti • R. Oelmueller
Friedrich-Schiller University of Jena, Biology and Pharmacy Faculty, Institute for General Botany and Plant Physiology, Domburger Street 159, 07743 Jena, Germany
e-mail: irena.sherameti@uni_jene.de; b7oera@hotmail.com

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_12,

12.1 Introduction

The fungus *Piriformospora indica* was originally isolated from the roots of different xerophytes in the Indian Thar desert (Verma et al. 1998; Varma et al. 1999). *P. indica* is easily culturable, lacks host specificity, and is colonizing roots of many different plants, mostly in an endophytic fashion (Varma et al. 2001). *Piriformospora indica* promotes nutrient uptake; allows plants to survive under water, temperature, and salt stress; confers (systemic) resistance to toxins, heavy metal ions, and pathogenic organisms; and stimulates growth and seed production (Oelmueller et al. 2009). The host range includes bryophytes (*Aneura pinguis*), pteridophytes (*Pteris ensiformis*), gymnosperms (*Pinus halepensis*), and large number of angiosperms (Varma et al. 2001). It turned out that this fungus also has a remarkable hardening effect when inoculated to micropropagated plants (Sahay and Varma 1999). However, it was also reported that under some circumstances, a transition from mutualism to antagonism between the fungus and the inoculated plant can occur.

Many mechanistic details were elucidated in the *Arabidopsis thaliana* model during the course of the interaction of *P. indica* (Oelmueller et al. 2009). Apparently, the *P. indica–Arabidopsis* interaction represents a novel type of beneficial fungus–root interaction with early protein alterations in the plasma membrane and the endocytoplasmic reticulum (Shahollari et al. 2005; Peskan-Berghofer et al. 2004). In *A. thaliana*, also a pronounced plant growth promotion and enhanced seed production occur in the presence of *P. indica*, which involves the synthesis of a leucine-rich protein repeat (Shahollari et al. 2007), the increased expression of nitrate reductase, and a starch-degrading enzyme (Sherameti et al. 2005) as well as the expression of a ß-glucosidase in the endoplasmic reticulum (Sherameti et al. 2008a, b). In addition, also changes in plant hormone levels of auxins and cytokinins are involved (Vadassery et al. 2009). Interestingly, the root endophytic fungus requires host cell death in the outer epidermal cell layers for the proliferation during the symbiosis of *P. indica* with barley (Deshmukh et al. 2006).

Inoculation with *P. indica* increases drought tolerance in *Arabidopsis* (Sherameti et al. 2008a, b) and barley (Waller et al. 2005). *P. indica* clearly increases the general stress tolerance of plants by increasing also antioxidative enzymes in this mutualistic interaction (Vadassery et al. 2009). The increase in growth performance induced by *P. indica* and *Sebacina vermifera* in *Nicotiana attenuata* was shown to be at the expense of herbivore resistance (Barazani et al. 2005). The inoculation with *P. indica* also increases the systemic resistance against fungal pathogen attack, like with *Blumera* in barley (Waller et al. 2005) and wheat (Serfling et al. 2007).

A comprehensive molecular phylogenetic analysis using the nuclear gene for the ribosomal large unit (nrLSU) shed light on the ecology and evolution of fascinating group of *Sebacinaceous* fungi whose striking biodiversity and ecological importance has only started to be recognized (Glen et al. 2002; Selosse et al. 2007; Urban et al. 2003; Kottke et al. 2003). Weiss et al. (2004) have presented well-resolved phylogeny of the main lineages of *Basidiomycetes* which shows that the

Sebacinaceae is the most basal group with known mycorrhizal members. Extrapolating from the known rDNA sequences in *Sebacinaceae*, it is evident that there is a cosm of mycorrhizal biodiversity yet to be discovered in this group. *Sebacinaceae* was recognized as a new family, with the new order *Sebacinales* and with *P. indica* an integral taxonomic genus (Weiss et al. 2004). The effect of bacteria on mycorrhiza, as described in the concept of mycorrhizal helper bacteria (Garbaye 1994), has not been documented yet in this particular mycorrhiza-like symbiotic system of *P. indica* interacting with a wide variety of plants with high practical importance. A tightly associated endofungal bacterium, *Rhizobium radiobacter*, has been identified in situ and localized using fluorescence in situ hybridization and confocal laser scanning microscopy in hyphae of *P. indica* cultures (Sharma et al. 2008). However, the functional role of this bacterium with *P. indica* and in the interaction with plant roots is not known yet. This bacterium can be grown in pure culture outside the fungus, while *P. indica* could not be cured yet from this bacterium. Other members of the Sebacinales have other endofungal bacteria, like *Paenibacillus* sp., in *S. vermifera*; however, this bacterium was not yet been successfully grown in pure culture without the fungus (Sharma et al. 2008).

An increasing demand for improving the sustainability of low-input agriculture has resulted in greater interest in rhizosphere microorganisms that increase soil fertility or improve plant nutrition and health (Ryan et al. 2009). Exploiting the potential of, e.g., fluorescent *Pseudomonads, Serratia* sp*p., Bacillus* spp., and *Burkholderia* spp. to act as crop protectants (biological control agent) has become the focus of many research groups. Their biocontrol capabilities result largely from their ability to produce a battery of antifungal metabolites which also can affect beneficial fungal–root symbioses (Raajimakers et al. 2009). On the other hand, e.g., *Azospirillum* and *Herbaspirillum* spp., known as diazotrophic, plant growth-promoting agents or green biofertilizers, are also gaining much importance to improve nutrition, growth, and yield of crop and energy plants (Franche et al. 2009). Research on the cellular and metabolome basis of the interaction of these plant-beneficial bacteria with *P. indica* should contribute to the understanding of the beneficial or deleterious associations between bacteria and fungi in general. In this communication, we report the intense interaction between *P. indica* and economically important rhizobacteria. While some rhizobacteria could promote growth and root colonization of the fungus or behave neutral in the interaction with *P. indica*, others severely inhibit its development. Extent and mechanisms of inhibition by *Pseudomonas fluorescens* SS101 *and* WS5, *Bacillus amyloliquefaciens* FZB42, and *Burkholderia cepacia* LA3 were studied in different working models including the effect on the metabolome of *P. indica* at sublethal inhibitory conditions.

12.2 Materials and Methods

12.2.1 Cultivation of Microorganisms

P. indica was maintained on modified *Aspergillus* medium (Garbaye 1994). Initial pH was adjusted to 6.5 and incubated at 28 ± 2 °C. Stock cultures of bacteria were maintained on King'B medium (g/l: Difco peptone 20.0, K_2HPO_4 1.5, $MgSO_4$.7 H_2O 1.5, and glycerol 10.0 ml). pH was maintained at 7.0–7.2. The other bacteria were grown on NB medium, supplemented with antibiotics as required.

12.2.2 Interaction of Fungus and Rhizobacteria on Roots of Gnotobiotic Barley Seedlings

Seeds of barley (*Hordeum vulgare*), cultivar Barke, were surface sterilized using 2% hypochlorite treatment followed by intense washing with sterile distilled water. After germination in the dark, the seedlings were checked for contamination on nutrient agar plates and placed then in autoclaved glass vials filled with quartz sand (5 cm in height) and containing sterile Hoagland solution. Incubations were performed at 22°C room temperature with day–night cycles of 16/8 h. *P. indica* and different rhizobacteria were inoculated at different combinations, and the biomass of the roots was determined 4 weeks after inoculation.

12.2.3 Cocultivation of Fungus and Rhizobacteria on Agar Plates

For coculture experiments, agar discs of 4 mm diameter from the margin of actively growing *P. indica* cultures were placed in the center of the plates containing modified *Aspergillus* medium or nutrient broth agar and allowed to grow for 2 days. The respective bacteria from the mid of the log phase were inoculated according to the experimental design at either side of the discs.

12.2.4 Staining and Microscopic Observation

Hyphae and chlamydospores were stained with trypan blue and examined with a Leica DM or Zeiss Axiophot microscope. The coated specimens were scanned (Philips electron microscope) at an anode potential of 15.00 KV. For studying the interspecific differences, the pictures were taken at different magnifications.

The stained sections were observed with Philips, CM—10 electron microscope. The microscope was operated at 60–80 KV.

The colonization of barley roots by *P. indica* and different rhizobacteria was followed using confocal laser scanning microscopy (LSM510, Zeiss Jena, Germany) after the roots had been stained using the fluorescence in situ hybridization (FISH) technique (Amann et al. 1990). The fluorescence-labeled oligonucleotide probes Eub339 I, II, and III—cy5 (blue in rgb image) for bacteria and EuK-cy3 (red in the rgb image) for the fungus—were applied (Amann et al. 1990).

12.2.5 Spectrometry

Absorption spectrophotometry, 200–900 nm, was carried out using Shimadzu 260. Spectrofluorimetry used was 270–400 nm (Carry Eclipse EL0035456); mass range monitored was from 100 *m/z* to 500 *m/z* values. The sample was diluted in methanol.

12.2.6 Column Chromatography

As column material, CP-Sil 8 CB-MS (30 m × 0.25 mm × 0.25 thickness film) was selected. Oven temperature was set at 80°C for 1 min, 10°C for 1 min, and 275°C for 10 min.; the injection temperature was 270°C. The helium gas flown 1 ml/min, and the trapping temperature was 200°C. The manifold temperature was set to 40°C, and the transfer line temperature was 260°C.

12.2.7 Mass Spectrometry

High-resolution mass spectra were acquired on a Bruker (Bremen, Germany) APEX Qe Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS), equipped with a 12 Tesla superconducting magnet and an Apollo II ESI source. The methanol extracts of the biomass were introduced into the micro-electrospray source at a flow rate of 120 μl/h with a nebulizer gas pressure of 20 psi and a drying gas pressure of 15 psi (at 250°C). Positive electrospray ionization mode was used. The required mass spectra were exported to a mass list at signal-to-noise ratio 3 and were submitted to MassTRIX (http://mips.gsf.de/proj/mbx/~masstrix/) (Suhre and Schmitt-Kopplin 2008).

Table 12.1 Influence of rhizobacteria on growth of *Piriformospora indica*

Strains	Source	Impact
Herbaspirillum frisingense GSF30^T	Muenchen	Neutral
H. lusitanum P6-12^T	Muenchen	Neutral
H. seropedicae Z67^T	Muenchen	Neutral
H. rubrisubalbicans LMG 2286^T	Muenchen	Neutral
Azospirillum brasilense Sp7^T	Muenchen	Neutral
A. brasilense Sp245	Muenchen	Neutral
Bacillus coagulans NCC235	New Delhi	Neutral
Bacillus subtilis NCC09	New Delhi	Neutral
Pseudomonas putida IsoF	Muenchen	Stimulatory
Serratia liquefaciens MG1	Muenchen	Inhibitory
Burkholderia cepacia LA3	Muenchen/Dharbanga	Inhibitory
Pseudomonas fluorescens WS5	Bangalore	Inhibitory
Gluconacetobacter sp. Comb19	Coimbatore	Inhibitory
Streptomyces lividans SL8	Jena	Inhibitory
Streptomyces coelicolor A 3(2)	Jena	Inhibitory
Bacillus amyloliquefaciens FZB42	Berlin	Inhibitory
" FZB42, mutant CH3[a]	"	No inhibition
" FZB42, mutant AK1[b]	"	No inhibition
" FZB42, mutant AK2[c]	"	Slight inhibition
" FZB42, mutant AK3[d]	"	No inhibition
Pseudomonas fluorescens SS101	Wageningen/Netherlands	Inhibitory
" SS101, mutant 10.24[e]	"	No inhibition

[a]lipopeptides- and polyketides-deficient mutant
[b]bacillomycin-deficient mutant
[c]fengycin-deficient mutant
[d]bacillomycin- and fengycin-deficient mutant
[e]biosurfactant- massetolideA-deficient mutant

12.3 Salient Observation/Results

12.3.1 Confrontation Assays on Plates

Stimulatory and inhibitory influences as a result of cocultivation of *P. indica* with diverse rhizobacteria were studied in confrontation assays on nutrient agar plates using a collection of strains and mutants. While *Pseudomonas putida* IsoF promoted the growth of the fungus, most of the *Pseudomonas* strains, like *P. fluorescens* WS5 and SS101, and the nitrogen-fixing *Burkholderia cepacia* LA3*, Gluconacetobacter sp.* Comb19, *and Streptomyces lividans* SL8 inhibited the growth of *P. indica* (Table 12.1). The commercially available plant growth enhancing strain *Bacillus amyloliquefaciens* FZB42 (Rhizo Plus^R, ABITEP, Berlin, Germany) was investigated in more detail concerning the nature of the inhibitory compound. In this bacterium, several lipopeptides and antibiotics are known, and knockout mutants are available (Chen et al. 2007; Koumoutsi et al. 2007). While the wild-type strain FZB42 severely inhibited the growth of *P. indica*, the mutants

CH3, AK1, and AK3, lacking lipopeptide and polyketide synthesis or bacillomycin D production resp., had no inhibitory effect any more (Table 12.1). In contrast, the mutant AK2, deficient only in fengying production, was still inhibitory. This pointed to the fact that bacillomycin is the most effective inhibitory metabolite in the interaction of FZB42 with *P. indica*. The biocontrol strain *Ps. fluorescens* SS101 (Raaijmakers et al. 2006) inhibited growth of *P. indica*, while its lipopeptide biosurfactant massetolide A-deficient mutant 10.24 (Mazzola et al. 2007) had clearly no inhibitory effect on hyphal growth of *P. indica*. Accordingly, the isolated cyclic lipopeptide massetolide A inhibited the growth of *P. indica* down to 1–10 µg concentrations in the confrontation assay (data not shown).

12.3.2 *Interaction of Rhizobacteria with* P. indica *in Axenic Barley Seedlings*

When *P. indica* is applied to barley roots in an axenic system, root development is enhanced already in the seedling stage (Fig. 12.1a). Selected rhizobacteria that showed stimulation or inhibition of the growth of *P. indica* in the confrontation plate assay influenced also the root growth stimulatory effect of *P. indica* in the gnotobiotic barley system. While *Azospirillum brasilense* Sp245 had no effect, *Serratia liquefaciens* MG1 clearly inhibited, and *Pseudomonas putida* IsoF enhanced the root growth stimulation of *P. indica* (Fig. 12.1a). FISH analysis using probes specific for the bacteria and fungi and confocal laser scanning microscopy demonstrated tight physical interactions between the *Pseudomonas putida* IsoF and *P. indica* (Fig. 12.1b), while in the case of inhibitory interactions, close contact was very rare (not shown).

12.3.3 *Growth Inhibition and Structural Changes of* P. indica *Induced by Inhibitory* Ps. fluorescence *WS5 and* Burkholderia cepacia *LA3*

In vitro inhibition assays on solid agar medium revealed that *Ps. fluorescens* WS5 and *Burkholderia cepacia* LA3 (Jha et al. 2009) were inhibitory to the growth of the fungus (Table 12.1). At days 3 and 8, a clear zone of growth inhibition of *P. indica* close to the bacterial growth zone was visible (Fig. 12.2a, b). When iron-limited Fe-"ve" medium was used, the inhibitory effect of the bacteria was also observed. However, after day 22, fluorescence development was observed (Fig. 12.2c), and the fluorescent zone was also showing inhibitory activity in the direct confrontation assay (not shown). It was further observed that after 3–4 days, the hyphal growth of the fungus on the surface of nutrient agar plates had a radius of 1.0–1.5 cm in the presence of *Pseudomonas* WS5, while in the presence of *Burkholderia cepacia*

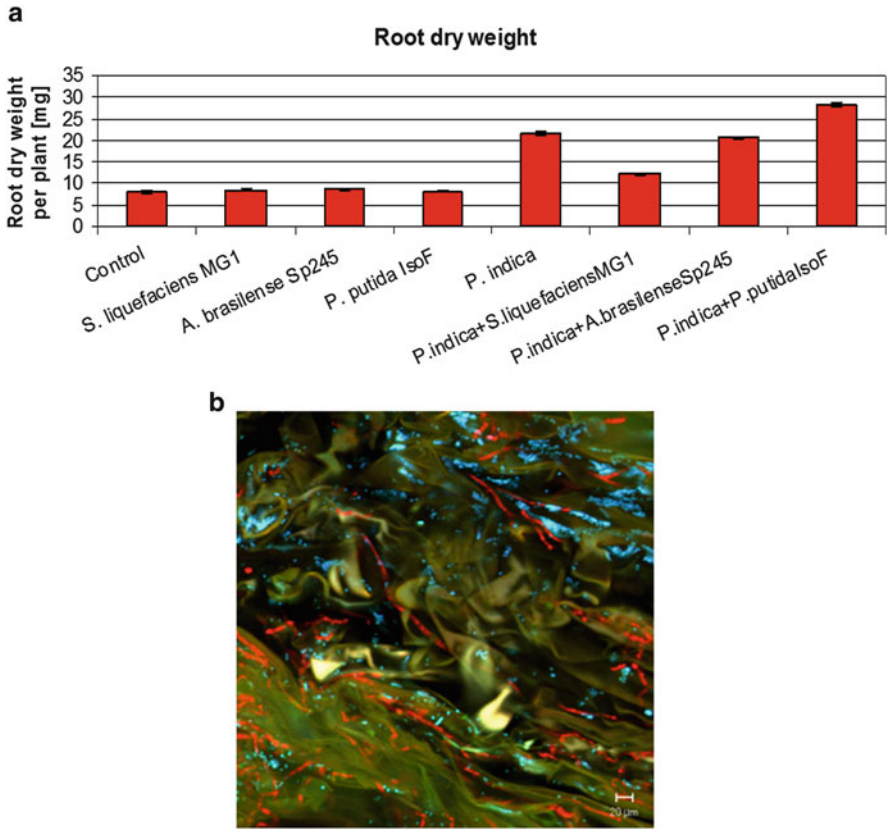

Fig. 12.1 (**a**) Root dry weight of axenically grown barley seedlings under the influence of *P. indica* and the rhizobacteria *Azospirillum brasilense* Sp245, *Serratia liquefaciens* MG1, and *Pseudomonas putida* IsoF (control: not inoculated). (**b**) Colonization of barley roots in an axenic system by *P. indica* and *Pseudomonas putida* IsoF. FISH analysis and confocal laser scanning microscopy (LSM510, Zeiss Jena, Germany) were performed. The fluorescence-labeled oligonucleotide probes Eub339 I, II, III—Cy5 (*blue* in rgb image) for bacteria (*Ps. putida* IsoF) and EuK-Cy3 (*red* in the rgb image) for the fungus *P. indica* were applied

LA3, growth of the fungus was suppressed even more already at day one, and the radius of hyphal growth never increased beyond 0.6 cm (Fig. 12.3).

The growth inhibition results were further confirmed by studying the hyphal morphology of the fungus under the influence of these rhizobacteria. In controls without rhizobacteria, the mycelial mat was dense with thin and densely interwoven hyphae of 0.5–2 µm in diameter. In the presence of *P. fluorescens* WS5, no observable mycelial mat was produced, the hyphae were thin, and lysis of some of the hyphae was observed. In controls, the chlamydospores produced were (12.5) 14–17(20) × 18–26 µm with cell walls of 0.5–1.5 (−2) µm thickness. *P. indica* interacting with *P. fluorescens* WS5 did not produce typical pear-shaped chlamydospores, although undulating hyphae were recorded, which are normally

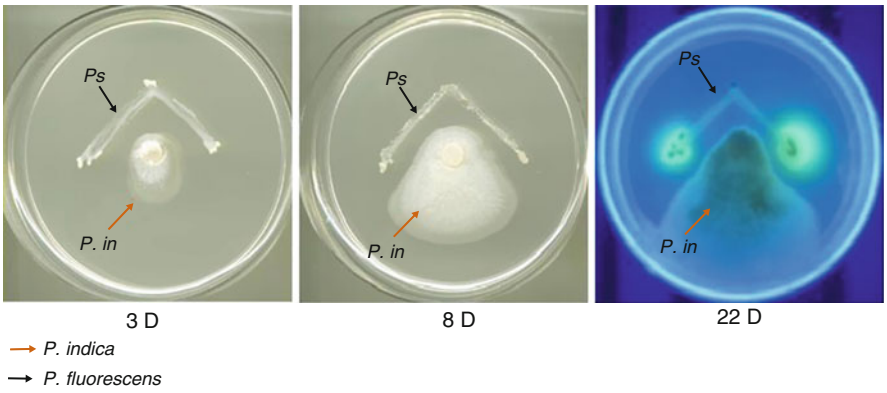

Fig. 12.2 Interaction of *P. indica* with rhizobacteria (*Pseudomonas fluorescens* WS5). (**a**–**c**) An agar disc (4 mm diam.) with mycelium and chlamydospores of *P. indica* was placed in the center of a petri-plate on Aspergillus medium (initial pH adjusted to 6.5) and incubated for 72 h. Thereafter, bacterial cultures from early log phase were inoculated in four equal distant points close to the margin of the plate. Incubation was carried out at 28 ± 2°C; growth after 3 days, (**a**) 8 days, (**b**) and 22 days with iron-deficient Fe-Ve-plates

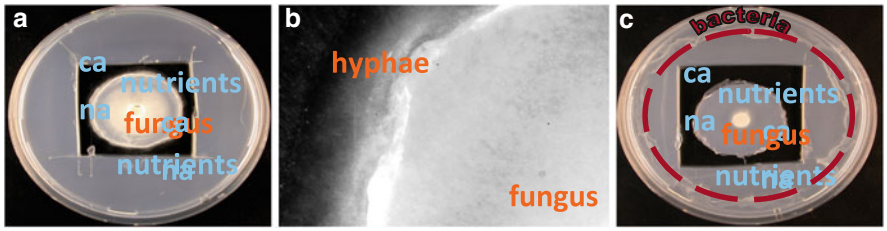

Fig. 12.3 *P. indica* vs. *Burkholderia cepacia* LA3 incubated on modified *Aspergillus* medium. An agar disc (4 mm diam.) with mycelium and chlamydospores of *P. indica* was placed in the center as described in Fig. 12.1. With a help of sterile scalpel 0.5 cm i.d., canal was made in which 100 µl nutrient medium was added. Thereafter, bacterial cultures from early log phase were streaked in a circular manner along the rim of the plate. Incubation was carried out at 28 ± 2°C for another 4 days. (**a**) Control (no bacteria added), (**b**) control where the fungus overgrown through nutrient canal (enlargement), and (**c**) coculture with *Burkholderia cepacia* LA3 where the fungus was completely inhibited

differentiated prior to the production of chlamydospores. Additionally, a moderate acidification of the medium (from pH 6.5 down to pH 5.5–6.0) was observed in *P. indica* control cultures, while in coculture with *P. fluorescens* WS5, the pH was reduced to pH <4.0.

Scanning electron micrograph of the control (*P. indica* only) showed healthy fungal hyphae with a smooth chitinous cell wall (at 8,000× magnification). The fungal hyphae were shown to be damaged when the bacterial cells were in the direct physical contact with the fungus. Certain dotted substances were released (Fig. 12.4a,b). Transmission electron micrographs further unraveled the

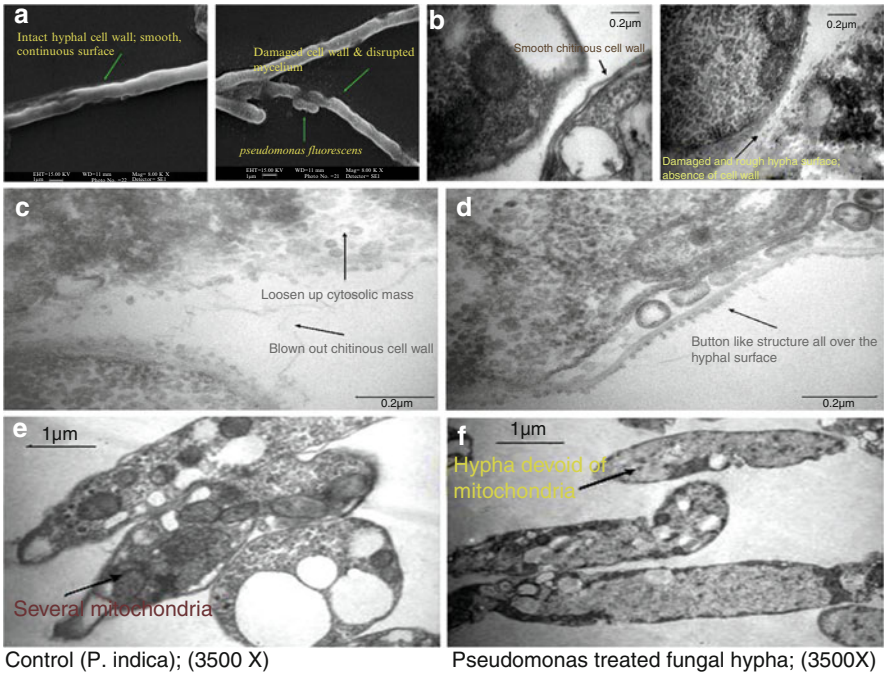

Fig. 12.4 (**a**) SEM showing fungal hypha (*control*) and *Ps. fluorescens* WS5-treated damaged hypha (magnification: ×8,000); (**b**) TEM (magnification ×11,000) showing the comparison between intact hyphae in control and disrupted hyphae after interaction with *Ps. fluorescens* WS5-interacted fungal hypha (in T.S. and L.S.); (**c**) TEM (magnification ×14,000) showing the surface anatomy of fungal hypha under the influence of *Ps. fluorescens* WS5 factors; (**d**) TEM (magnification ×14,000) showing the presence of strange globular structures at the surface of mycelium (**e**): TEM (magnification ×3,500) showing number of mitochondria in control and WS5-treated fungal hypha; (**f**) Pseudomonas-treated fungal hypha (×3,500)

morphology of the dotted substance released during *Ps. fluorescens* WS5 interaction. At 1,100× magnification, the control (i.e., only fungal hyphae) showed smooth surfaces, while *Pseudomonas*-treated samples showed dotted structures exuding out into the medium as well as a tuberculated surface without a chitinous cell wall layer (Fig. 12.4c,d).

The *P. indica* hyphae had an average diameter of 2.26 μm in the untreated control (i.e., fungus without *Ps. fluorescens* WS5). Using 5 μm as unit length of hypha and the formula $\pi r^2 h$ (where $\pi = 3.14$, $r =$ radius of the hypha, $h =$ unit length of the hypha), the unit volume of hyphae was calculated to be 20.2 μm^3. In several micrographs, it was observed that the number of mitochondria varies from 2 to 7 (considering longitudinal as well as transverse sections) per unit volume hyphae without the influence of *Ps. fluorescens* WS5. In the case of *P. fluorescens* WS5-treated fungus, the hyphal diameter was 1.74 μm wide, which is considerably less than in the control; this calculated to a unit volume of 11.9 μm^3. In the bacteria-treated

hyphae, the number of mitochondria was considerably reduced to 0–3 per 5 μm^3 hyphal length (Fig. 12.3e,f). Transmission electron microscopy studies showed the absence of cell wall material suggesting the high potency of this antifungal substance.

Thermal stability test suggested that the antifungal substance is thermolabile; the protease test (pancreatic trypsin, Poly A, protease EC 3.4.24.32, proteinase K, and pepstatin A) indicated a protease-resistant nature of the compound. Dialysis tests revealed a molecular weight more than 12,000 kDa for this biomolecule, and inhibition test using this substance demonstrated a "fungistatic" as well as "fungicidal" nature. Gas chromatogram–mass spectrometry ion fragmentation pattern studies of the green fluorescent compound produced by *Ps. fluorescens* WS5 under iron-limiting conditions (Fig. 12.2c) suggested that the substance could be pyoverdine—a well-known potent siderophore of Pseudomonads.

12.3.4 Metabolome Analysis of P. indica

The inhibitory influence of *B. cepacia* LA3 was even more intense (0.6 cm growth diameter) as compared to *Ps. fluorescens* WS5 (1.3 cm growth diameter). Therefore, it was decided to continue with further studies on *B. cepacia* LA3. The growth of the control was normal, and the fungi passed through the nutrient canal and went to the edges of the plate. In contrast, the fungus growth was completely restricted at the center of the source of the inoculum in the presence of *B. cepacia* LA3. Since there was no physical contact between the bacteria and the fungal hyphae, the inhibiting effect was caused by the excreted metabolite(s) of *B. cepacia*. Changes in the metabolome of the fungal biomass as caused by the bacterial metabolites were investigated using high-resolving mass spectrometry. To be able to investigate a wide range of small molecular weight components, positive electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry with direct ion infusion (ESI + −FT-ICR-MS) at broad mass range was applied. Due to the high resolution (100,000–500,000) and high mass accuracy (less than 0.5 ppm at *m*/*z* range of 150–1,000), 3,000–6,000 peaks were detected from the extract of the biomass (Fig. 12.5). The peaks then were annotated to the metabolites listed in KEGG database applying an online software (http://mips.gsf.de/proj/mbx/~masstrix/) that gives an overview of the possible presented and detectable metabolites within the characterized pathways and could be used for nontargeted metabolome screening but cannot differentiate the isomers (Amann et al. 1990). Since only a few fungal species have been investigated in this manner, and *P. indica* has not been characterized in the KEGG database, a model organism had to be selected. The basidiomycetes yeasts/fungi *Saccharomyces* and *Aspergillus* species were investigated to characterize the metabolome of *P. indica*. The highest matches within the pathways were found when *S. cerevisiae* was used as a model organism. The metabolome pattern of the methanol extract did not show dependency on the age of the *P. indica* biomass.

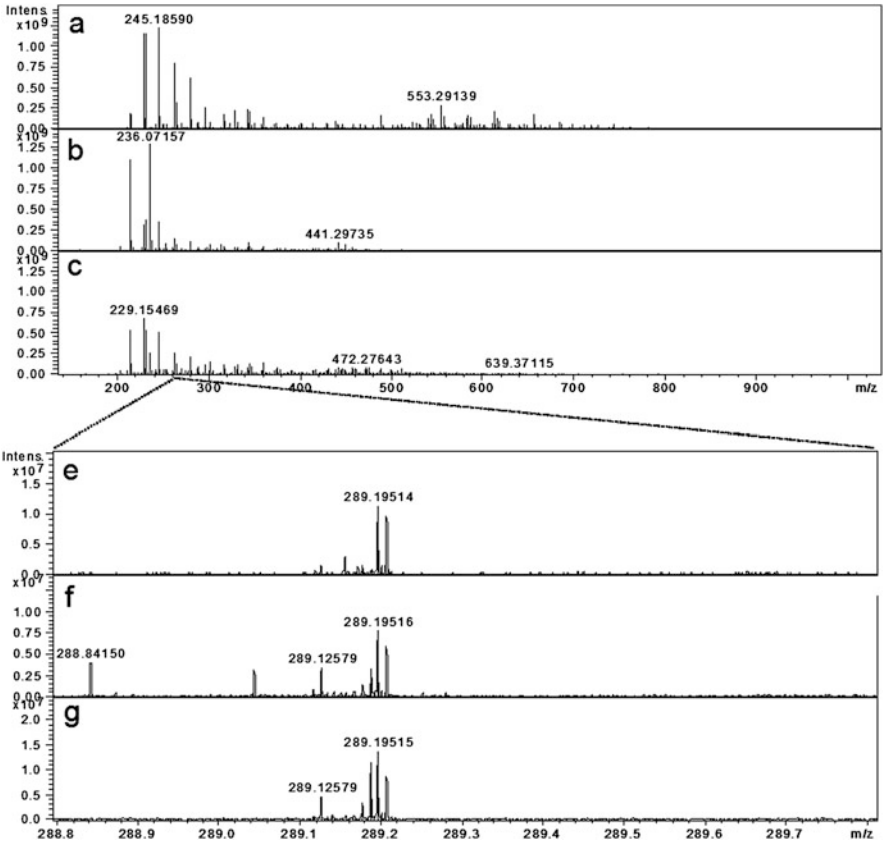

Fig. 12.5 Broad mass range (150–1,000) FT-ICR-MS spectra of the methanol extract of (**a**) *Burkholderia cepacia* LA3, (**b**) *Piriformospora indica,* and (**c**) *Burkholderia cepacia* LA3 and *Piriformospora indica* grown together as described in Fig. 12.4. Mass spectra at m/z of 288.8–289.8 of the methanol extract of (**e**) *Burkholderia cepacia* LA3, (**f**) *Piriformospora indica,* and (**g**) *Burkholderia cepacia* LA3 and *Piriformospora indica* grown together as described in Fig. 12.3 are shown as example for the complex metabolome analysis

The number and type of metabolites within the pathways present in *S. cerevisiae* were compared with the metabolites in the extract of fungal biomass grown with or without the influence of *B. cepacia* LA3 (Fig. 12.6). Out of 110 pathways, 42 were further investigated, since these had more than 3 annotated metabolites. Changes in the number of annotated metabolites were observed in several pathways, but in some of them, like the phenylalanine metabolism and biosynthesis or sphingolipid metabolism, the metabolites were not influenced by the interaction with *B. cepacia* LA3.

The number of annotated metabolites decreased in number of pathways, like several amino acid metabolism or biosynthesis pathways. A decrease in the annotated metabolites of the pentose phosphate and glycolysis was observed in

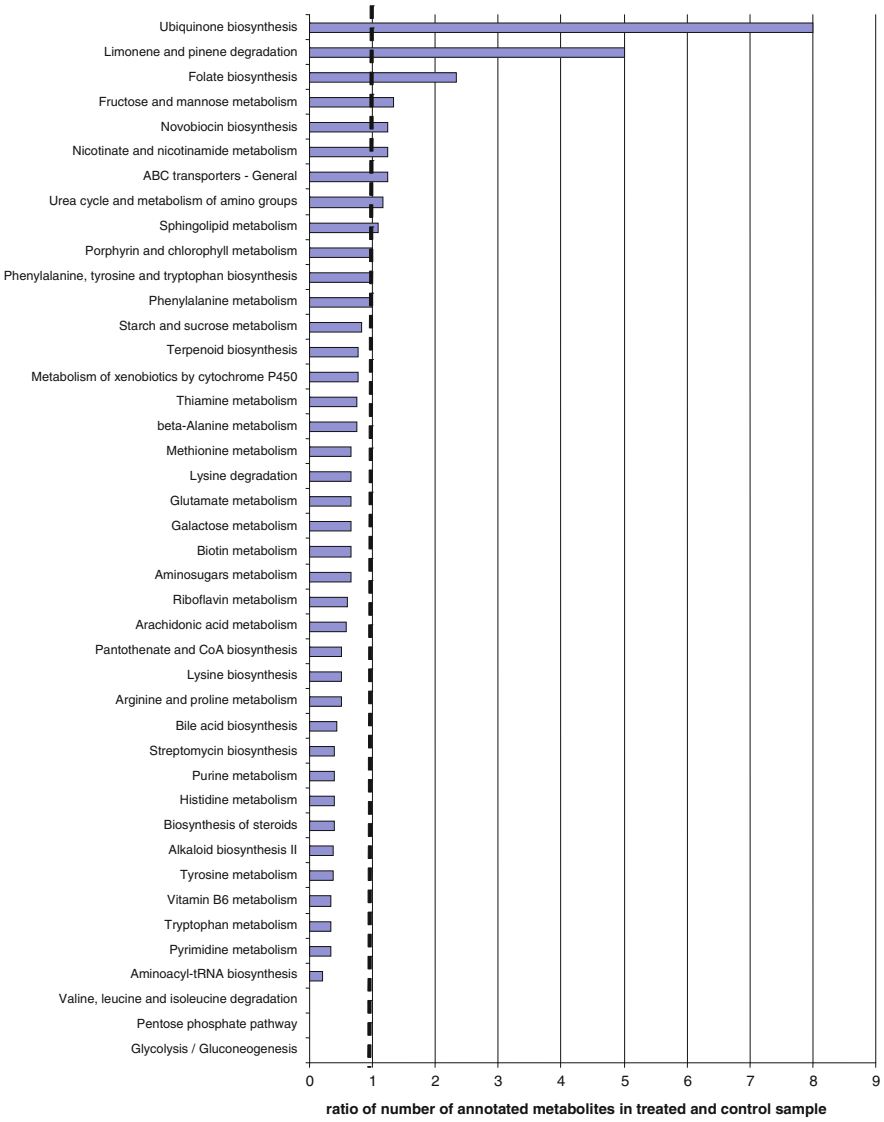

Fig. 12.6 Ratio of number of annotated metabolites within a pathway in fungal biomass treated or not treated with bacteria

the treated fungi compared to the control indicating a reduction in the activities of these pathways. In contrast, the number of annotated metabolites in the ubiquinone biosynthesis, limonene and pinene degradation, and folate biosynthesis highly increased under the influence of the inhibitory bacterium. A maximum was observed in the number of annotated metabolites within the ubiquinone biosynthesis in the 14-day-old biomass when the *P. indica* was interacting with the external metabolites of *B. cepacia* LA3.

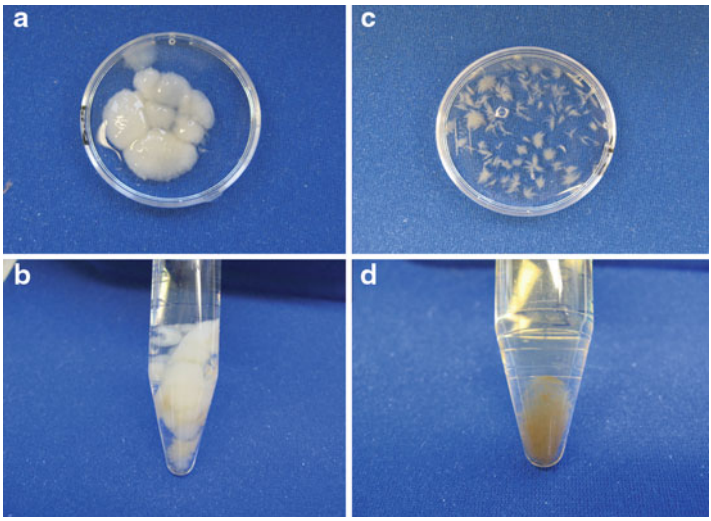

Fig. 12.7 *P. indica* grown in the absence (**a**, **b**) and presence (**c**, **d**) of 0.5 % (w/v) saponin on agar plate (**a**, **c**) and in liquid medium (**b**, **d**). Inhibition of growth was observed in the presence of saponin at the applied concentrations

The diffusible inhibitory metabolites of the *B. cepacia* LA3 exerted a complex influence on the metabolomic pathways of *P. indica*. Therefore, a mixture of small molecular weight and bioactive components were taken to compare the pathway differences of inhibited growth of the fungus. Saponin was selected as a model component mixture produced by plants, since it is a complex mixture of biomolecules like steroids and terpenes having surfactant and inhibitory activity. Also in biocontrol *Bacillus* and *Pseudomonas* bacteria, surfactant compounds like cyclic lipopeptides are known as potent inhibitors of pathogenic fungi (Onega and Jacques 2007; Raaijmakers et al. 2006). The growth of *P. indica* grown in medium containing saponin was clearly inhibited, and the suppression was concentration dependant (Fig. 12.7). The suppression of fungal growth was dependant on the concentration of the saponin in the range from 0.1% to 1.0%. At 0.1 and 0.5% saponin concentrations, the fungus grew slowly until at 1 % the growth was completely blocked. The identical metabolome annotation procedure was applied as described above for the annotation of metabolite pathways affected by inhibitory rhizobacteria. As was observed for the inhibition by diffusible bacterial metabolites, the number of annotated metabolites in the glycolysis, pentose phosphate pathway, purine and pyrimidine metabolism decreased with the increase of the saponin amount. In contrast to the treatment with inhibitory bacteria, the number of annotated metabolites in ubiquinone biosynthesis and limonene and pinene degradation was not influenced by the saponin content. Thus, the activation of these metabolic pathways was unique for the inhibitory effect of diffusible bacterial inhibitors.

12.4 Interpretation and Conclusion

Several mechanisms like the production of siderophores, antifungal metabolites, HCN, ammonia, and lytic enzymes like chitinases and β-1,3-glucanases by rhizobacterial isolates have been implicated in suppression of fungal growth (Hampp and Maier 2004; Raajimakers et al. 2009). Frey-Klett et al. (2007) proposed an enhancement of fungal growth by certain mycorrhiza helper bacteria (MHBs), like *P. fluorescens,* chiefly due to trophic stimulations. It involves production of metabolites by the bacterium directly used by the fungus as nutrients or enhancing its anabolism. MHBs associated with *Hebeloma crustuliniforme*, *Paxillus involutus,* and *Laccaria laccata* excrete some organic acids (predominantly malic and citric acid) serving as an effective carbon source for the fungal growth (Pham et al. 2004; Barea et al. 2004). Mycorrhiza helper bacteria can also suppress the plant defense response and thus add to support mycorrhizal colonization (Lehr et al., 2007). Bacterial volatiles have also been shown to facilitate communication between microbes (Bassler 2002; Persello-Cartieaux et al. 2003). It has been reported that mainly volatiles, like 2,3-butanediol and acetoin, promote the growth of *Arabidopsis thaliana* (Ryu et al. 2003). In the present study, the differential response of *P. indica* to different rhizobacteria leads us to postulate the existence of an ecological balance among the microbial communities in the rhizosphere/rhizoplane (mycorrhizosphere), which allows diverse microbial functional groups to coexist and share common resources.

The antifungal factor produced by the bacteria is potent enough to degrade the chitinous cell wall of the fungus and bring down the mitochondrial count within the hyphae which is one step beyond the findings of Thrane et al. (1999) who showed alterations in mitochondrial organization. When the fungus is growing normally, it requires energy for its growth and development. The lower diameter of hypha in the *P. fluorescens* WS5-treated sample suggests the fungistatic nature of the "inhibitory compound." Spectrofluorimetry, gas chromatography data, and mass spectra fragmentation patterns show the *m/z* value to be 416 suggesting thereby that one of the inhibitory substances could be pyoverdine (and its derivatives)—a potent siderophore (Fuchs and Budzikiewicz 2000, 2001). The data suggest the siderophore is a chromopeptide molecule. The presence of unusual modified amino acids (ornithine) and D-amino acids prevents the cell wall from proteolytic degradation. The "nonfluorescent phase" inhibition of *P. fluorescens* WS5 is under investigation and could be due to a lipopeptide. This antifungal factor(s) has its action on fungal cell wall, plasmalemma, mitochondria, and probably cytoskeletons.

Two contrasting observations, i.e., differential response of *P. indica* to the presence of rhizobacteria and its ability to survive in soil and colonize plant roots, led to the hypothesis that microbial communities interact through diffusible metabolites to counteract the inhibitory or stimulatory factor(s), thereby maintaining the delicate balance between diverse soil microorganisms. To prove this concept, in vitro assays using axenic barley seedlings were carried out using the widely distributed PGPR *Azospirillum brasilense* Sp245, *Pseudomonas putida*

IsoF, and *Serratia liquefaciens* MG1. The results demonstrated a wide range of possible interactions—from inhibition to stimulation—taking place on the root surface (Fig. 12.1). The possible molecular mechanisms of stimulatory interactions could be manifold, but these are not the focus of this communication. Concerning the inhibitory effects, antibiotics and lipopeptides produced and excreted by many biocontrol active rhizobacteria were shown to be responsible for the observed inhibition of *P. indica* by the plant growth enhancing inoculant *B. amyloliquefaciens* FZB42 and the biocontrol rhizobacterium *Ps. fluorescens* SS101. This inhibition of *P. indica* by diffusible bacterial metabolites was demonstrated in more morphological and metabolomic details for the interaction with *P. fluorescens* WS5 and *Burkholderia cepacia* LA3.

B. cepacia LA3, a Gram-negative nitrogen-fixing bacterium isolated from the roots of rice, showed stronger inhibition on the fungus than *Ps. fluorescens* WS5. Therefore, we focused on the metabolome change in *P. indica* caused by the excreted metabolite(s) of the bacterium. A general screening approach using direct infusion FT-ICR-MS and MassTRIX was applied where the metabolic pathways were characterized. Although some pathways were independent on the bacterial metabolites, many of them were clearly affected. The glycolysis and the pentose phosphate pathways were deactivated in the treated fungus compared to the control. Since both pathways play important roles in the energy maintenance, a decreased number of metabolites in the pathways responsible for energy production, and in consequence fungal growth, were greatly reduced. The other primary function of the pentose phosphate pathway is to provide the cell ribose-5-phosphate (R5P) for the synthesis of the nucleotides and nucleic acids. The number of annotated metabolites within the pyrimide and purine metabolism was reduced to half in the extract from the treated fungus compared to the control for 14-day-old biomass of control fungus. In contrast, *P. indica* under the influence of *B. cepacia* LA3 showed stronger activity in the ubiquinone biosynthesis, limene degradation, and folate biosynthesis. Ubiquinone is an essential component of the mitochondrial respiratory chain (Kilz et al. 1999). Increased numbers of annotated metabolites in the ubiquinone biosynthesis suggest its increased activity and thus higher concentration of free radicals and radical oxygen species subsequent upon a decreased longevity (Szkopinska 2000). Terpenes are the largest class of plant secondary metabolites, and thus, the increased number of metabolites in the pathway of limonene and pinene degradation by the *B. cepacia* LA3 excluded metabolites may be important in the sense of bioremediation and plant–microbe interaction. However, only a few metabolites in the limonene and pinene degradation could be annotated; therefore, more studies are required on these metabolomic aspects of growth inhibition.

To investigate if similar mixture has identical influence on the growth of the *P. indica*, saponin was used as a model component since it is a complex mixture of secondary metabolites with, e.g., surfactant nature of several plants. In contrast to the influence with diffusible inhibitory metabolites of certain rhizobacteria, the ubichinon biosynthesis as well as limonene and pinene degradation pathways were not affected by saponin, although growth of the fungus was suppressed.

The diversity of mycorrhiza-like strategies present in axenically cultivable *P. indica* is unique (44). The studies may shed light on the ecology and evolution of a fascinating group of fungi whose striking biodiversity and ecological importance has only recently started to be recognized (6, 14). *P. indica* has been documented to benefit plant growth and increase resistance against pathogens in a broad range of host plants (10). This study has special significance as the fungus is being exploited for biotechnological applications in the area of agriculture, forestry, arboriculture, and flori-horticulture in field and also hydroponics cultivation of several vegetables and aromatic hosts. For a possible combined application of *P. indica* with plant growth-promoting rhizobacteria, it has to be tested beforehand that an inhibitory effect of these rhizobacteria on *P. indica* does not occur.

Acknowledgements Authors cordially thank Prof. Dr. Rainer Borriss (Humboldt University Berlin) for the provision with the *Bacillus amyloliquefaciens* FZB42 mutants and Prof. Dr. Jos Raaijimakers (Wageningen University, The Netherlands) for the kind supply with *Ps. fluorescens* SS101, its mutant 10.24, and a sample of pure massetolide A. Critical comments received from Prof. Paola Bonfante (Turin, Italy) are highly appreciated. Ajit Varma is thankful to Department of Biotechnology, New Delhi, for partial financial assistance.

References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56:1919–1925

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. Oecologia 146:234–243

Barea JM, Azcon R, Azcon-Aguilar C (2004) Mycorrhizal fungi and plant growth promoting rhizobacteria. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin, Germany, pp 351–362

Bassler BL (2002) Small talk. Cell-to-cell communication in bacteria. Cell 109:421–424

Chen XH, Vater J, Piel J, Franke P, Scholz R, Schneider K, Koumoutsi A, Hitzeroth G, Grammel N, Strittmatter AW, Gottschalk G, Süssmuth RD, Borriss R (2007) Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB42. J Bacteriol 188:4024–4036

Deshmukh S, Hueckelhoven R, Schaefer P, Imani J, Sharma M, Weiss M, Waller F, Kogel KH (2006) The root endophytic fungus Piriformospora indica requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Franche C, Lindström K, Elmerich C (2009) Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321:35–59

Frey-Klett P, Garbaye J, Tarkka M (2007) The mycorrhiza helper bacteria revisited. New Phytol 176:22–36

Fuchs R, Budzikiewicz H (2000) Structural studies of pyoverdines with cyclopeptidic substructures by electrospray ionization and collision induced fragmentation. Spectroscopy 14:229–246

Fuchs R, Budzikiewicz H (2001) Structural studies of pyoverdines by mass spectrometry. Curr Organ Chem 5:265–288

Garbaye J (1994) Tansley Review No. 76 Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–200

Glen M, Tommerup IC, Bougher NL, O'Brien PA (2002) Are *Sebacinaceae* common and widespread ectomycorrhizal associates of *Eucalyptus* spp. Mycorrhiza 12:243–247

Hampp R, Maier A (2004) Interaction between soil bacteria and ectomycorrhiza-forming fungi. In: Varma A, Abbott LK, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin, Germany

Jha B, Thakur MC, Gontia I, Albrecht V, Stoffels M, Schmid M, Hartmann A (2009) Isolation, partial identification and application of diazotrophic rhizobacteria from traditional Indian rice cultivars. Eur J Soil Biol 45:62–72

Kilz S, Lenz C, Fuchs R, Budzikiewicz H (1999) A fast screening method for the identification of siderophores from fluorescent *Pseudomonas* spp. by liquid chromatography/electrospray mass spectrometry. J Mass Spectrometry 34:281–290

Kottke I, Beiter A, Weiss M, Haug I, Oberwinkler F, Nebel M (2003) Basal hymenomycetes belonging to the *Sebacinaceae* are ectomycorrhizal on temperate deciduous trees. Mycol Res 107:957–968

Koumoutsi A, Chen X-H, Vater J, Borriss R (2007) DegU and YczE positively regulate the synthesis of Bacillomycin D by *Bacillus amyloliquefaciens* strain FZB42. Appl Environ Microbiol 73:6953–6964

Lehr NA, Schrey SD, Bauer R, Hampp R, Tarkka MT (2007) Suppression of plant defence response by a mycorrhizal helper bacterium. New Phytol 174:892–903

Mazzola M, Zhao X, Cohen MF, Raaijmakers JM (2007) Cyclic lipopeptide surfactant production by *Pseudomonas fluorescens* SS101 is not required for suppression of complex *Pythium* spp. populations. Phytopathology 97:1348–1355

Oelmueller R, Sherameti I, Tripathi S, Varma A (2009) *Piriformospora indica*: a novel multifunctional symbiotic fungus. Symbiosis 49:1–18

Onega M, Jacques P (2007) Bacillus lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16:115–125

Persello-Cartieaux F, Nussaume L, Robaglia C (2003) Tales from the underground: molecular plant–rhizobacteria interactions. Plant Cell Environ 26:189–199

Peskan-Berghofer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmueller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represent a novel system to study beneficial plant–microbe interactions and involve in early plant protein modifications in the endocytoplasmic reticulum and in the plasma membrane. Physiol Plant 122:465–477

Pham HG, Kumari R, Singh A, Malla R, Prasad R, Sachdev M, Kaldorf M, Buscot F, Oelmueller R, Hampp R, Saxena AK, Rexer KH, Kost G, Varma A (2004) Axenic cultures of *Piriformospora indica*. In: Varma A, Abbott LK, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin, Germany, pp 593–613

Raaijmakers JM, de Bruijn I, de Kock MJD (2006) Cyclic lipopeptide production by plant-associated *Pseudomonas* species: diversity, activity, biosynthesis and regulation. Mol Plant Microbe Interact 19:699–710

Raajimakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moenne-Loccoz Y (2009) The rhizosphere. A playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil 321:341–361

Ryan PR, Dessaux Y, Thomashow LS, Weller DM (2009) Rhizosphere engineering and management for sustainable agriculture. Plant Soil 321:363–383

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW (2003) Bacterial volatiles promote growth in Arabidopsis. Proc Natl Acad Sci USA 100:4927–4932

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Selosse MA, Setaro S, Glatard F, Richard F, Urcelay C, Weiss M (2007) *Sebacinales* are common mycorrhizal associates of *Ericaceae*. New Phytol 174:864–878

Serfling A, Wirsel SGR, Lind V, Deising HB (2007) Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field conditions. Phytopathology 97:523–531

Shahollari B, Vadassery J, Varma A, Oelmueller R (2007) A leucine rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 50:1–13

Shahollari B, Varma A, Oelmueller R (2005) Expression of a receptor kinase in Arabidopsis roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol 162:945–958

Sharma M, Schmid M, Rothballer M, Hause G, Zuccaro A, Imani J, Schäfer P, Hartmann A, Kogel K-H (2008) Detection and identification of mycorrhiza helper bacteria intimately associated with representatives of the order *Sebacinales*. Cell Microbiol 10:2235–2246

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmueller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. J Biol Chem 280:26241–26247

Sherameti I, Tripathi S, Varma A, Oelmuller R (2008a) The root-colonizing endophyte *Pirifomospora indica* confers drought tolerance in Arabidopsis by stimulating the expression of drought stress–related genes in leaves. Mol Plant Microbe Interact 21:799–807

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmueller R (2008b) PYK10, a β-glucosidase located in the endoplasmic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 54:428–439

Suhre K, Schmitt-Kopplin P (2008) MassTRIX: mass translator into pathways. Nucl Acid Res 36: W481–W484

Szkopinska A (2000) Ubiquinone biosynthesis of quinone ring and its isoprenoid side chain. Intracellular localization. Acta Biochimica Polonica 47:469–480

Thrane C, Olsson S, Nielsen TH, Sorensen J (1999) The use of vital fluorescent probes for the detection of stress in the fungi *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamide from *Pseudomonas fluorescens* DR54. FEMS Microbiol Ecol 30:11–23

Urban A, Weiss M, Bauer R (2003) Ectomycorrhizas involving sebacinoid mycobionts. Mycol Res 107:3–14

Vadassery J, Tripathi S, Prasad R, Varma A, Oelmueller R (2009) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and *Arabidopsis*. J Plant Physiol 165:60–70

Varma A, Singh A, Sudha M, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) The mycota 9. Springer, Berlin, Germany, pp 125–150

Varma A, Verma S, Sudha, Sahay N, Butehorn B, Franken P (1999) *Piriformospora indica*—a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:2741–2744

Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Butehorn B, Franken P (1998) *Piriformospora indica* gen. nov., a new root-colonizing fungus. Mycologia 90:896–903

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hueckelhoven R, Neumann C, von Wettstein D et al (2005) The endophytic fungus Piriformospora indica reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) *Sebacinales*: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Part III
Resistance Against Biotic and Abiotic Stress Factors

Chapter 13
Root-Based Innate Immunity and Its Suppression by the Mutualistic Fungus *Piriformospora indica*

Sophie Jacobs, Karl-Heinz Kogel, and Patrick Schäfer

13.1 Introduction

Plants are permanently confronted with environmental cues throughout their lifetime. As result of evolution, plants achieved adaptation to environmental constraints, which even allowed certain plant species to conquer hostile environments such as saline or arid habitats. The invasion by pathogens is another environmental hazard that can affect plant health and might even result in plant lethality. In addition to evolution-driven and genetically determined adaptation, plant acclimatization to a broad versatility of natural habitats relies on their ability to establish alliances with mutualistic organisms (Weiß et al. 2004; Parniske 2008; Schäfer and Kogel 2009; Luo et al. 2009). These associations either base on an extracellular coverage of roots (e.g., by ectomycorrhizas) or the intracellular colonization of root tissue by symbionts as known for endosymbionts such as N_2-fixing rhizobia or various endomycorrhizas (e.g., arbuscular mycorrhizas). In either case, these association are mutualistic as the microbes obtain plant metabolites (e.g., carbohydrates), while plants gain an improved nutrient and water acquisition through fungal hyphae and/or an enhanced abiotic stress tolerance. Notably, the establishment of endosymbioses is based on successive reprogramming of host cells by the mutualistic microbes (Oldroyd and Downie 2008; Parniske 2008). The underlying processes of root colonization are highly

S. Jacobs • K.-H. Kogel
Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany
e-mail: sophie.jacobs@agrar.uni-giessen.de; Karl-Heinz.Kogel@agrar.uni-giessen.de

P. Schäfer (✉)
The School of Life Sciences, University of Warwick, Gibbet Hill Campus, Coventry CV4 7AL, United Kingdom
e-mail: p.schafer@warwick.ac.uk

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_13,

similar to pathogenic plant colonization. The most intriguing difference is the transient or even nonactivation of plant innate immune responses. These observations raise the question whether plants discriminate between mutualistic and parasitic invasion.

In order to counter biotic stressors, plants have evolved an efficient innate immune system making disease outbreak, as result of successful microbial colonization, an exception (Schulze-Lefert and Panstruga 2011). Plants possess a two-layered innate immune system. The first line of plant immunity bases on the recognition of conserved microbial molecules (e.g., chitin, flagellin) summarized as microbe-associated molecular patterns (MAMPs) (Jones and Dangl 2006) or cell wall-derived polysaccharides (part of damage-associated molecular patterns, DAMPs) released during microbial attack (Boller and Felix 2009). In general, MAMPs such as bacterial flagellin or fungal chitin are not present in host cells and are required for microbial vitality. Plants sense these patterns by plasma membrane-localized pattern recognition receptors (PRRs) thereby initiating the activation of basal defenses known as MAMP-triggered immunity (MTI) (Felix et al. 1999; Gómez-Gómez and Boller 2000; Zipfel et al. 2006; Petutschnig et al. 2010). MTI strongly relies on elevated defense gene induction via mitogen-activated protein kinase (MAPK) and Ca^{2+}-dependent protein kinase (CDPK) pathways (Asai et al. 2002; Boudsocq et al. 2010; Boutrot et al. 2010), on rapid production of the stress hormone ethylene, increase of ion fluxes (e.g., Ca^{2+}), and immediate production of reactive oxygen species measurable as the oxidative burst (Gómez-Gómez et al. 1999; Liu et al. 2004). Ca^{2+} participates in defense signaling by activating CDPKs (Boudsocq et al. 2010) and by inducing SA synthesis via CBP60g (Wang et al. 2009). It is further thought to activate NADPH oxidases thereby affecting the oxidative burst (Zhang et al. 2007a; Mersmann et al. 2010). As an outcome of defense signaling is the activation of transcription factors such as various WRKYs, which regulate genes involved in the synthesis of antimicrobial proteins and metabolites like camalexin and glucosinolates (Glazebrook and Ausubel 1994; Navarro et al. 2004,; Clay et al. 2009). The phytohormones ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) influence and modulate various immune processes and thus substantiate MTI (Glazebrook 2005; Asselbergh et al. 2008; Tsuda et al. 2009). Gibberellic acid (GA) balance SA–JA metabolism and influence oxidative stress tolerance, thereby affecting resistance against pathogens (Achard et al. 2006; Navarro et al. 2008). Some pathogens try to camouflage their presence by modifying their cell surface as certain fungi replace chitin by chitosan during tissue colonization (El Gueddari et al. 2002), while a bacterial strategy is the structural modification of LPS (Silipo et al. 2008). Plants obviously penetrated such strategies. For instance, chitosan is recognized by CERK1 and inducing MTI (Petutschnig et al. 2010). Successful pathogens counter MTI by delivering effectors into host cells. Effectors were found to impair MTI by targeting receptor kinases, MAP kinases, as well as components involved in ubiquitination and vesicle trafficking (Janjusevic et al. 2006; Nomura et al. 2006; He et al. 2006; Shan et al. 2008; Xiang et al. 2008; Bartetzko et al. 2009). As a result of these microbial activities, plants have evolved a second layer of defense called effector-triggered immunity (ETI). Another class of receptors, the

so-called resistance (R) proteins, monitor the presence or action of effectors thereby triggering a fast and more accentuated immune response often associated with the development of a localized programmed cell death (Chisholm et al. 2006; Jones and Dangl 2006).

The present review aimed to provide current knowledge regarding MAMP-triggered immune signaling in roots during pathogenic and mutualistic interactions. In addition, we will concentrate on the ability of mutualistic microbes to suppress root immunity. In this respect, we will introduce the colonization strategy of the mutualistic root endophyte *Piriformospora indica* whose colonization success greatly depends on its ability to suppress root innate immunity.

13.2 Root Innate Immunity During Pathogenic Interactions

Due to the microbial density at rhizospheres, it was commonly believed that roots might not possess a MTI system as described for leaves. Recent studies have redrawn this picture, as roots showed almost identical MAMP response patterns as reported for leaves (Millet et al. 2010; Jacobs et al. 2011). Notably, the amplitude of MAMP-triggered immune responses is apparently lower in roots (Jacobs et al. 2011). In leaves, the phytohormones SA, JA, and ET are well-characterized components of MTI. In general, JA- and ET-dependent signaling inhibits pathogens with necrotrophic lifestyle, whereas the activation of the SA pathway inhibits pathogens with a biotrophic lifestyle (Glazebrook 2005; Pieterse et al. 2009). Consistent with this model, roots of mutants impaired in JA signaling were more susceptible against the oomycetes *Pythium irregulare* and *Pythium mastophorum* (Adie et al. 2007; Vijayan et al. 1998) and the ascomycete *Verticillium longisporum* (Johansson et al. 2006). However, this is a very simplified view and more complex antagonistic, additive, and synergistic interactions between the various hormones exist to effectively halt plant-colonizing microbes (Pieterse et al. 2009). As expected, root colonization by the necrotrophic pathogen *Fusarium oxysporum* was reduced in plants constitutively overexpressing the transcription factor *Ethylene Response Factor 1* (*ERF1*), which is involved in JA and ET defense (Berrocal-Lobo and Molina 2004). In contrast, Thatcher et al. (2009) found that roots of plants lacking the central regulator of JA signaling, Coronatine Insensitive 1 (COI1), were more resistant against *F. oxysporum*. The oomycete *Phytophthora arabidopsidis* colonizes *Arabidopsis* roots in a biphasic (hemibiotrophic) manner in which cells are initially biotrophically colonized prior to switching to a later necrotrophic lifestyle. Attard et al. (2010) demonstrated that a parallel activation of the SA and JA signaling occurs during early penetration stages, while solely ET signaling was elevated during biotrophic and necrotrophic growth. Subsequent mutant studies revealed a significant contribution of SA, JA, and ET to defense as respective mutants displayed enhanced susceptibility.

Despite the observed overlap in certain processes (Jacobs et al. 2011), immune signaling in leaves and roots obviously exhibits some differences. The pathogenic

bacteria *P. syringae* pv. *tomato* DC3000 (*Pst*) is able to colonize leaves and roots. *Pst* requires its type-III secretion system (T3SS) to achieve full susceptibility in leaves as result of effector-mediated manipulation of host immunity and metabolism (Alfano and Collmer 2004; Nomura et al. 2005; He et al. 2006). In contrast, suppression of root MTI and thus full root susceptibility does not rely on the T3SS but is solely dependent on coronatine, which is a mimic of JA (Melotto et al. 2006; Millet et al. 2010). In leaves, coronatine suppresses MAMP responses by antagonizing SA defense (Brooks et al. 2005). Although the molecular basis of coronatine-mediated suppression of root MTI is currently unknown, it was shown to be independent on the JA–SA antagonism (Millet et al. 2010). Further evidence for differences in immune signaling in roots and leaves was provided by transcriptomic studies, in which several genes activated in roots had no homology to genes activated in leaves during colonization in beech infected with *Phytophthora* sp. However in case of beech–*Phytophthora* sp. interaction, defense suppression by the hemibiotrophic oomycete was observed and accompanied by a negative crosstalk between JA and SA signaling in roots and shoots (Schlink 2010).

In sum, these studies provide evidence for a root surveillance system that is similar to leaf MTI although the subsequent signaling processes also show tissue-specific differences. Interestingly, the existence of root-based ETI is less clear. The rice blast pathogen *Magnaporthe grisea* is a well-known pathogen of rice shoots, and infection can be stopped by MTI and ETI. Sesma and Osbourn (2004) could demonstrate that the *R* gene *Pi-CO39* did not only confer leaf resistance but also root resistance against *M. grisea* strains carrying *AVR1-CO39*. This indicated the presence of ETI in roots against fungi and ETI might be more commonly distributed as was reported for incompatible plant root–nematode interactions (Fuller et al. 2008).

13.3 Root Innate Immunity During Mutualistic Root Interactions

Mutualistic fungi (arbuscular mycorrhizas, AM) and bacteria (e.g., rhizobia), like pathogenic microbes, are confronted with root MTI and have to overcome it in order to colonize plants. For instance, a pea *myc*$^{-}$ mutant showed defense induction in response to mycorrhizal colonization, which blocked the penetration at the level of appressorium development (Golotte et al. 1993, 1995). Transcriptional profiling in response to colonization by AM revealed a complex alteration in gene expression in mycorrhizal plants (Hohnjec et al. 2005; Deguchi et al. 2007). Commonly observed induction of plant defense responses such as the accumulation of PR proteins and enhancement of phytoalexin biosynthesis were generally less vigorous and only transiently induced in mycorrhizal plants as compared to pathogenic interactions (Lambais and Mehdy 1995 Blee and Anderson 1996; Gianinazzi-Pearson 1996; Salzer and Boller 2000; Deguchi et al. 2007). Although in general

a sustained defense response is not induced during AM symbiosis, there are some exceptions. The expression of defense genes, e.g., *PR-1*, *ß-1,3-glucanase*, was observed and localized to arbuscule-containing cells (Lambais and Mehdy 1998). It indicated that the induction of defense-related genes does occur and might represent a host strategy in arbuscule-containing cells to control fungal development and spread (Lambais and Mehdy 1998). However, such responses might be interaction stage-specific and defense response in host roots are thought to be rapidly suppressed by AM fungi.

Root colonization by mutualistic fungi and bacteria is, as observed in pathogenic interactions, also affected by phytohormones. SA, ET, ABA, cytokinin, and auxin affect root colonization by arbuscular mycorrhizas (Hause et al. 2007; Herrera-Medina et al. 2007). However, the molecular background of phytohormonal action on host metabolism or even immunity is almost unknown. JA is among the best studied hormones and known to support mycorrhization (García-Garrido and Ocampo 2002; Zhao and Qi 2008; Gutjahr and Paszkowski 2009). It is thought that JA regulates the synthesis of secondary metabolites (e.g., flavonoids) as well as the supply of the fungus with carbohydrates (Hause and Schaarschmidt 2009). As reported for mycorrhization, early events associated with root nodulation are supported by the stimulatory effect of JA on isoflavonoid synthesis (Scervino et al. 2007; Zhang et al. 2007b). JA further participates in the regulation of *NOD* genes of rhizobacteria (Rosas et al. 1998; Mabood et al. 2006). In contrast, subsequent pro-symbiotic Ca^{2+} spiking and thus nodulation are disturbed by JA as well as ET and ABA (Sun et al. 2006, Ding and Oldroyd 2009). Together with cytokinin, GA, and auxin, ABA has an additionally regulatory effect on nodule organogenesis in the cortex (Ding and Oldroyd 2009). Whether these hormones have an additional impact on plant immune signaling and thus nodulation, as reported for SA, is currently unknown (Ding and Oldroyd 2009).

13.4 Plant Root Colonization by the Mutualistic Fungus *Piriformospora indica*

The basidiomycete *Piriformospora indica* establishes mutualistic interactions with a broad variety of plant species. Colonized plants exhibit increased biomass and yield, elevated abiotic stress tolerance, and disease resistance (Varma et al. 1999; Peskan-Berghöfer et al. 2004; Waller et al. 2005; Stein et al. 2008, Camehl et al. 2011). The growth-promoting effect was shown to depend on phosphate supply of the plant through the fungus (Yadav et al. 2010). P. indica has been sequenced and is accessible to genetic tranformation (Zuccaro et al. 2009, Zuccaro et al. 2011, Hilbert et al. 2012). The fungus is grouped within the order Sebacinales, which comprises a great variety of mycorrhizal fungi other than arbuscular mycorrhizas (Weiß et al. 2004, 2011). Root colonization studies in barley (Deshmukh et al. 2006; Schäfer et al. 2009, Zuccaro et al. 2011) and in *Arabidopsis* (Jacobs et al.

2011) revealed similar colonization patterns. The fungus either colonizes roots intracellularly via rhizodermal cells or penetrates cortical cells after an initial intercellular growth phase. Root colonization gradiently increases with tissue age as the maturation zone is predominantly colonized, while the fungus is almost absent at the meristematic zone. Accordingly, sporulation is mainly found at extracellular regions of the maturation zone in addition to intracellular sporulation at rhizodermal and cortical cells of the same tissue. Cytological studies indicated a cell death dependency in root colonization by *P. indica* (Deshmukh et al. 2006; Jacobs et al. 2011). In *Arabidopsis* and barley, cytological studies revealed a biotrophic colonization stage preceding cell death-associated colonization (Jacobs et al. 2011, Zuccaro et al. 2011) and subsequent biochemical and genetic studies revealed an endoplasmic stress-induced vacuolar-dependent programmed cell death (Qiang et al. 2012).

Several determinants of root colonization have been identified. In accordance to its lifestyle, *P. indica* colonization of barley roots was accompanied with the suppression of the negative cell death regulator *BAX inhibitor-1* (Hv*BI-1*). Consistent with this, fungal proliferation was strongly inhibited in transgenic barley overexpressing Hv*BI-1* (Deshmukh et al. 2006). The significance of root innate immunity for controlling *P. indica* colonization was evident from studies with mutants altered in MAMP and defense-related hormone signaling. Plants lacking a functional version of the chitin receptor CERK1 were more susceptible to *P. indica* as were *sid2* and *eds1*, which are disturbed in SA synthesis or signaling, respectively. As it demonstrates the relevance of chitin recognition and SA defense in controlling *P. indica* colonization, it indicates conserved patterns in leaf to root immunity. Furthermore, *jin1* and *jar1* mutants that are impaired in JA signaling or synthesis, respectively, were more resistant against *P. indica*. In accordance to the synergistic activity of JA and ET, mutants showing an enhanced ET synthesis (*eto1*) and signaling (*ctr1*) were more susceptible. The inefficiency of ET defense in stopping the fungus was finally indicated by the enhanced colonization of 35S::*ERF1* roots (Khatabi et al. 2012). Recent studies revealed the significance of auxin for the establishment of biotrophic colonization of barley roots by P. indica (Hilbert et al. 2012).

Secondary metabolites are central components of basal defense and nonhost resistance. Among these, camalexin and glucosinolates were shown to exhibit antimicrobial activity (Lipka et al. 2005; Clay et al. 2009; Bednarek et al. 2009). Interestingly, while camalexin was not affecting *P. indica* colonization, *pen2* and *pyk10* mutants that are impaired in glucosinolate metabolism displayed an enhanced susceptibility against the mutualist (Sherameti et al. 2008; Jacobs et al. 2011). These analyses clearly indicated that despite the general susceptibility of plants, components of the root innate immune system control *P. indica* colonization.

13.5 Colonization Success of *P. indica*: A Question of Efficient Suppression of Root Innate Immunity

P. indica has a broad host range among monocotyledonous and dicotyledonous plants, and a nonhost plant has not been identified (Verma et al. 1998; Blechert et al. 1999; Varma et al. 1999; Waller et al. 2005). As a consequence of biotrophism, *P. indica* colonizes living cells by invaginating the plasma membrane, which provokes its recognition by plasma membrane-localized PRRs (e.g., CERK1). An early event after MAMP recognition is Ca^{2+} influx, reactive oxygen species (ROS) burst, (Felix et al. 1999; Miya et al. 2007; Aslam et al. 2009), apoplastic alkalization (Felix et al. 1999), MAPK and CDPK activation, and defense gene induction (Asai et al. 2002; Boudsocq et al. 2010). These responses were observed in roots treated with MAMPs (Vadassery et al. 2009; Millet et al. 2010; Jacobs et al. 2011). In addition, application of cell wall extracts of *P. indica* induced a transient Ca^{2+} response in *Arabidopsis* roots (Vadassery et al. 2009), and a rapid alkalization response was recorded in barley roots after *P. indica* chlamydospores inoculation (Felle et al. 2009). Based on these findings, one would expect an activation of immune responses as a result of *P. indica* recognition. The studies of Deshmukh and Kogel (2007) showed a transient induction of *PR* genes in barley roots at later interaction steps implying an active suppression by the fungus. This was confirmed by microarray studies of the barley root–*P. indica* interaction, which indicated that almost all defense-responsive genes were transiently induced (Schäfer et al. 2009). These finding draw similarities to mycorrhizas and rhizobial interactions and their defense suppressing activities (Salzer et al. 1999; Salzer and Boller 2000; García-Garrido and Ocampo 2002, Kloppholz et al. 2011).

Further studies in *Arabidopsis* roots revealed that *P. indica* was able to suppress MAMP-triggered immune responses (see below) induced by bacterial and fungal MAMPs (Jacobs et al. 2011). This immune-suppressing activity was required for successful root colonization. Roots pretreated with flg22, thereby activating root MTI, showed resistance against *P. indica* as did plants lacking the plant U-box proteins PUB22, PUB23, and PUB24 (Jacobs et al. 2011). The triple mutant *pub22/23/24* does not exert a constitutive immune response under non-stressed conditions, but MTI is hyperactivated after MAMP application (Trujillo et al. 2008). Hence, PUB22-24 are thought to act as negative regulators of MTI. This triple mutant was more resistant against *P. indica*, and root MTI was hyperactivated in *P. indica*-colonized mutant roots (Jacobs et al. 2011). These studies indicated the significance of root MTI suppression by *P. indica* but also suggest the release of immune-activating MAMPs by *P. indica*.

13.6 Regulation of Plant Defense Responses in Plant Root–*P.indica* Interactions

Mechanisms to attenuate plant defense responses can be achieved at several sites of the signaling cascade (e.g., MAMP recognition, gene expression). The ROS burst is known as one of the earliest cell responses after MAMP recognition (Boller and Felix 2009). During pathogen attack in leaves, the ROS burst is involved in defense signaling and defense gene induction (Gómez-Gómez et al. 1999; Zipfel et al. 2006; Miya et al. 2007). Comparable to leaf responses (Felix et al. 1999), *Arabidopsis* roots respond with the induction of a ROS burst to various MAMPs, e.g., flg22 (active epitope of bacterial flagellin), elf18 (active epitope of *Agrobacterium*-derived elongation factor TU), and fungal chitin (Jacobs et al. 2011). From mycorrhizal studies, ROS accumulation has been observed in arbuscular mycorrhiza-colonized plants (e.g., *Medicago truncatula, Nicotiana tabacum*, and *Zea mays*) (Salzer et al. 1999; Fester and Hause 2005) and had been proposed to balance AM development and infestation (Fester and Hause 2005; Puppo et al. 2005). In the *P. indica* interactions, a complete different situation emerges. It could be demonstrated that *P. indica* suppresses the root oxidative burst in response to diverse MAMPs such as flg22, elf18, and fungal chitin in *Arabidopsis*. In addition, *P. indica* colonization resulted in the suppression of flg22-induced expression of defense marker genes (e.g., *WRKY22*, *VSP2*, *CBP60g*). Lastly, *P. indica*-colonized seedlings were insensitive to elf18- and flg22-induced seedling growth inhibition (Jacobs et al. 2011). This growth inhibition is well known in *Arabidopsis* seedlings treated with respective MAMPs and probably reflects a physiological switch from growth to a defense program (Boller and Felix 2009). flg22-induced seedling growth inhibition was also abolished in *quadruple DELLA* mutants, which exerts a constitutive GA signaling (Navarro et al. 2008). GA signaling balances SA–JA antagonism by elevating SA synthesis/defense but suppressing JA defense (Navarro et al. 2008). It further regulates ROS production in response to abiotic stress (Achard et al. 2008). GA deficiency abolished oxidative stress symptoms in contrast to the enhanced stress-induced ROS production in mutants displaying constitutive GA signaling (Achard et al. 2008). GA was found to support colonization of barley and *Arabidopsis* roots by *P. indica*. Barley mutants defective in GA signaling or synthesis exhibited lower colonization levels (Schäfer et al. 2009). Similarly, we observed reduced colonization in GA synthesis mutant *ga1-6* and enhanced colonization in *quintuple DELLA* mutant. However, *P. indica* was unable to suppress defense gene expression in respective barley and both *Arabidopsis* mutants (*ga1-6*, *quintuple DELLA*) (Schäfer et al. 2009; Jacobs et al. 2011). Further studies in *Arabidopsis* implied that improved colonization of *quintuple DELLA*, despite the elevated defense response in this mutant, might be related to a cell death-supportive activity of GA (Jacobs et al. 2011). Interestingly, mutants disturbed in JA synthesis (*jar1-1*) or signaling (*jin1-1*) showed a reduced colonization by *P. indica*. In these mutants, *P. indica* was unable to abolish flg22-induced oxidative burst or to

suppress defense gene expression (Jacobs et al. 2011). It means that *P. indica* requires JA signaling for MTI suppression.

13.7 Conclusions

Based on studies of the past years, roots undoubtedly possess a root innate immune system that recognizes various MAMPs (e.g., chitin, flg22, elf18) (Millet et al. 2010; Jacobs et al. 2011). However, plants are enabled to discriminate between parasitic and mutualistic invaders. This implicates a distinct communication between plants and microbes. While mutualists appear to be provided with a hospital environment accompanying root colonization, pathogens are confronted with a concerted immune response soon after recognition. These different plant responses might be associated with the release of strigolactones or flavonoids by roots to attract mycorrhizas or rhizobium, respectively. By sensing these metabolites, the mutualists release Myc or Nod factors, respectively, to prepare root accommodation. Interestingly, weeds of the Striga family have successfully adapted a strigalactone-assisted root colonization strategy (Wigchert et al. 1999). Pathogens might sense plants similar to mutualists but might have developed another strategy to access roots. Pathogens strongly rely on the initial release of effectors whose primary function is to suppress defense and avoid recognition. Nevertheless, suppression of root innate immunity is obviously also pivotal for mutualistic microbes. There is certainly a need for root–microbe model systems that exhibit distinct interactions types and microbial lifestyles and that allow the performance of genetic studies. This would elevate our current understanding of defense networks in roots. Such studies are especially important for breeding efforts toward plants with selective resistance against root and shoot pathogens without affecting compatibility to mutualistic root microbes.

References

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science 6:91–94

Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell 8:2117–29

Adie BAT, Perez-Perez J, Perez-Perez MM, Godoy M, Sanchez-Serrano J, Schmelz EA, Solano R (2007) ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses *in Arabidopsis*. Plant Cell 19:1665–1681

Alfano JR, Collmer A (2004) Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu Rev Phytopathol 42:385–414

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature 415:977–983

Aslam SN, Erbs G, Morrissey KL, Newman MA, Chinchilla D, Boller T, Molinaro A, Jackson RW, Cooper RM (2009) Microbe-associated molecular pattern (MAMP) signatures, synergy, size and charge: influences on perception or mobility and host defence responses. Mol Plant Pathol 10:375–87

Asselbergh B, De Vleesschauwer D, Höfte M (2008) Global switches and fine-tuning-ABA modulates plant pathogen defense. Mol Plant Microbe Interact 21:709–19

Attard A, Gourgues M, Callemeyn-Torre N, Keller H (2010) The immediate activation of defense responses in *Arabidopsis* roots is not sufficient to prevent *Phytophthora parasitica* infection. New Phytol 187:449–60

Bartetzko V, Sonnewald S, Vogel F, Hartner K, Stadler R, Hammes UZ, Börnke F (2009) The Xanthomonas campestris pv. vesicatoria type III effector protein XopJ inhibits protein secretion: evidence for interference with cell wall-associated defense responses. Mol Plant Microbe Interact 22:655–64

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A, Schulze-Lefert P (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 2:101–6

Berrocal-Lobo M, Molina A (2004) Ethylene response factor 1 mediates *Arabidopsis* resistance to the soilborne fungus *Fusarium oxysporum*. Mol Plant Microbe Interact 17:763–70

Blechert O, Kost G, Hassel A, Rexer RH, Varma A (1999) First remarks on the symbiotic interactions between *Piriformospora indica* and terrestrial orchid. In: Varme A, Hock B (eds) Mycorrhizae, 2nd edn. Springer, Germany

Blee KA, Anderson AJ (1996) Defense related transcript accumulation in *Phaseolus vulgar L.* colonized by the arbuscular mycorrhizal fungus *Glomus intraradices*. Plant Physiol 10:675–688

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60:379–406

Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng SH, Sheen J (2010) Differential innate immune signalling via $Ca^{(2+)}$ sensor protein kinases. Nature 464:418–22

Boutrot F, Segonzac C, Chang KN, Qiao H, Ecker JR, Zipfel C, Rathjen JP (2010) Direct transcriptional control of the *Arabidopsis* immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. Proc Natl Acad Sci USA 107:14502–7

Brooks DM, Bender CL, Kunkel BN (2005) The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. Mol Plant Pathol 6:629–639

Camehl I, Drzewiecki C, Vadassery J, Shahollari B, Sherameti I, Forzani C, Munnik T, Hirt H, Oelmüller R (2011) The OXI1 kinase pathway mediates Piriformospora indica-induced growth promotion in Arabidopsis. PLoS Pathog 5:e1002051

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host–microbe interactions: shaping the evolution of the plant immune response. Cell 24:803–14

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an *Arabidopsis* innate immune response. Science 2:95–101

Deguchi Y, Banba M, Shimoda Y, Chechetka SA, Suzuri R, Okusako Y, Ooki Y, Toyokura K, Suzuki A, Uchiumi T, Higashi S, Abe M, Kouchi H, Izui K, Hata S (2007) Transcriptome profiling of *Lotus japonicus* roots during arbuscular mycorrhiza development and comparison with that of nodulation. DNA Res 14:117–33

Deshmukh S, Kogel KH (2007) *Piriformospora indica* protects barley from root rot caused by *Fusarium graminearum*. J Plant Dis Protect 114:263–268

Deshmukh S, Hückelhoven R, Schäfer P, Imani J, Sharma M, Weiß M, Waller F, Kogel KH (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Ding Y, Oldroyd GE (2009) Positioning the nodule, the hormone dictum. Plant Signal Behav 4:89–93

El Gueddari NE, Rauchhaus U, Moerschbacher BM, Deising HB (2002) Developmentally regulated conversion of surface-exposed chitin to chitosan in cell walls of plant pathogenic fungi. New Phytol 156:103–112

Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 18:265–76

Felle HH, Waller F, Molitor A, Kogel KH (2009) The mycorrhiza fungus *Piriformospora indica* induces fast root-surface pH signaling and primes systemic alkalinization of the leaf apoplast upon powdery mildew infection. Mol Plant Microbe Interact 9:1179–85

Fester T, Hause G (2005) Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza 5:373–9

Fuller VL, Lilley CJ, Urwin PE (2008) Nematode resistance. New Phytol 180:27–44

García-Garrido JM, Ocampo JA (2002) Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. J Exp Bot 53:1377–86

Gianinazzi-Pearson V (1996) Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. Plant Cell 8:1871–1883

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43:205–27

Glazebrook J, Ausubel FM (1994) Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc Natl Acad Sci USA 19:8955–9

Golotte A, Gianinazzi-Pearson V, Giovanetti M, Sbrana C, Avio L, Gianinazzi S (1993) Cellular localization and cytochemical probing of resistance reactions to arbuscular mycorrhizal fungi in a 'locus a' myc − mutant of *Pisum sativum* L. Planta 191:112–122

Golotte A, Gianinazzi-Pearson V, Gianinazzi S (1995) Immunodetection of infection thread glycoprotein and arabinogalactan protein in wild type *Pisum sativum* (L.) or an isogenic mycorrhiza-resistant mutant interacting with *Glomus mosseae*. Symbiosis 18:69–85

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell 5:1003–11

Gómez-Gómez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J 3:277–84

Gutjahr C, Paszkowski U (2009) Weights in the balance: jasmonic acid and salicylic acid signaling in root-biotroph interactions. Mol Plant Microbe Interact 22:763–72

Hause B, Schaarschmidt S (2009) The role of jasmonates in mutualistic symbioses between plants and soil-born microorganisms. Phytochemistry 70:1589–99

Hause B, Mrosk C, Isayenkov S, Strack D (2007) Jasmonates in arbuscular mycorrhizal interactions. Phytochemistry 68:101–10

He P, Shan L, Lin NC, Martin GB, Kemmerling B, Nürnberger T, Sheen J (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in *Arabidopsis* innate immunity. Cell 125:563–575

Herrera-Medina MJ, Steinkellner S, Vierheilig H, Ocampo Bote JA, García Garrido JM (2007) Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. New Phytol 175:554–64

Hilbert M, Voll LM, Ding Y, Hofmann J, Sharma M, Zuccaro A (2012) Indole derivative production by the root endophyte Piriformospora indica is not required for growth promotion but for biotrophic colonization of barley roots. New Phytol 196:520–534

Hohnjec N, Vieweg MF, Pühler A, Becker A, Küster H (2005) Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different Glomus fungi provide

insights into the genetic program activated during arbuscular mycorrhiza. Plant Physiol 137:1283–301

Jacobs S, Zechmann B, Molitor A, Trujillo M, Petutschnig E, Lipka V, Kogel KH, Schaefer P (2011) Broad spectrum suppression of innate immunity is required for colonization of *Arabidopsis thaliana* roots by the fungus *Piriformospora indica*. Plant Physiol. doi:10.1104/pp. 111.176446

Janjusevic R, Abramovitch RB, Martin GB, Stebbins CE (2006) A bacterial inhibitor of host programmed cell death defenses is an E3 ubiquitin ligase. Science 311:222–226

Johansson A, Staal J, Dixelius C (2006) Early responses in the *Arabidopsis-Verticillium longisporum* pathosystem are dependent on NDR1, JA- and ET-associated signals via cytosolic NPR1 and RFO1. Mol Plant Microbe Interact 9:958–69

Jones JD, Dangl JL (2006) The plant immune system. Nature 16:323–9

Khatabi B, Molitor A, Lindermayr C, Pfiffi S, Durner J, von Wettstein D, Kogel KH, Schäfer P (2012) Ethylene supports colonization of plant roots by the mutualistic fungus Piriformospora indica. PLoS One 7:e35502

Kloppholz S, Kuhn H, Requena N (2011) A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. Curr Biol 21:1204–1209

Lambais MR, Mehdy MC (1995) Differential expression of defense-related genes in arbuscular mycorrhiza. Can J Bot 3:533–540

Lambais MR, Mehdy MC (1998) Spatial distribution of chitinases and 1,3-glucanase transcripts in bean arbuscular mycorrhizal roots under low and high soil phosphate conditions. New Phytol 140:33–42

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science 310:1180–3

Liu Y, Schiff M, Dinesh-Kumar SP (2004) Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to tobacco mosaic virus. Plant J 38:800–9

Luo ZB, Janz D, Jiang X, Göbel C, Wildhagen H, Tan Y, Rennenberg H, Feussner I, Polle A (2009) Upgrading root physiology for stress tolerance by ectomycorrhizas: insights from metabolite and transcriptional profiling into reprogramming for stress anticipation. Plant Physiol 151:1902–17

Mabood F, Souleimanov A, Khan W, Smith DL (2006) Jasmonates induce Nod factor production by *Bradyrhizobium japonicum*. Plant Physiol Biochem 44:759–765

Melotto M, Underwood W, Koczan J, Nomura K, He SY (2006) Plant stomata function in innate immunity against bacterial invasion. Cell 8:969–80

Mersmann S, Bourdais G, Rietz S, Robatzek S (2010) Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. Plant Physiol 154:391–400

Millet YA, Danna CH, Clay NK, Songnuan W, Simon MD, Werck-Reichhart D, Ausubel FM (2010) Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. Plant Cell 22:973–990

Miya A, Albert P, Shiny T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc Natl Acad Sci USA 104:19613–19618

Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JD (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. Plant Physiol 135:1113–28

Navarro L, Bari R, Achard P, Lison P, Nemri A, Harberd NP, Jones JDG (2008) DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. Curr Biol 18:650–655

Nomura K, Melotto M, He SY (2005) Suppression of host defense in compatible plant-*Pseudomonas syringae* interactions. Curr Opin Plant Biol 4:361–8

Nomura K, Debroy S, Lee YH, Pumplin N, Jones J, He SY (2006) A bacterial virulence protein suppresses host innate immunity to cause plant disease. Science 313:220–223

Oldroyd GE, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59:519–46

Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 6:763–75

Peskan-Berghöfer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plant 122:465–477

Petutschnig EK, Jones AM, Serazetdinova L, Lipka U, Lipka V (2010) The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. J Biol Chem 37:28902–11

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5:308–16

Puppo A, Groten K, Bastian F, Carzaniga R, Soussi M, Lucas MM, de Felipe MR, Harrison J, Vanacker H, Foyer CH (2005) Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process. New Phytol 3:683–701

Qiang X, Zechmann B, Reitz MU, Kogel KH, Schäfer P (2012) The mutualistic fungus Piriformospora indica colonizes Arabidopsis roots by inducing an endoplasmic reticulum stress-triggered caspase-dependent cell death. Plant Cell 24:794–809

Rosas S, Soria R, Correa N, Abdala G (1998) Jasmonic acid stimulates the expression of nod genes in rhizobium. Plant Mol Biol 38:1161–1168

Salzer P, Boller T (2000) Elicitor-induced reactions in mycorrhizae and their suppression. In: Podila GK, Douds DD Jr (eds) Current advances in mycorrhizae research. APS Press, St. Paul, Minnesota

Salzer P, Corbiere H, Boller T (1999) Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the arbuscular mycorrhiza-forming fungus *Glomus intraradices*. Planta 208:319–325

Scervino JM, Ponce MA, Erra-Bassells R, Bompadre J, Vierheilig H, Ocampo JA, Godeas A (2007) The effect of flavones and flavonols on colonization of tomato plants by arbuscular mycorrhizal fungi of the genera Gigaspora and Glomus. Can J Microbiol 53:702–9

Schäfer P, Kogel KH (2009) The sebacinoid fungus *Piriformospora indica*: an orchid mycorrhiza which may increase host plant reproduction and fitness. In: Deising HB, Esser K (eds) The mycota, vol 5, Plant relationships. Springer, Heidelberg, pp 99–112

Schäfer P, Pfiffi S, Voll LM, Zajic D, Chandler PM, Waller F, Scholz U, Pons-Kühnemann J, Sonnewald S, Sonnewald U, Kogel KH (2009) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. Plant J 59:461–74

Schlink K (2010) Down-regulation of defense genes and resource allocation into infected roots as factors for compatibility between *Fagus sylvatica* and *Phytophthora citricola*. Funct Integr Genomics 2:253–64

Schulze-Lefert P, Panstruga R (2011) A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. Trends Plant Sci 16:117–25

Sesma A, Osbourn AE (2004) The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. Nature 431:582–586

Shan L, He P, Li J, Heese A, Peck SC, Nürnberger T, Martin GB, Sheen J (2008) Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. Cell Host Microbe 17:17–27

Sherameti I, Tripathi S, Varma A, Oelmüller R (2008) The root-colonizing endophyte *Piriformospora indica* confers drought tolerance in *Arabidopsis* by stimulating the expression of drought stress-related genes in leaves. Mol Plant Microbe Interact 6:799–807

Silipo A, Sturiale L, Garozzo D, Erbs G, Jensen TT, Lanzetta R, Dow JM, Parrilli M, Newman MA, Molinaro A (2008) The acylation and phosphorylation pattern of lipid A from *Xanthomonas campestris* strongly influence its ability to trigger the innate immune response in *Arabidopsis*. Chembiochem 14:896–904

Stein E, Molitor A, Kogel KH, Waller F (2008) Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. Plant Cell Physiol 49:1747–51

Sun J, Cardoza V, Mitchell DM, Bright L, Oldroyd GED, Harris JM (2006) Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. Plant J 46:961–970

Thatcher LF, Manners JM, Kazan K (2009) *Fusarium oxysporum* hijacks COI1-mediated jasmonate signaling to promote disease development in *Arabidopsis*. Plant J 58:927–939

Trujillo M, Ichimura K, Casais C, Shirasu K (2008) Negative regulation of PAMP-triggered immunity by an E3 ubiquitin ligase triplet in *Arabidopsis*. Curr Biol 18:1396–401

Tsuda K, Sato M, Stoddard T, Glazebrook J, Katagiri F (2009) Network properties of robust immunity in plants. PLoS Genet 12:e1000772

Vadassery J, Ranf S, Drzewiecki C, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R (2009) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J 2:193–206

Varma A, Verma S, Sudha SN, Bütehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Verma S, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:898–905

Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J (1998) A role for jasmonate in pathogen defense of *Arabidopsis*. Proc Natl Acad Sci USA 95:7209–7214

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neuman C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Wang L, Tsuda K, Sato M, Cohen JD, Katagiri F, Glazebrook J (2009) *Arabidopsis* CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. PLoS Pathog 5:e1000301

Weiß M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Weiß M, Sýkorová Z, Garnica S, Riess K, Martos F, Krause C, Oberwinkler F, Bauer R, Redecker D (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS One 6:e16793

Wigchert SC, Kuiper E, Boelhouwer GJ, Nefkens GH, Verkleij JA, Zwanenburg B (1999) Dose–response of seeds of the parasitic weeds Striga and Orobanche toward the synthetic germination stimulants GR 24 and Nijmegen 1. J Agric Food Chem 47:1705–10

Xiang T, Zong N, Zou Y, Wu Y, Zhang J, Xing W, Li Y, Tang X, Zhu L, Chai J, Zhou JM (2008) *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. Curr Biol 8:74–80

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. J Biol Chem 20:26532–44

Zhang J, Shao F, Li Y, Cui H, Chen L, Li H, Zou Y, Long C, Lan L, Chai J, Chen S, Tang X, Zhou JM (2007a) A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. Cell Host Microbe 1:175–185

Zhang J, Subramanian S, Zhang Y, Yu O (2007b) Flavone synthases from *Medicago truncatula* are flavanone-2-hydroxylases and are important for nodulation. Plant Physiol 144:741–751

Zhao SZ, Qi XQ (2008) Signaling in plant disease resistance and symbiosis. J Integr Plant Biol 50:799–807

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 19:749–60

Zuccaro A, Basiewicz M, Zurawska M, Biedenkopf D, Kogel KH (2009) Karyotype analysis, genome organization, and stable genetic transformation of the root colonizing fungus Piriformospora indica. Fungal Genet Biol 46:543–550

Zuccaro A, Lahrmann U, Güldener U, Langen G, Pfiffi S, Biedenkopf D, Wong P, Samans B, Grimm C, Basiewicz M, Murat C, Martin F, Kogel KH (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont Piriformospora indica. PLoS Pathog 7:e1002290

Chapter 14
Role of Defense Compounds in the Beneficial Interaction Between *Arabidopsis thaliana* and *Piriformospora indica*

Iris Camehl, Irena Sherameti, Eileen Seebald, Joy Michael Johnson, and Ralf Oelmüller

14.1 Defense in Beneficial Plant/Microbe Interactions: An Introduction

In nature, plants are exposed to a large number of quite diverse microorganisms, which can be beneficial (mutualistic interaction), harmful (pathogenic interaction), or neutral (commensalistic interaction) for plant performance. Root-colonizing rhizobacteria, mycorrhizal fungi, or beneficial endophytes often promote plant growth and biomass production and establish tolerance against biotic and abiotic stresses. They compete with soil-borne microbes which are harmful for the plant (Johnson and Oelmüller 2009). These two types of symbiotic interactions are extremes. Therefore, it is not surprising that the mode of interaction between two symbionts is never stable, and all types of transitions have been observed in nature, depending on environmental conditions and genetic programs. Genetic studies have uncovered that single gene loci in both plants and microbes determine the mode of interaction and manipulation of crucial genes may cause severe alterations in the symbiosis (Johnson and Oelmüller 2009 and references therein).

We study the beneficial interaction between the growth- and biomass-promoting endophyte *Piriformospora indica* and plant roots. The endophytic fungus, a basidiomycete of the Sebacinaceae family, interacts with many plant species, including *Arabidopsis*. Like other members of the Sebacinales, *P. indica* colonizes the roots, grows inter- and intracellularly, and forms pear-shaped spores in the roots as well as on the root surface. The endophyte promotes nutrient uptake, allows plants to survive under abiotic (water and salt) stress, confers resistance to toxins, heavy metal ions, and pathogenic organisms, and stimulates growth and seed production

I. Camehl • I. Sherameti • E. Seebald • J. Michael Johnson • R. Oelmüller (✉)
Institute of General Botany and Plant Physiology, Friedrich-Schiller-University of Jena, Dornburger Str. 159, 07743 Jena, Germany
e-mail: iris.camehl@gmx.de; irena.sherameti@uni-jena.de; Eileen.Seebald@uni-jena.de; joymkau@gmail.com; b7oera@hotmail.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_14,

(cf. Oelmüller et al. 2004, 2005, 2009; Peškan-Berghöfer et al. 2004; Pham et al. 2004; Sahay and Varma 1999; Shahollari et al. 2005, 2007; Sherameti et al. 2005; Varma et al. 1999, 2001). *P. indica* is a cultivable fungus and can grow on synthetic media without a host (Peškan-Berghöfer et al. 2004; Varma et al. 2001; Verma et al. 1998). The host range includes trees, agricultural, horticultural, and medicinal plants, monocots, dicots, and mosses (Barazani et al. 2005; Glen et al. 2002; Peškan-Berghöfer et al. 2004; Shahollari et al. 2005, 2007; Sherameti et al. 2005; Varma et al. 2001; Waller et al. 2005; Weiss et al. 2004), suggesting that the interaction is based on general recognition and signaling processes.

Using a genetic approach, we identified genes and proteins which are required for the beneficial interaction between the two symbionts (Oelmüller et al. 2004; Shahollari et al. 2005, 2007; Sherameti et al. 2008; Camehl et al. 2010). For this screen, we used independent plant responses which are induced by *P. indica* in *Arabidopsis* roots and leaves (e.g. growth promotion, seed yield, resistance against drought or leaf pathogens, marker gene expression, protein phosphorylation pattern, spore germination) and identified those mutants in which these responses are not induced by the fungus. We isolated two classes of mutants: those which do not respond to *P. indica* and grow like uncolonized plants in the presence of the fungus and those for which *P. indica* was pathogenic (Camehl et al. 2010). For some of these mutants, the genes were identified by map-based cloning strategies. Interestingly, several of these mutants were defective in defense compounds. Their analysis uncovered that mutualism depends on a balanced activation of defense mechanisms. For some of the defense compounds, we also discovered novel functions in this beneficial symbiosis.

Defense responses have been intensively studied in beneficial microbial and mycorrhizal communications. As long as the microbial partner is not recognized as a friend (e.g. during early phases of mycorrhizal interaction, when the plants have not yet benefited from the fungus and nutrient exchange has not yet started), the plant often initiates defense responses against the symbiont. Güimil et al. (2005) have shown that over 40% of the genes in the roots of rice seedlings respond to beneficial and non-beneficial fungi and many of them are involved in plant defense. When the establishment of a beneficial symbiosis proceeds and the plant recognizes the microbial partner as a friend, the expression of defense genes becomes downregulated. The molecular mechanism which causes the shutdown of defense responses is unclear at present. It can be an active process initiated by either the plant or the fungus or simply a passive process since defense activating compounds are no longer present in the beneficial symbiosis (Harrison 2005).

Plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play major roles in regulating plant defense responses. Simplified, SA is involved in the reaction against biotrophic and hemi-biotrophic pathogens while JA and ET are associated with defense against necrotrophic pathogens and herbivorous insects. The two main defense mechanisms in plants are the systemic acquired resistance (SAR) where SA is essential and the induced systemic resistance (ISR) which is mainly based on ET and JA signaling. ISR results from colonization of roots by certain nonpathogenic bacteria (van Loon et al. 1998) but

also friendly fungi such as *Trichoderma* spp. (Shoresh et al. 2010) or *P. indica* (Stein et al. 2008).

Furthermore, reactive oxygen species (ROS), in particular H_2O_2, are important signaling compounds in plants dealing with pathogenic microorganisms (Apel and Hirt 2004) but also with rhizobia (Puppo et al. 2005) or arbuscular mycorrhizal fungi (Fester and Hause 2005). Tanaka et al. (2006) have shown the important role of ROS in regulating the mutualistic interaction between a clavicipitaceous fungal endophyte, *Epichloë festucae*, and its grass host, *Lolium perenne*. *E. festucae* grows systemically in intercellular spaces of leaves as infrequently branched hyphae parallel to the leaf axis. A fungal mutant defective in a NADPH oxidase gene (*noxA*) altered the interaction from mutualistic to antagonistic. Plants infected with the *noxA* mutant lose apical dominance, become severely stunted, show precocious senescence, and eventually die. The fungal biomass in these associations is increased dramatically. ROS accumulation was detected cytochemically in the endophyte extracellular matrix and at the interface between the extracellular matrix and host cell walls of meristematic tissue in wild-type but not in *noxA* mutant associations. These results demonstrate that not only plant-synthesized ROS but also fungal ROS production is critical in mutualistic fungus–plant interactions, presumably by restricting root colonization.

These examples demonstrate that defense gene activation is an important aspect in beneficial symbioses, which is further highlighted by the identification of genes involved in defense processes in the *P. indica*/*Arabidopsis* screen outlined above. Here, we discuss four defense-related processes which are required for the beneficial interaction between *P. indica* and *Arabidopsis*.

14.2 PYK10

Glucosinolate biosynthesis plays an important role in plant/pathogen interactions (Halkier and Gershenzon 2006). Many genes for glucosinolate biosynthesis and/or degradation are upregulated in *P. indica*-colonized *Arabidopsis* roots. Several of them appear to be involved in establishing a mild defense response against the fungus. One of the genes codes for the putative myrosinase PYK10 (Nitz et al. 1999), an abundant protein in the roots of Brassicaceae. The putative β-glucosidase of 65 kDa is located in so-called endoplasmic reticulum (ER) bodies and contains the ER-retention signal KDEL (Matsushima et al. 2003b). ER bodies are spindle-shaped structures of ~10 μm in length and ~1 μm in width (cf. Matsushima et al. 2003a; Haseloff et al. 1997; Hawes et al. 2001; Hayashi et al. 1999; Ridge et al. 1999) which have been found in more than 50 plant species (Behnke and Eschlbeck 1978; Bones et al. 1989; Bonnett and Newcomb 1965; Gunning 1998; Iversen 1970). ER bodies are surrounded by ribosomes (Hayashi et al. 1999) and are highly enriched in the roots of young seedlings (Matsushima et al. 2002). Interestingly, ER bodies can also be induced in rosette leaves by JA (McConn et al. 1997), and the JA-insensitive *coronatine insensitive1* (*coi1*; Xie et al. 1998) mutant does not form

ER bodies (Matsushima et al. 2002). This suggests that PYK10 might be involved in JA-induced defense responses.

PYK10 has been identified as a target of *P. indica* in *Arabidopsis* roots (Peškan-Berghöfer et al. 2004). Within minutes after the contact of the roots with the fungus, a shift in the electrophoretic mobility of PYK10 can be observed on two-dimensional gels, suggesting that the protein becomes modified in response to signals from the fungus. We identified an EMS and a T-DNA insertion line, which are defective in *PYK10* expression. These mutants do not respond to *P. indica,* indicating that PYK10 is required for the establishment of the beneficial interaction between the two symbionts (Sherameti et al. 2008). This observation is further supported by an independent mutant with a lesion in the transcription factor NAI1. The basic helix-loop-helix domain-containing transcription factor NAI1 is responsible for *PYK10* expression. Closer inspection of plants with altered PYK10 levels uncovered that the putative myrosinase controls the degree of root colonization: lower *PYK10* mRNA levels result in higher root colonization, while plants overexpressing *PYK10* under the control of the 35S promoter are less colonized. Although the physiological role and natural substrate(s) of PYK10 are unknown at present, these observations suggest that enzymatic activities associated with PYK10 may restrict root colonization. Apparently, the beneficial interaction between *Arabidopsis* and *P. indica* is based on a highly sophisticated balance between the two symbiotic partners. It is conceivable that increasing quantities of fungal hyphae lead to a degree of root colonization that provokes plant defense responses and represses beneficial responses, whereas decreasing quantities of hyphae in the root environment result in suboptimal exchanges of information and nutrients between the two partners. This resembles mycorrhizal symbioses, in which initially activated defense responses against the symbiont are reduced during later phases of the interaction or are even actively repressed (cf. Pozo and Azcón-Aguilar 2007). Although not studied in detail, Zeng et al. (2003) have also shown that myrosinase activity controls the growth of ectomycorrhiza fungi.

β-glucosidases and myrosinases hydrolyze β-glucosidic bonds of aryl and alkyl β-D-glucosides, as well as glucosides with carbohydrate moieties such as cellobiose and other β-linked oligosaccharides (Esen 1993). In particular, myrosinases hydrolyze nontoxic glucosinolates to biologically active isothiocyanates, thiocyanates, nitriles, or epithionitriles (cf. Bones and Rossiter 1996; Poulton 1990; Rask et al. 2000; Wittstock and Halkier 2002), and the biological function of a myrosinase depends upon the nature of the aglycon moieties released from the substrates. A well-studied role of these agylcons is their involvement in plant defense against herbivores and microbes (Rask et al. 2000; Stotz et al. 1999, 2000; Tierens et al. 2001; Sanchez-Vallet et al. 2010). PYK10 is released from the endosomal system and reacts with PBP1, forming a multimeric complex. Thus, the substrate(s) of PYK10 is likely to be separated from the enzyme through membranes (cf. references in Nagano et al. 2005), and destruction of the cell and cellular compartments is required to bring these components together. One might speculate that this occurs during root colonization after the two organisms come into contact

with each other. Overcolonization might result in more damage to the root cells and thus more activation of glucosinolate-based defense responses.

Although the role of PYK10 in the interaction between *Arabidopsis* and *P. indica* is unclear at present, the observation that *Arabidopsis* lines with reduced PYK10 protein levels are more susceptible to fungal colonization/association supports the idea that the enzyme is involved in defending the root cells against an excess of invading hyphae, which could result in a disturbance of the balanced mutualistic interaction. PYK10 exhibits striking sequence similarities to PEN2, a glycosyl hydrolase, which restricts pathogen entry of two ascomycete powdery mildew fungi into *Arabidopsis* leaf cells (Lipka et al. 2005). Like PEN2, PYK10 belongs to the class of glycosyl hydrolase family 1. Both proteins are located in intracellular organellar structures (PYK10 in ER bodies and PEN2 in peroxisomes), and both proteins share a high degree of sequence similarity. The catalytic domains of both proteins contain two conserved nucleophilic glutamates. Lipka et al. (2005) have shown that glutamate^{183} is required for PEN2 function in vivo, which suggests that PEN2 catalytic activity is required for restricting pathogen entry. Thus, PYK10 might have a similar biological function in the *P. indica*/*Arabidopsis* system.

An important task for the future will be to understand the function of PYK10, one of the most abundant protein in Brassica roots. It is not known whether the enzyme has myrosinase activity. Furthermore, the appropriate substrate(s) and product(s) need to be identified.

14.3 ET Signaling Components

ET and JA often function synergistically in plant defense response. Defense genes such as *PLANT DEFENSIN 1.2* (*PDF1.2*) and *PATHOGENESIS-RELATED PROTEIN* (*PR*)*-3* encoding the basic chitinase are activated against necrotrophic fungi primarily by the ET/JA pathway. Both hormones are also required for the ISR which is triggered by beneficial rhizobacteria and fungi (Pieterse et al. 1998; van Wees et al. 2008). In contrast, biotrophic pathogens are more efficiently countered by SA-controlled defense mechanisms (Thomma et al. 1998, 1999) and the activation of *PR-1*, *PR-2*, and *PR-5*.

We identified *Arabidopsis* mutants which are smaller in the presence of the fungus compared to the uncolonized control. This suggests that these mutants consider *P. indica* as a foe and that the interaction is shifted from mutualism to parasitism. Several mutated genes were identified as components of the ET signaling pathway (Camehl et al. 2010).

ET is perceived by a family of membrane-associated two-component systems at the ER, including ETR1/ETR2, ET response sensor (ERS) 1 and 2, and EIN4 in *Arabidopsis* (Chang et al. 1993; Hua et al. 1995; Hua and Meyerowitz 1998; Sakai et al. 1998). EIN2, EIN3, EIN5, and EIN6 are positive regulators of ET responses, acting downstream of CTR1. CTR1 derepresses EIN2, and this leads to the activation of EIN3 and EIN3-like (EIL) transcription factors. EIN2 is an integral

membrane protein of unknown function with similarities to NRAMP metal transporters (Alonso et al. 1999). Growth of *etr1*, *ein2*, and *ein3*/*eil1* plants is not promoted or even inhibited by the fungus. The plants produce less seeds, and the roots are more colonized compared to the wild type roots. This results in a mild activation of defense responses. These results clearly demonstrate that restriction of fungal growth by ET signaling components is required for the beneficial interaction between the two symbionts. Furthermore, overexpression of the *ETHYLENE RESPONSE FACTOR1* (ERF1) constitutively activates defense responses, which also abolishes the benefits for the plants. Therefore, ET signaling components and ET-targeted transcription factors are required for balancing beneficial and non-beneficial traits in the symbiosis. Manipulation of signaling components of this pathway, including crucial target transcription factors, results in an unstable symbiosis which is no longer beneficial for the plant (Camehl et al. 2010).

Several questions remain unanswered. The exact target genes of the ET signaling pathway, which are required for the establishment of the beneficial interaction, are still unknown. Furthermore, it is interesting to note that growth promotion is abolished at the seedlings level. Thus, ET signaling is already required during early phases of the interaction. Again, a link between ET signaling and the control of the growth response is unknown. Finally, we and others have demonstrated that leaves of *P. indica*-colonized plants are more resistant against leaf pathogens. It is likely that the information flow from the roots to the leaves is mediated by a mechanism that resembles an ISR response (Pieterse and Van Loon 2004). Stein et al. (2008) have shown that *P. indica* SAR in *Arabidopsis* requires JA signaling and the cytoplasmic function of NPR1. Since the ISR in *Arabidopsis* depends on ET and JA signaling, a putative role of ET in the *P. indica*-induced ISR needs to be defined.

14.4 OXI1/PDK1

One of the mutants which do not respond to *P. indica* has a lesion in *OXI1*. OXI1 is a serine/threonine kinase which is necessary for oxidative burst-mediated signaling in *Arabidopsis* roots (Anthony et al. 2004; Rentel et al. 2004). The enzyme is a member of the AGC protein kinase family and was originally identified because its expression was induced by H_2O_2 in vivo (Rentel et al. 2004). OXI1 is required for full activation of the two MITOGEN-ACTIVATING PROTEIN KINASES 3 and 6 after treatment with ROS or elicitors and for different ROS-mediated processes including basal resistance to *Peronospora parasitica* infection and root hair growth (Rentel et al. 2004). Besides ROS, OXI1 is also activated by the PHOSPHOLIPID-BINDING KINASE (PDK)1 (Anthony et al. 2004). The main phospholipid in plants is phosphatidic acid (PA) which functions as a second messenger in many stress response pathways. The active OXI1 phosphorylates and thus activates the downstream serine/threonine kinase PTI1-2 in response to ROS and PA signals (Anthony et al. 2006), and many of these signals derive from microbial pathogens

or elicitors, such as cell wall fragments or specific protein factors released by pathogens (van der Luit et al. 2000; Yamaguchi et al. 2005).

P. indica does not induce ROS production in *Arabidopsis* roots (Vadassery et al. 2009a), while the PA level is stimulated. Furthermore, under beneficial, growth-promoting cocultivation conditions, defense genes are downregulated in *Arabidopsis*. Genetic studies established the PA-activated PDK1-OXI1 pathway as a novel signaling event which is crucial for a beneficial interaction between the two symbionts. Thus, like in mammalian systems, this pathway is required for *P. indica*-induced growth promotion and proliferation rather than activation of defense processes. Even under non-beneficial cocultivation conditions of the two symbionts, activation of defense genes is independent of the PA-PDK1-OXI1 pathway. This novel function of the originally identified defense pathway and the role of AGC kinase in beneficial plant/microbe interactions may be of general importance (cf. Pislariu and Dickstein 2007).

14.5 AtHSPRO2

In our screen for *Arabidopsis* mutants which recognizes *P. indica* as a pathogen rather than a beneficial fungus, we identified *hspro2*. HSPRO2 is required for basal resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. The *Arabidopsis* protein exhibits striking sequence similarities to a nematode resistance protein from *Beta procumbens* (Cai et al. 1997). HSPRO2 appears to function downstream of SA and is negatively regulated by signaling through JA and ET (Murray et al. 2007). We are only at the beginning to understand the role of this protein in pathogenic and beneficial plant–microbe interactions.

14.6 Conclusions

The initially activated defense response of a plant against beneficial microbes resembles that against pathogenic microbes. The same genes and signaling pathways are activated by microbe-associated molecular patterns from pathogens and beneficial fungi. We show that defense gene activation plays a crucial role in the beneficial symbiosis between *Arabidopsis* and *P. indica*. These defense responses appear to function at different levels (Fig. 14.1). Glucosinolates and enzymes involved in their breakdown appear to be activated only after cell damage. This may occur, at least to some extent, if the fungus enters the plant cell. It is conceivable that this defense strategy becomes important if uncontrolled hyphal growth occurs in the roots. Reduced levels of PYK10, for instance, allows overcolonization of the root cells, presumably because the roots cannot restrict hyphal growth. Quite similar, ET signaling controls the interaction between the two symbionts at early stages. Inactivation of components of the ET pathway has a

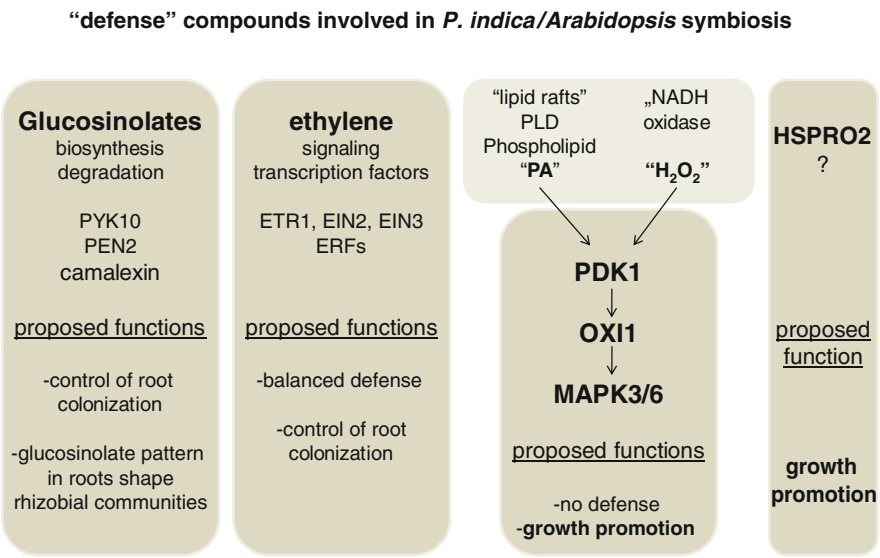

Fig. 14.1 Plant defense compounds identified in the beneficial interaction between *P. indica* and *Arabidopsis*

severe impact on root colonization, whereas higher ET signaling represses and lower ET signaling promotes hyphal growth. Overcolonization of the roots is associated with a mild defense response for the restriction of fungal growth. On the other hand, overexpression of *ERF1* induces a mild defense response, which also restricts root colonization. As a consequence, the roots are less colonized as wild-type roots, which—in turn—is less beneficial for the plant. These two examples support classical concepts developed for mycorrhizal symbiosis that some defense strategies are necessary for long-term harmony between symbionts.

In contrast, the PDK1/OXI1 pathway, previously identified to activate defense responses against pathogens, appears to have a different function in beneficial interactions. The kinases are required for long-term harmony between the two symbionts, and they are not involved in defense gene activation against or restriction of hyphal growth of *P. indica*. Finally, the role of HSPRO2 in the *P. indica*/*Arabidopsis* interaction is unclear. All available information suggests that HSPRO2 has another/additional function than activating defense processes against *P. indica*.

Taken together, a sophisticated network of defense responses which need to be active in a time- and space-dependent manner is required to maintain a beneficial *P. indica*/*Arabidopsis* symbiosis. The relative large number of mutants which have been identified in our screen demonstrates that defense components are crucial for this beneficial symbiosis. While some of them activate mild defense responses, the function of other is not yet understood.

Acknowledgements We thank IMPRS and DFG for funding and Corinna Drzewiecki for support.

References

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. Science 284:2148–2152

Anthony RG, Henriques R, Helfer A, Mészáros T, Rios G, Testerink C, Munnik T, Deák M, Koncz C, Bögre L (2004) A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in *Arabidopsis*. EMBO J 23:572–581

Anthony RG, Khan S, Costa J, Pais MS, Bögre L (2006) The *Arabidopsis* protein kinase PTI1-2 is activated by convergent phosphatidic acid and oxidative stress signaling pathways downstream of PDK1 and OXI1. J Biol Chem 281:37536–3746

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. Oecologia 146:234–243

Behnke H-D, Eschlbeck G (1978) Dilated cisternae in Capparales—an attempt towards the characterization of a specific endoplasmic reticulum. Protoplasma 97:351–363

Bones AM, Rossiter JT (1996) The myrosinase-glucosinolate system, its organization and biochemistry. Physiol Plant 97:194–208

Bones AM, Evjen K, Iversen T-H (1989) Characterization and distribution of dilated cisternae of the endoplasmic reticulum in intact plants, protoplasts, and calli of *Brassicaceae*. Isr J Bot 38:177–192

Bonnett HT Jr, Newcomb EH (1965) Polyribosomes and cisternal accumulations in root cells of radish. J Cell Biol 27:423–432

Cai D, Kleine M, Kifle S, Harloff HJ, Sandal NN, Marcker KA, Klein-Lankhorst RM, Salentijn EM, Lange W, Stiekema WJ, Wyss U, Grundler FM, Jung C (1997) Positional cloning of a gene for nematode resistance in sugar beet. Science 275:832–834

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Oelmüller R (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and non-beneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol 185:1062–1073

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science 262:539–544

Esen A (1993) β-glucosidases: overview. In: Esen A (ed) B-Glucosidases. Biochemistry and molecular biology. American Chemical Society, Washington, DC, pp 1–14

Fester T, Hause G (2005) Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza 15:373–379

Glen M, Tommerup IC, Bougher NL, O'Brien PA (2002) Are Sebacinaceae common and widespread ectomycorrhizal associates of Eucalyptus species in Australian forests? Mycorrhiza 12:243–247

Güimil S, Chang HS, Zhu T, Sesma A, Osbourn A, Roux C, Ioannidis V, Oakeley EJ, Docquier M, Descombes P, Briggs SP, Paszkowski U (2005) Comparative transcriptomics of rice reveals an ancient pattern of response to microbial colonization. Proc Natl Acad Sci USA 102:8066–8070

Gunning BES (1998) The mystery organelles in *Arabidopsis* expressing GFP. Trends Plant Sci 3:417

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol 57:303–333

Harrison MJ (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 59:19–42

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc Natl Acad Sci USA 94:2122–2127

Hawes C, Saint-Jore C, Martin B, Zheng H-Q (2001) ER confirmed as the location of mystery organelles in *Arabidopsis* plants expressing GFP. Trends Plant Sci 6:245–246

Hayashi M, Toriyama K, Kondo M, Hara-Nishimura I, Nishimura M (1999) Accumulation of a fusion protein containing 2S albumin induces novel vesicles in vegetative cells of *Arabidopsis*. Plant Cell Physiol 40:263–272

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94:261–271

Hua J, Chang C, Sun Q, Meyerowitz EM (1995) Ethylene insensitivity conferred by *Arabidopsis ERS* gene. Science 269:1712–1714

Iversen T-H (1970) Cytochemical localization of myrosinase (β-thioglucosidase) in root tips of *Sinapis alba*. Protoplasma 71:451–466

Johnson JM, Oelmüller R (2009) Mutualism and parasitism: life in an unstable continuum. What can we learn from the mutualistic interaction between *Piriformospora indica* and *Arabidopsis thaliana*? Endocytobiol Cell Res 19:81–111

Lipka V, Dittgen J, Bednarek P et al (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science 310:1180–1183

Matsushima R, Hayashi Y, Kondo M, Shimada T, Nishimura M, Hara-Nishimura I (2002) An endoplasmic reticulum-derived structure that is induced under stress conditions in *Arabidopsis*. Plant Physiol 130:1807–1814

Matsushima R, Hayashi Y, Yamada K, Shimada T, Nishimura M, Hara-Nishimura I (2003a) The ER body, a novel endoplasmic reticulum-derived structure in *Arabidopsis*. Plant Cell Physiol 44:661–666

Matsushima R, Kondo M, Nishimura M, Hara-Nishimura I (2003b) A novel ER-derived compartment, the ER body, selectively accumulates a β-glucosidase with an ER retention signal in *Arabidopsis*. Plant J 33:493–502

McConn M, Creelman RA, Bell E, Mullet JE, Browse J (1997) Jasmonate is essential for insect defense in *Arabidopsis*. Proc Natl Acad Sci USA 94:5473–5477

Murray SL, Ingle RA, Petersen LN, Denby KJ (2007) Basal resistance against *Pseudomonas syringae* in *Arabidopsis* involves WRKY53 and a protein with homology to a nematode resistance protein. Mol Plant Microbe Interact 20:1431–1438

Nagano AJ, Matsushima R, Hara-Nishimura I (2005) Activation of an ER-body-localized β-glucosidase *via* a cytosolic binding partner in damaged tissues of *Arabidopsis thaliana*. Plant Cell Physiol 46:1140–1148

Nitz I, Berkefeld H, Puzio PS, Grundler FMW (1999) *Pyk10*, a seedling and root specific gene and promoter from *Arabidopsis thaliana*. Plant Sci 161:337–346

Oelmüller R, Shahollari B, Peškan-Berghöfer T, Trebicka A, Giong PH, Sherameti I, Oudhoff M, Venus Y, Altschmied L, Varma A (2004) Molecular analyses of the interaction between *Arabidopsis* roots and the growth-promoting fungus *Piriformospora indica*. Endocytobiosis Cell Res 15:504–517

Oelmüller R, Peškan-Berghöfer T, Shahollari B, Sherameti I, Varma A (2005) MATH-domain containing proteins represent a novel gene family in *Arabidopsis thaliana* and are involved in plant/microbe interactions. Physiol Plant 124:152–166

Oelmüller R, Sherameti I, Tripathi S, Varma A (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Peškan-Berghöfer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V, Varma A, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plant 122:465–477

Pham GH, Kumari R, An S et al (2004) Axenic cultures of *Piriformospora indica*. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin, Heidelberg, pp 593–616

Pieterse CM, Van Loon LC (2004) NPR1: the spider in the web of induced resistance signaling pathways. Curr Opin Plant Biol 7:456–464

Pieterse CM, van Wees SC, van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, van Loon LC (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell 10:1571–1580

Pislariu CI, Dickstein R (2007) An IRE-like AGC kinase gene, MtIRE, has unique expression in the invasion zone of developing root nodules in *Medicago truncatula*. Plant Physiol 144:682–694

Poulton JE (1990) Cyanogenesis in plants. Plant Physiol 94:401–405

Pozo MJ, Azcón-Aguilar C (2007) Unraveling mycorrhiza-induced resistance. Curr Opin Plant Biol 10:393–398

Puppo A, Groten K, Bastian F, Carzaniga R, Soussi M, Lucas MM, de Felipe MR, Harrison J, Vanacker H, Foyer CH (2005) Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process. New Phytol 165:683–701

Rask L, Andreasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol 42:93–113

Rentel MC, Lecourieux D, Ouaked F, Usher SL, Petersen L, Okamoto H, Knight H, Peck SC, Grierson CS, Hirt H, Knight MR (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. Nature 427:858–861

Ridge RW, Uozumi Y, Plazinski J, Hurley UA, Williamson RE (1999) Developmental transitions and dynamics of the cortical ER of *Arabidopsis* cells seen with green fluorescent protein. Plant Cell Physiol 40:1253–1261

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Sakai H, Hua J, Chen QG, Chang C, Medrano LJ, Bleecker AB, Meyerowitz EM (1998) *ETR2* is an *ETR1*-like gene involved in ethylene signaling in *Arabidopsis*. Proc Natl Acad Sci USA 95:5812–5817

Sanchez-Vallet A, Ramos B, Bednarek P, López G, Piślewska-Bednarek M, Schulze-Lefert P, Molina A (2010) Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. Plant J 63:115–127

Shahollari B, Varma A, Oelmüller R (2005) Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol 162:945–958

Shahollari B, Vadassery J, Varma A, Oelmüller R (2007) A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 50:1–13

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. J Biol Chem 280:2641–2647

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmüller R (2008) PYK10, a β-glucosidase located in the endoplasmic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 50:1–17

Shoresh M, Harman GE, Mastouri F (2010) Induced systemic resistance and plant responses to fungal biocontrol agents. Annu Rev Phytopathol 48:21–43

Stein E, Molitor A, Kogel KH, Waller F (2008) Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. Plant Cell Physiol 49:1747–1751

Stotz HU, Kroymann J, Mitchell-Olds T (1999) Plant–insect interactions. Curr Opin Plant Biol 2:268–272

Stotz HU, Pittendrigh BR, Kroymann J, Weniger K, Fritsche J, Bauke A, Mitchell-Olds T (2000) Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of *Arabidopsis* against cotton worm but not diamondback moth. Plant Physiol 124:1007–1017

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B (2006) Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell 18:1052–1066

Thomma B, Eggermont K, Penninckx I, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Separate jasmonate dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci USA 95:15107–15111

Thomma BP, Nelissen I, Eggermont K, Broekaert WF (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J 19:163–171

Tierens KF, Thomma BP, Brouwer M, Schmidt J, Kistner K, Porzel A, Mauch-Mani B, Cammue BP, Broekaert WF (2001) Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of *Arabidopsis* to microbial pathogens. Plant Physiol 125:1688–1699

Vadassery J, Ranf S, Drzewiecki C, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R (2009a) A cell wall extract from *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J 59:193–206

van der Luit AH, Piatti T, van Doorn A, Musgrave A, Felix G, Boller T, Munnik T (2000) Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. Plant Physiol 123:1507–1516

van Loon LC, Bakker PA, Pieterse CM (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483

van Wees SC, Van der Ent S, Pieterse CM (2008) Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol 11:443–448

Varma A, Verma S, Sudha S, Sahay NS, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sudha S et al (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) Mycota IX. Springer, Berlin, pp 123–150

Verma SA, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:898–905

Waller F, Achatz B, Baltruschat H et al (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Wittstock U, Halkier BA (2002) Glucosinolate research in the *Arabidopsis* era. Trends Plant Sci 7:263–270

Xie D-X, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science 280:1091–1094

Yamaguchi T, Minami E, Ueki J, Shibuya N (2005) Elicitor-induced activation of phospholipases plays an important role for the induction of defense responses in suspension-cultured rice cells. Plant Cell Physiol 46:579–587

Zeng RS, Mallik AU, Setliff E (2003) Growth stimulation of ectomycorrhizal fungi by root exudates of Brassicaceae plants: role of degraded compounds of indole glucosinolates. J Chem Ecol 29:1337–1355

Chapter 15
A Mixed Bag: The Plant Growth-Promoting *Sebacina vermifera* Impairs Defense Mechanisms Against Herbivores

Oz Barazani and Ian Thomas Baldwin

15.1 Introduction

Endophytic root-colonizing microorganisms, either bacteria or fungi, may possess beneficial effect on plant growth (Glick 1995; Varma et al. 1999; Barazani et al. 2005; Waller et al. 2005; Long et al. 2008). This group of plant growth-promoting microorganisms (PGPM) can affect plant development directly—by the secretion of plant growth substances (Bottini et al. 2004; Strobel et al. 2004), increasing the availability of nutrients (Vessey 2003; Orhan et al. 2006) or indirectly by manipulation of the plant's phytohormone balance (Arkhipova et al. 2005; Glick 2005). For example, by the cleavage of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor for ethylene (ET) biosynthesis, PGPM are able to utilize ACC as nitrogen source, reduce ET synthesis, and indirectly promote plant growth (Glick 1995).

ET is a gaseous phytohormone that plays an important role in all stages of plant development, from seed germination to flowering, fruit ripening, and senescence, as well as in wounding responses and in defense against pathogenic microorganisms (Kepczynski and Kepczynska 1997; Bleecker and Kende 2000; Bari and Jones 2009). More recently, ET's involvement in defense against herbivores has been added to the list of ET's functions from work in *Nicotiana attenuata* (von Dahl and Baldwin 2007). In this model plant system, jasmonic acid (JA) and ET positively and negatively regulate nicotine synthesis and accumulation, respectively (Kahl et al. 2000; Shoji et al. 2000). ET and JA are also known to synergistically regulate the accumulation of the anti-nutritional proteinase inhibitors in response to

O. Barazani
Institute of Plant Sciences, Agricultural Research Organization, Bet Dagan 50250, Israel
e-mail: barazani@agri.gov.il

I.T. Baldwin (✉)
Department of Molecular Ecology, Max Planck Institute for Chemical Ecology,
Hans-Knöll-Strasse 8, 07745 Jena, Germany
e-mail: baldwin@ice.mpg.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_15,

wounding in tomato plants (O'Donnell et al. 1996). These studies that point to the importance of cross talk among phytohormones also suggest that in addition to ET's effect on plant growth, the manipulation of ET by belowground microbial interactions will also affect a plant's aboveground interactions with herbivores. Such multiple-level interactions may dramatically influence the potential value of microorganisms in both ecological and agronomical ecosystems.

The *N. attenuata* model plant system and its respective transgenic lines impaired in several genes of its defense-related signaling pathways offer a unique opportunity to study multilevel organism interactions. *N. attenuata*, a native species of the Great Basin Desert of the USA, germinates after fires in a nitrogen-rich soil from long-lived seed banks (Baldwin and Morse 1994; Baldwin et al. 1994) to establish monoculture populations that are attacked by a diverse assemblage of mammalian and insect herbivores. The belowground interactions of *N. attenuata* with the microbial community include association with *Glomus* sp. mycorrhizal fungi (Riedel et al. 2008) and beneficial endophytic bacteria (Long et al. 2008, 2010). In addition, a Sebacinales-like fungus, genetically similar to *Piriformospora indica* and *Sebacina vermifera*, was identified in a clone library of DNA extracted from native soil samples, collected from the rhizosphere of *N. attenuata* (Barazani et al. 2005).

Previous studies demonstrated the growth-promoting effects of *P. indica* for several plant species including several cultivated crops (Sahay and Varma 1999; Varma et al. 1999; Singh et al. 2000; Kumari et al. 2003; Rai and Varma 2005), and this work motivated our study on the ecological consequences of the interaction between *N. attenuata* with *P. indica* and *S. vermifera*. As expected, both *P. indica* and *S. vermifera* induced seed germination, promoted plant growth, and increased plant height. In addition, inoculated plants bolted and flowered earlier and produced a larger number of seed capsules (Barazani et al. 2005). However, the enhanced fitness came at the expense of important resistance traits against herbivores (Barazani et al. 2005), questioning whether the endophytic fungi are true mutualists and suggested a new mechanism for the growth-promoting effect of Sebacinales species.

15.2 Sebacinaceae Interactions with Host Plants Involve Modification of Plant Hormones

Beneficial endophytic bacteria and fungi can promote plant growth by changing the hormonal balance in the plant by secretion of growth regulators (Xie et al. 1996; Barazani and Friedman 1999; Gutierrez-Manero et al. 2001; Arkhipova et al. 2005; Ryu et al. 2005; Wang et al. 2005; Madhaiyan et al. 2006) or suppression of ET synthesis in the plant (Glick 1995). In the latter case, ACC deaminase-producing microorganisms (both bacteria and fungi) can utilize the ethylene precursor ACC as a nitrogen source, resulting in the reduction of ET synthesis in the plant and increasing plant growth and height (Glick 1995, 2005; Glick et al. 1998; Jia et al. 1999).

It was suggested that by secretion of plant growth regulators, PGPM can benefit from elevated amounts of nutrients by increasing exudation from the roots (Meharg and Killham 1995). Suppression of ET in stressful environments may provide plants with an ecological advantage. It was shown that ET emission can inhibit the growth of neighboring plants (i.e., allopathic effect), while shade avoidance and increased height growth in high-density plant communities have also been shown to be mediated by ET synthesis (Pierik et al. 2003, 2004). In contrast, experiments with transgenic plants expressing the PGPM ACC deaminase showed that by reducing ET synthesis, tomato plants were more tolerant of abiotic heavy metals and flooding (Grichko et al. 2000; Grichko and Glick 2001) and canola became better able to tolerate salt stress (Sergeeva et al. 2006). Thus, it was suggested that by suppressing ET synthesis, ACC deaminase-producing PGPM may increase a plant's ability to cope with harsh environments (Glick et al. 2007). Accordingly, while the suppression of ET synthesis by PGPM under stressful environments may at first appear to be maladaptive, the increase in growth that results from ET suppression by PGPM suggests that a plant's normal hormonal responses to biotic or abiotic stress may have evolved to become too conservative, in that it restricts growth, reducing plant fitness more than an optimal response to the stress would require. In this way, the interactions between PGPM and their host plants can become mutualistic if the plant's final physiological responses to abiotic stresses that result from the PGPM–plant interaction increase plant fitness (Long et al. 2010). As such, it was shown that several strains of PGP bacteria significantly increased tomato growth and development under salt and drought conditions (Mayak et al. 2004a, b) as well as flooding stress for *Brassica napus* (Farwell et al. 2007), and inoculation of several plant species, i.e., *Lolium perenne*, *Festuca arundinacea*, *Secale cereale*, and *Hordeum vulgare*, with ET-suppressing ACC deaminase-producing bacteria, proved to be a useful practice for phytoremediation of contaminated soil in Ontario, Canada (Gurska et al. 2009).

In its natural habitats, *N. attenuata* is associated with Sebacinales endophytic fungi as well as ACC deaminase-producing bacteria and symbiotic *Glomus* spp. (Barazani et al. 2005; Riedel et al. 2008; Long et al. 2010), suggesting that in the harsh desert environment, PGPM may offer a fitness advantage to the plant, both in helping plants to cope with drought stress as well as increasing plant growth and allowing for early reproduction—assisting plants to escape the abiotic stress and return to the seed bank. Results of the study by Waller et al. (2005) supported this hypothesis, demonstrating that inoculation of barley with the endophytic *P. indica* increased resistance to salt stress, but a different mechanism was implicated, that of elevated antioxidative activity (Waller et al. 2005).

Previously, we had shown that by downregulating transcripts and activity of trypsin proteinase inhibitors (TPIs), *S. vermifera* and *P. indica* increased *N. attenuata*'s susceptibility to the larvae of *Manduca sexta* (Barazani et al. 2005, 2007). These defensive anti-nutritional proteins are regulated by JA and its amino acid-conjugated (JA–isoleucine: JA-Ile) and methylated (methyl jasmonate) forms

(Halitschke and Baldwin 2003, 2005; Kang et al. 2006). Since JA-mediated plant defenses incur fitness costs (Zavala et al. 2004a), it was hypothesized that the growth-promoting effects of this apparently mutualistic fungi result from its interference with the JA signaling pathway. The use of transgenic lines of *N. attenuata* silenced in JA signaling and TPI biosynthetic genes provided the tools to examine this hypothesis by testing the prediction that *S. vermifera's* growth-promoting effects would disappear in interactions with these transgenic lines. However, the results indicated that *S. vermifera* significantly increased the growth and fitness of both JA- and TPI-deficient lines—rejecting our principal hypothesis [(Barazani et al. 2007), Fig. 15.1].

More recent studies have taken a similar approach and employed transgenic and mutant lines to investigate the nature of the relations between plants and beneficial microorganisms; Camehl et al. (2010) demonstrated the involvement of ET perception in the interactions of *A. thaliana* with *P. indica*. By using a JA-deficient line, Isayenkov et al. (2005) showed that JA is crucial for the establishment of AM in *Medicago truncatula* roots.

JA and ET act in concert in regulating defensive-related genes against pathogens and herbivores (Bari and Jones 2009). Alterations in the triple response assay provided the first indication that ET is involved in relations between *N. attenuata* and *S. vermifera* and suggested that inoculated seedlings were less affected by increased ET exposure than uninoculated seedlings. Furthermore, the association of *S. vermifera* with *N. attenuata* downregulated ET biosynthesis genes in WT plants and did not show any observed phenotypic effects in a transgenic line impaired in ET biosynthesis by RNAi of ACC oxidase (*ACO*) (Fig. 15.1). These results provided further evidence that the interactions of *N. attenuata* with *S. vermifera* involved ET biosynthesis but also provided direct evidence that reduced ET synthesis was involved in the growth promotion phenotype (Barazani et al. 2007). Similarly, it was shown that the interactions of the AM, *Glomus intraradices*, with *N. attenuata* decreased ET synthesis in the plant (Riedel et al. 2008). However, the effect of ET depends on the interacting plant and fungal species; Camehl et al. (2010) demonstrated that *P. indica* promoted the growth of *A. thaliana* lines producing higher level of ET, but did not promote the growth of lines impaired in ET perception. In addition, inoculation of an IAA-overproducing *A. thaliana* line with *P. indica* rescued its dwarf phenotype, by increasing the formation of inactive IAA conjugates (Vadassery et al. 2008). Based on the results of our studies on *N. attenuata–S. vermifera* interactions, we presume that a signaling compound secreted by the fungus other than ACC deaminase (Barazani et al. 2007) negatively regulates ET synthesis genes (Fig. 15.1). Thus, the above reports suggest that the mechanisms by which fungal-mediated growth promotion occurs are highly species specific. Moreover, the fact that changes in defense-related signaling molecules are associated with the growth-promoting effects of Sebacinales suggests that the

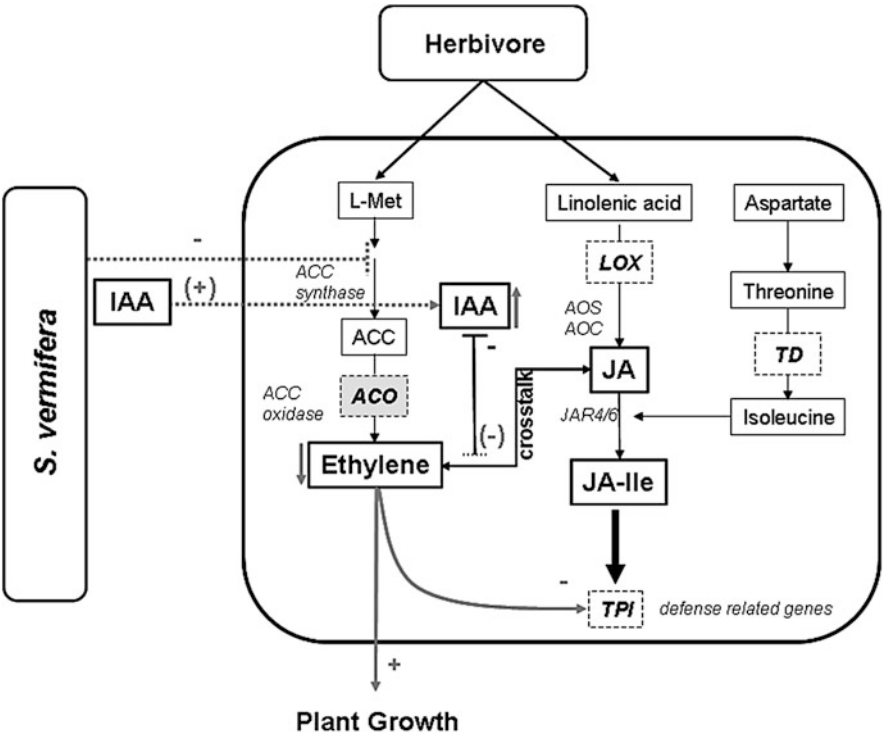

Fig. 15.1 Schematic model of the possible effect of *S. vermifera* on the defense-related signaling pathway of *N. attenuata*. Transgenic lines that were used to test the hypothesis that *S. vermifera* interferes with defense-related pathways are presented in *dashed boxes*. The presumed effect of *S. vermifera* on the phytohormones are denoted in *gray dashed lines*, the resulting suppression/increase in their respective in-planta concentrations in *up*/*down facing arrows*, and the consequent regulation of plant defense and growth in *gray arrows*.

A burst of JA and ET after herbivore attack induce plant defense against herbivores. The cross talk between this two signaling compounds also involves downregulation of IAA which is responsible for *N. attenuata* phenotypic response to herbivores (Onkokesung et al. 2010). *S. vermifera* promoted the growth of transgenic lines impaired in JA, JA-Ile, and TPI (*dashed boxes*), but did not have any effect on transgenic line impaired in ET synthesis (*dashed-gray box*), presumably by interfering with the ET synthesis pathway upstream of *ACO*. We suggest that by negative regulation of ET (Barazani et al. 2007) and the ability to secrete low levels of IAA (Vadassery et al. 2008), *Sebacinales* endophytic fungi can increase plant growth. In addition, in manipulating ET biosynthesis and influencing auxin concentrations and JA-ET cross talk, Sebacinales can reduce the induced levels of defense-related compounds and as a consequence increase the plant susceptibility to herbivores; considering the growth-defense trade-off that results from producing TPIs, downregulation of TPIs may additionally increase plant growth and fitness

fungus is able to manipulate plant defense against microorganisms, which can also imply that this belowground interaction might have effects on aboveground plant–insect interactions.

15.3 Does a Plant's Belowground Interactions with Sebacinales Fungi Influence Aboveground Interactions?

Plant hormones are used as signaling compounds and act together in the regulation of developmental processes and stress responses. Many of these processes that involve antagonistic or synergistic interactions among different phytohormones imply that by fine-tuning hormonal responses, plant can coordinate and activate defense responses against different stresses (Pieterse and Dicke 2007; Koornneef and Pieterse 2008; Bari and Jones 2009). Thus, it is reasonable to assume that trade-offs among traits might occur under natural conditions, in which plants are more often than not simultaneously exposed to different biotic stimuli. As an example, JA and ET act synergistically in the response to necrotrophic pathogens, while defense mechanisms against biotrophic pathogens are mainly regulated by salicylic acid (SA) (Glazebrook 2005). Accordingly, the antagonistic nature of JA and SA interactions as mediated by *NPR1* increased the susceptibility of *A. thaliana* to secondary infestation by necrotrophic fungus *Alternaria brassicicola* after a prior infestation with the biotrophic *Pseudomonas syringae* (Spoel et al. 2007). In contrast, beneficial interactions with PGPM can improve mycorrhizal establishment (Frey-Klett et al. 1999; Deveau et al. 2007) and increase plant resistance to pathogens (van Loon et al. 1998; Pieterse et al. 2001; Bakker et al. 2005, 2007). In addition, the association of *A. thaliana* with PGP bacteria increased the plant's resistance to secondary exposures to pathogen and salt stresses (Barriuso et al. 2008). It is interesting to note that in the latter report, the researchers used *Arabidopsis* mutant lines to show that SA is involved in secondary exposure to biotrophic pathogen in PGP-inoculated salt-challenged plants.

However, in addition to their involvement in beneficial and harmful interactions with microorganisms, ET and JA are key regulators of aboveground plant–insect interactions (Kessler et al. 2004; De Vos et al. 2005; von Dahl and Baldwin 2007). This dual function of phytohormones in microbial and herbivory interactions motivated us to determine if the beneficial associations with Sebacinales fungi influence defense against *M. sexta*. As previously mentioned, inoculation with *P. indica* and *S. vermifera* reduced *N. attenuata's* resistance to *M. sexta* attack, as a result of the suppression of induced levels of TPI (Barazani et al. 2005, 2007).

Induced defenses against herbivores are activated by mechanical wounding and signals from the insect itself or can be mimicked by chemical treatment with jasmonates and include an arsenal of metabolites that function in the direct and indirect plant responses. Among these, TPIs inhibit proteases in the insect gut and reduce herbivore development as a consequence of nutritional deficiency (Zavala et al. 2004b). As with many of the other induced defenses, TPIs are regulated by JA-Ile signaling and can be elicited by external treatments of JA and methyl JA (Zavala et al. 2004a; Kang et al. 2006) (which are rapidly converted into JA-Ile). During *M. sexta* larval attack, the introduction of oral secretions (OS) into wounds during feeding is essential to induce the TPI defensive response (Halitschke et al. 2003). However, while OS elicits the accumulation of TPI via JA signaling, it also

suppresses nicotine by eliciting an ET burst (Kahl et al. 2000), which negatively regulates putrescine *N*-methyltransferase and results in reduced nicotine levels (Kahl et al. 2000; Winz and Baldwin 2001).

It was recently suggested that the accumulation of ET and JA upon herbivory may reduce IAA metabolism and consequently suppress cell expansion at the wound site (Onkokesung et al. 2010). To test this hypothesis, a genetic cross between the JA-deficient *LOX3*-silenced plants and the ET-insensitive *ETR1* lines was used to test the plant's response to simulated herbivory, providing strong evidence for the genetic trade-off between plant growth and defense (Onkokesung et al. 2010). It was also recently shown that both ET and IAA are involved in shade avoidance responses in *A. thaliana* (Pierik et al. 2009). The results of these studies highlight the significance of the overall in-planta phytohormone balance in sculpting responses to biotic stimuli and specifically suggest that the ability of Sebacinales fungi to produce IAA (Vadassery et al. 2008) and suppress ET synthesis (Barazani et al. 2007) allows this group of endophytic fungi to increase plant growth (Fig. 15.1).

Other reports on plant–microbial interactions suggest that the cross talk among phytohormones plays an essential role in determining the outcome of secondary infections after insect herbivory. Feeding experiments showed that *Trichoplusia ni* performance on a pathogen-sensitive *Arabidopsis* line impaired in SA synthesis and mutants insensitive to SA was lower than on WT, while larvae feeding on lines with increased SA levels gained more mass than those feeding on WT plants (Cui et al. 2002). These results are consistent with the results of Thaler et al. (2002) demonstrating that larvae of *Spodoptera exigua* gained more mass when fed on tomato plants treated with both the SA-agonist BTH and JA-deficient mutant and are consistent with a general model of SA/JA cross talk being beneficial for herbivore performance. Lately, we had shown that the secretion of bacterial quorum signaling compounds had an effect on plant–insect interactions, reducing *N. attenuata* resistance to *M. sexta* caterpillars, presumably by downregulation of *TPI* gene (Heidel et al. 2010). How these quorum signals interact with phytohormone cross talk needs to be explored further, but the results are consistent with trade-offs between a plant's response to belowground PGPM and aboveground insect herbivore attack. Thus, the results of our studies emphasize the importance of investigating multiple plant interactions and the likely trade-offs that occur among the interactions that a plant has with its multitude of biotic and abiotic stresses.

References

Arkhipova TN, Veselov SU, Melentiev AI, Martynenko EV, Kudoyarova GR (2005) Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. Plant Soil 272:201–209

Bakker PAHM, Pieterse CMJ, Van Loon LC (2005) Induction of systemic resistance by *Pseudomonas* spp. Phytopathology 95:S145

Bakker PAHM, Pieterse CMJ, van Loon LC (2007) Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology 97:239–243

Baldwin IT, Morse L (1994) Up in smoke. 2. Germination of *Nicotiana attenuata* in response to smoke-derived cues and nutrients in burned and unburned soils. J Chem Ecol 20:2373–2391

Baldwin IT, Staszakkozinski L, Davidson R (1994) Up in smoke. 1. Smoke-derived germination cues for postfire annual, *Nicotiana attenuata* Torr Ex Watson. J Chem Ecol 20:2345–2371

Barazani O, Friedman J (1999) Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? J Chem Ecol 25:2397–2406

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. Oecologia 146:234–243

Barazani O, Von Dahl CC, Baldwin IT (2007) *Sebacina vermifera* promotes the growth and fitness of *Nicotiana attenuata* by inhibiting ethylene signaling. Plant Physiol 144:1223–1232

Bari R, Jones J (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69:473–488

Barriuso J, Solano BR, Manero FJG (2008) Protection against pathogen and salt stress by four plant growth-promoting rhizobacteria isolated from *Pinus* sp on *Arabidopsis thaliana*. Phytopathology 98:666–672

Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol 16:1–18

Bottini R, Cassan F, Piccoli P (2004) Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. Appl Microbiol Biotechnol 65:497–503

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Oelmuller R (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol 185:1062–1073

Cui JP, Jander G, Racki LR, Kim PD, Pierce NE, Ausubel FM (2002) Signals involved in *Arabidopsis* resistance to *Trichoplusia ni* caterpillars induced by virulent and avirulent strains of the phytopathogen *Pseudomonas syringae*. Plant Physiol 129:551–564

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Metraux JP, Van Loon LC, Dicke M, Pieterse CMJ (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. Mol Plant Microbe Interact 18:923–937

Deveau A, Palin B, Delaruelle C, Peter M, Kohler A, Pierrat JC, Sarniguet A, Garbaye J, Martin F, Frey-Klett P (2007) The mycorrhiza helper *Pseudomonas fluorescens* BBc6R8 has a specific priming effect on the growth, morphology and gene expression of the ectomycorrhizal fungus *Laccaria bicolor* S238N. New Phytol 175:743–755

Farwell AJ, Vesely S, Nero V, Rodriguez H, McCormack K, Shah S, Dixon DG, Glick BR (2007) Tolerance of transgenic canola plants (*Brassica napus*) amended with plant growth-promoting bacteria to flooding stress at a metal-contaminated field site. Environ Pollut 147:540–545

Frey-Klett P, Churin JL, Pierrat JC, Garbaye J (1999) Dose effect in the dual inoculation of an ectomycorrhizal fungus and a mycorrhiza helper bacterium in two forest nurseries. Soil Biol Biochem 31:1555–1562

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43:205–227

Glick BR (1995) The enhancement of plant-growth by free-living bacteria. Can J Microbiol 41:109–117

Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol Lett 251:1–7

Glick BR, Penrose DM, Li JP (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J Theor Biol 190:63–68

Glick BR, Todorovic B, Czarny J, Cheng ZY, Duan J, McConkey B (2007) Promotion of plant growth by bacterial ACC deaminase. Crit Rev Plant Sci 26:227–242

Grichko VP, Glick BR (2001) Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35S, rolD or PRB-1b promoter. Plant Physiol Biochem 39:19–25

Grichko VP, Filby B, Glick BR (2000) Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb, and Zn. J Biotechnol 81:45–53

Gurska J, Wang WX, Gerhardt KE, Khalid AM, Isherwood DM, Huang XD, Glick BR, Greenberg BM (2009) Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste. Environ Sci Technol 43:4472–4479

Gutierrez-Manero FJ, Ramos-Solano B, Probanza A, Mehouachi J, Tadeo FR, Talon M (2001) The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiol Plantarum 111:206–211

Halitschke R, Baldwin IT (2003) Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata*. Plant J 36:794–807

Halitschke R, Baldwin IT (2005) Jasmonates and related compounds in plant-insect interactions. J Plant Growth Regul 23:238–245

Halitschke R, Gase K, Hui DQ, Schmidt DD, Baldwin IT (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. Plant Physiol 131:1894–1902

Heidel AJ, Barazani O, Baldwin IT (2010) Interaction between herbivore defense and microbial signaling: bacterial quorum-sensing compounds weaken JA-mediated herbivore resistance in *Nicotiana attenuata*. Chemoecology 20:149–154

Isayenkov S, Mrosk C, Stenzel I, Strack D, Hause B (2005) Suppression of allene oxide cyclase in hairy roots of *Medicago truncatula* reduces jasmonate levels and the degree of mycorrhization with *Glomus intraradices*. Plant Physiol 139:1401–1410

Jia YJ, Kakuta Y, Sugawara M, Igarashi T, Oki N, Kisaki M, Shoji T, Kanetuna Y, Horita T, Matsui H, Honma M (1999) Synthesis and degradation of 1-aminocyclopropane-1-carboxylic acid by *Penicillium citrinum*. Biosci Biotechnol Biochem 63:542–549

Kahl J, Siemens DH, Aerts RJ, Gaebler R, Kuehnemann F, Preston CA, Baldwin IT (2000) Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. Planta 210:336–342

Kang JH, Wang L, Giri A, Baldwin IT (2006) Silencing threonine deaminase and JAR4 in *Nicotiana attenuata* impairs jasmonic acid-isoleucine-mediated defenses against *Manduca sexta*. Plant Cell 18:3303–3320

Kepczynski J, Kepczynska E (1997) Ethylene in seed dormancy and germination. Physiol Plantarum 101:720–726

Kessler A, Halitschke R, Baldwin IT (2004) Silencing the jasmonate cascade: induced plant defenses and insect populations. Science 305:665–668

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiol 146:839–844

Kumari R, Kishan H, Bhoon YK, Varma A (2003) Colonization of cruciferous plants by *Piriformospora indica*. Curr Sci 85:1672–1674

Long HH, Schmidt DD, Baldwin IT (2008) Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PLoS One 3:e2702

Long HH, Sonntag DG, Schmidt DD, Baldwin IT (2010) The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception. New Phytol 185:554–567

Madhaiyan M, Poonguzhali S, Ryu J, Sa T (2006) Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. Planta 224:268–278

Mayak S, Tirosh T, Glick BR (2004a) Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Sci 166:525–530
Mayak S, Tirosh T, Glick BR (2004b) Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiol Biochem 42:565–572
Meharg AA, Killham K (1995) Loss of exudates from the roots of perennial ryegrass inoculated with a range of microorganisms. Plant Soil 170:345–349
O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ (1996) Ethylene as a signal mediating the wound response of tomato plants. Science 274:1914–1917
Onkokesung N, Galis I, von Dahl CC, Matsuoka K, Saluz HP, Baldwin IT (2010) Jasmonic acid and ethylene modulate local responses to wounding and simulated herbivory in *Nicotiana attenuata* leaves. Plant Physiol 153:785–798
Orhan E, Esitken A, Ercisli S, Turan M, Sahin F (2006) Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. Sci Hort 111:38–43
Pierik R, Visser EJW, De Kroon H, Voesenek LACJ (2003) Ethylene is required in tobacco to successfully compete with proximate neighbours. Plant Cell Environ 26:1229–1234
Pierik R, Voesenek LACJ, de Kroon H, Visser EJW (2004) Density-induced plant size reduction and size inequalities in ethylene-sensing and ethylene-insensitive tobacco. Plant Biol 6:201–205
Pierik R, Djakovic-Petrovic T, Keuskamp DH, de Wit M, Voesenek LACJ (2009) Auxin and ethylene regulate elongation responses to neighbor proximity signals independent of gibberellin and DELLA proteins in *Arabidopsis*. Plant Physiol 149:1701–1712
Pieterse CMJ, Dicke M (2007) Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends Plant Sci 12:564–569
Pieterse CMJ, Van Pelt JA, Van Wees SCM, Ton J, Leon-Kloosterziel KM, Keurentjes JJB, Verhagen BWM, Knoester M, Van der Sluis I, Bakker PAHM, Van Loon LC (2001) Rhizobacteria-mediated induced systemic resistance: triggering, signalling and expression. Eur J Plant Pathol 107:51–61
Rai M, Varma A (2005) Arbuscular mycorrhiza-like biotechnological potential of *Piriformospora indica*, which promotes the growth of *Adhatoda vasica* Nees. Electronic J Biotechnol 8:107–112
Riedel T, Groten K, Baldwin IT (2008) Symbiosis between *Nicotiana attenuata* and *Glomus intraradices*: ethylene plays a role, jasmonic acid does not. Plant Cell Environ 31:1203–1213
Ryu CM, Hu CH, Locy RD, Kloepper JW (2005) Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*. Plant Soil 268:285–292
Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302
Sergeeva E, Shah S, Glick BR (2006) Growth of transgenic canola (*Brassica napus* cv. Westar) expressing a bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene on high concentrations of salt. World J Microbiol Biotechnol 22:277–282
Shoji T, Nakajima K, Hashimoto T (2000) Ethylene suppresses jasmonate-induced gene expression in nicotine biosynthesis. Plant Cell Physiol 41:1072–1076
Singh A, Sharma J, Rexer KH, Varma A (2000) Plant productivity determinants beyond minerals, water and light: *Piriformospora indica* - a revolutionary plant growth promoting fungus. Curr Sci 79:1548–1554
Spoel SH, Johnson JS, Dong X (2007) Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. Proc Natl Acad Sci USA 104:18842–18847
Strobel G, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. J Nat Prod 67:257–268
Thaler JS, Karban R, Ullman DE, Boege K, Bostock RM (2002) Cross-talk between jasmonate and salicylate plant defense pathways: effects on several plant parasites. Oecologia 131:227–235

Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B, Novak O, Strnad M, Ludwig-Müller J, Oelmüller R (2008) The role of auxins and cytokinins in the mutualistic interaction between *Arabidopsis* and *Piriformospora indica*. Mol Plant Microbe Interact 21:1371–1383

van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483

Varma A, Verma S, Sudha SN, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586

von Dahl CC, Baldwin IT (2007) Deciphering the role of ethylene in plant-herbivore interactions. J Plant Growth Regul 26:201–209

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Huckelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Wang YQ, Ohara Y, Nakayashiki H, Tosa Y, Mayama S (2005) Microarray analysis of the gene expression profile induced by the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5 in *Arabidopsis*. Mol Plant Microbe Interact 18:385–396

Winz RA, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. IV. Insect-induced ethylene reduces jasmonate-induced nicotine accumulation by regulating putrescine N-methyltransferase transcripts. Plant Physiol 125:2189–2202

Xie H, Pasternak JJ, Glick BR (1996) Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* CR12-2 that overproduce indoleacetic acid. Curr Microbiol 32:67–71

Zavala JA, Patankar AG, Gase K, Baldwin IT (2004a) Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*. Proc Natl Acad Sci USA 101:1607–1612

Zavala JA, Patankar AG, Gase K, Hui DQ, Baldwin IT (2004b) Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. Plant Physiol 134:1181–1190

Chapter 16
Piriformospora indica Versus Salt Stress

Mohammad Javad Zarea, Pooja Chordia, and Ajit Varma

16.1 Introduction

Increased incidences of abiotic and biotic stresses impacting productivity in principal crops are being witnessed all over the world. The problem of soil drought and salinity presents one of the greatest obstacles to enabling agriculture to meet the need of the world's growing population (Killham 1994). Shannon (1997) estimated that 10 % of the world's cropland and as much as 27 % of the irrigated land may already be affected by salinity, and one-third of the world's arable land resources are affected by salinity (Qadir et al. 2000). Many keys to agricultural success in arid and semiarid areas are to use adequate plant species and to use the soil biology potential to maintain soil fertility, and to guard against erosion and water stress (Zarea 2010).

Scientists have searched for new salt-tolerant crop plants (Glenn and O'Leary 1985), developed salt-tolerant crops through breeding (Shannon 1984), and continued to investigate the physiology of genetic alterations involved in salt tolerance (Apse et al. 1999). Other attempts to deal with saline soils have involved leaching of excessive salts (Hamdy 1990) or desalinizing seawater for use in irrigation (Muralev et al. 1997). Although these approaches have been successful, most are beyond the economic means of developing nations (Cantrell and Linderman 2001). Plant breeding may be available for some plant species in these areas but not for all crops being grown (Cantrell and Linderman 2001). An experimental alternative is to alleviate salt stress by inoculating crops seeds and

M.J. Zarea (✉)
Faculty of Agriculture, Ilam University, P.O. Box 69315516, Ilam, Iran
e-mail: mj.zarea@ilam.ac.ir; mjzarea@ymail.com

P. Chordia • A. Varma (✉)
Amity Institute of Microbial Technology, Amity University, Uttar Pradesh Sector 125, New Super Highway, Noida 201303, UP, India
e-mail: pooja_angel29@yahoo.co.in; ajitvarma@amity.edu

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_16,

seedlings with various plant growth-promoting bacteria (PGPB), such as Rhizobium and *Azospirillum* spp. and also with mycorrhizal fungi (Zarea et al. 2011b).

The use of symbiotic mycorrhizal fungi has proved useful in developing strategies to facilitate plant growth in saline soils (Zarea et al. 2011b). Fungi tend to be among the most tolerant microbes to water stress (Killham 1994). The improved growth of mycorrhizal plants has been attributed to enhanced nutrient uptake, particularly of N and P, and subsequent increased growth (Jeffries et al. 2003). However, in some cases plant salt tolerance was not related to P concentration (Ruiz-Lozano and Azcon 2000). Thus, it has been proposed that salt tolerance mechanisms, such as enhanced osmotic adjustment and leaf hydration, increased intrinsic water use efficiency, reduced oxidative damage, or improved nutritional status, can explain the contribution of AM symbioses to the salinity resistance of host plants (Augé 2001).

The present review captures the recent work on the role of *Piriformospora indica* in helping crops cope with salt stress, the most common stresses caused due to climate change. There is a lack of literature regarding the use of *P. indica* to protect agricultural plants from salt-induced damage. *P. indica* is a novel endophytic fungus and a new champion of symbiosis (Kharkwal et al. 2008). Thus, while numerous reports have highlighted the beneficial role of arbuscular mycorrhizal (AM) fungi in facilitation plant growth in saline soils, only a few of them have focused on the positive effects induced by endophytic fungus *P. indica* which are typically found in association with many plant species in nature. Surprisingly, to our knowledge, only three reports (Waller et al. 2005; Baltruschat et al. 2008; Zarea et al. 2011b) and a short information presented in the review by Oelmüller et al. (2009) deal with the alleviation of salt stress by the employment of *P. indica* fungus. The mechanisms by which *P. indica* fungus enhance plant tolerance to salinity are described in the present review.

16.2 Salt Stress

Salinity is the concentration of dissolved mineral salts present in the soils (soil solution) and waters. The dissolved mineral salts consist of the electrolytes of cations and anions. The major cations in saline soil solutions consist of Na^+, Ca^{2+}, Mg^{2+}, and K^+, and the major anions are Cl^-, $SO_4{}^{2-}$, $HCO_3{}^-$, $CO_3{}^{2-}$, and $NO_3{}^-$ (Manchanda and Garg 2008). Other constituents contributing to salinity in hypersaline soils and waters include B, $Sr_2{}^+$, SiO_2, Mo, Ba^{2+}, and Al^{3+} (Hu and Schmidhalter 2002). The composition and concentration of soluble salts in root-zone medium solution are known to influence plant growth, both by creating osmotic imbalance and via specific physiological toxicity of ions. Osmotic stress lowers the potential energy of the solution and causes reduced growth, due to the additional energy required by plants to take up water (Ben-Gal et al. 2009).

Salinity dominated by Na^+ and Cl^- not only reduces Ca^{2+} and K^+ availability but also reduces $Ca_2{}^+$ and K^+ mobility and transport to the growing parts of plants,

affecting the quality of both vegetative and reproductive organs (Kohler et al. 2009). The decline of K concentration under salinity conditions has been reported by Munns and Termaat (1986) and Devitt et al. (1981). Moreover, many studies have shown that high concentrations of NaCl in the soil solution may increase the ratios of Na^+/Ca^{2+} and Na^+/K^+ ratios in plants, which would then be more susceptible to osmotic and specific ion injury as well as to nutritional disorders that result in reduced yield and quality (Sivritepe et al. 2003). Saline stress induces P deficiency by reducing P uptake or translocation (Martinez and Lauchli 1991). Saline stress decreased nitrogen concentration in pepper plants (Kaya et al. 2009).

Salt stress has been shown to affect carbohydrate partitioning and metabolism, leading to the synthesis of new compounds (Sharma et al. 1990). In plants exposed to salinity, the total nonstructural carbohydrate content in the leaves was reduced significantly compared with plants not exposed to salinity (Kohler et al. 2009). The decrease in total soluble carbohydrates due to salinity could be related also to limited carbohydrate availability as a consequence of a decline in photosynthesis (Goicoechea et al. 2005).

Increasing salinity stress significantly increased the antioxidant enzyme activities of lettuce leaves, including those of peroxidases and catalase, of lettuce leaves compared to their respective nonstressed controls (Kohler et al. 2009). Salt stress may induce a combination of negative effects on salt-sensitive plants including osmotic stress, ion toxicity, and oxidative stress (Kohler et al. 2009). High salinity may induce imbalances in the soil: plant osmotic relationships (Wyn Jones and Gorham 1983) and in plant metabolism (Singh and Jain 1982).

Increasing salinity stress significantly decreased germination percentage (Barassi et al. 2006) High (60 mM) NaCl in nutrient solution strongly affected root elongation and mature vegetative growth of both spinach and lettuce, but especially in vegetables (Kaya et al. 2002; Barassi et al. 2006). Shoot growth is affected more than root growth in lettuce exposed to low salinities (Shannon 1997; Barassi et al. 2006).

The reduction in photosynthesis in the salinity-treated plants has been reported by many researchers (Downton 1977; Ball and Farquhar 1984; Behboudian et al. 1986). The adverse effects of high NaCl on chlorophyll concentration have previously been shown in rice (Yeo et al. 1990), barley (Belkhodja et al. 1994), tomato (Kaya et al. 2001), and pepper (Kaya et al. 2009). Kaya et al. (2009) reported that salt stressed caused a significant increase in electrolyte leakage compared to that in the nonstressed pepper plants. Similar results were obtained by Lutts et al. (1996) for NaCl-sensitive rice varieties wherein high salt concentration increased membrane permeability.

16.3 Plant Defences Mechanisms *Versus* Salinity Stress

Many effective protection systems exist in plants that enable them to perceive, respond to, and properly adapt to various stress signals (Chen et al. 2009), and a variety of genes and gene products have been identified that involve responses to

drought and high-salinity stress (Chen et al. 2009). An increase in concentration of K^+ in plants under salt stress could ameliorate the deleterious effects of salinity on growth and yield (Giri et al. 2007). Potassium is supposed to be responsible for stomatal movements in response to changes in bulk leaf water status (Caravaca et al. 2004). For example, the resistance mechanism of salt-tolerant olive cultivars is probably related to the ability to maintain an appropriate K/Na ratio in actively growing tissue (Tattini 1994). The induction of antioxidant enzyme such as catalase and peroxidase can be considered as one mechanism of salt tolerance in plants (Hernandez et al. 1995, 2000). Antioxidant enzymes are involved in eliminating H_2O_2 from salt-stressed roots (Kim et al. 2005). Salt stress induces proline accumulation in legumes (Ashraf 1989; Sharma et al. 1990; Rabie and Almadini 2005), in which accumulation of this amino acid is thought to be involved in osmotic adjustment of stressed tissues (Delauney and Verma 1993; Ashraf and Foolad 2006). Root proline accumulation under salinity is reported in other plant species (Sudhakar et al. 1993; Cusido et al. 1987; Sharifi et al. 2007). Proline acts as a major reservoir of energy and nitrogen for utilization during salinity stress (Goas et al. 1982). Changes in the composition of carbohydrates of the host plant may play a role in increasing salt tolerance (Rosendahl and Rosendahl 1991). Salinity reduced soluble carbohydrates (Sharma et al. 1990).

16.4 *Piriformospora indica*

P. indica, which belongs to the Sebacinales in Basidiomycota (Oelmüller et al. 2009), is a newly described root endophyte (Verma et al. 1998) with AMF-like characteristics (Varma et al. 2001). Fungi belonging to the order Sebacinales comprise a wide spectrum of lifestyles ranging from ectomycorrhizal (ECM), ericoid (EM), and orchid mycorrhizal (OM) to root endophytic. Sebacinales may be endophytic in many angiosperm roots, and this condition is plesiomorphic in Sebacinales. They bridge the gap between physiological studies, inoculating Sebacinales (*P. indica* or *Sebacina vermifera*) on diverse plants and molecular ecology, hitherto restricting Sebacinales to mycorrhizal interactions (Selosse et al. 2009). The endophytic fungus *P. indica* colonizes the roots of many plant species, promotes their growth and seed yield, and confers tolerance against biotic and abiotic stress (Verma et al. 1998, 1999; Oelmüller et al. 2009). The basidiomycete *P. indica* was isolated from the rhizospheres of bushes in the Thar Desert in India. It is a root endophyte that is easily cultivable in axenic culture (Verma et al. 1998). Inoculation of a broad variety of plants with *P. indica* had a positive effect on their general biomass production (Varma et al. 1999), and subsequent analyses showed that *P. indica* exerts a variety of physiological effects. It promotes the establishment of micropropagated plants (Sahay and Varma 1999), adventitious root formation in cuttings (Druege et al. 2007), and lignan production of hairy root cultures (Kumar et al. 2011); enhances flowering (Rai et al. 2001; Barazani et al. 2005); increases yield; and induces higher tolerance of and resistance to abiotic and

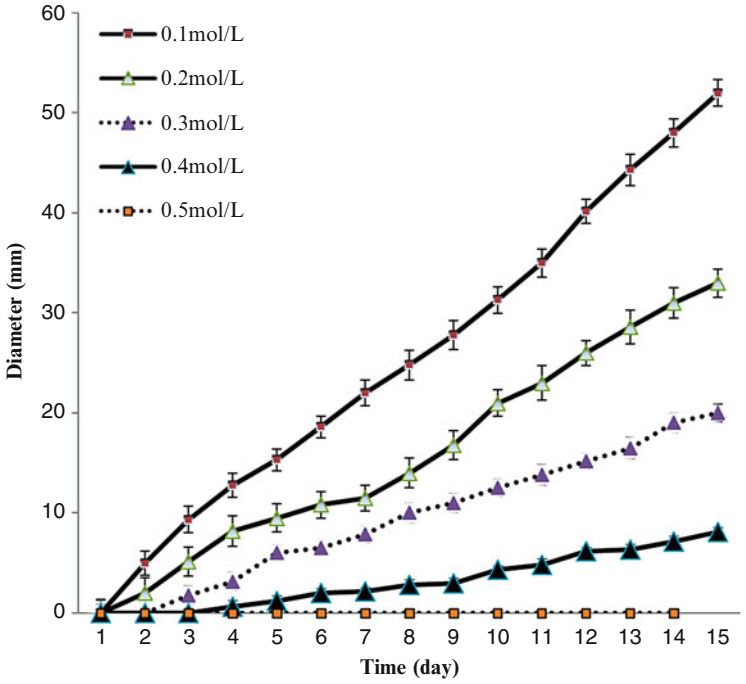

Fig. 16.1 Dose–response curves for the growth models of *Piriformospora indica* in the presence of NaCl at a range of concentrations in solidified Kafer medium over 15 days (Zarea et al. 2011a, b)

biotic stresses (Waller et al. 2005). *Piriformospora indica* improves plant growth and/or stress tolerance (Varma et al. 2001), such as on the non-mycorrhizal *Arabidopsis thaliana* (Peškan-Berghöfer et al. 2004) and Nicotiana tabacum (Barazani et al. 2005), but also on Fabaceae and Rhamnaceae (Varma et al. 2001), Asteraceae and Solanaceae (Rai et al. 2001), Geraniaceae and Euphorbiaceae (Druege et al. 2007), as well as Poaceae (Waller et al. 2005; Baltruschat et al. 2008). In vitro essay also has relevance to the ability of *P. indica* to produce extracellular enzymes such as cellulase, peroxidase, and proteinase (Basiewicz et al. 2011). Fungi tend to be among the most tolerant microbes to water stress (Killham 1994). Basidiomycotina, which are involved in most of the ectomycorrhizal symbioses, appears to have a tolerance to water stress, based solely on the salt concentration of a liquid culture medium, of up to about −70 bar (−7 MPa) (Killham 1994). *Piriformospora indica* had a great threshold for salinity tolerance via modified physiological and biochemical attributes that allows them to withstand a wide range of environmental saline stress (Zarea et al. 2011b). *Piriformospora indica* could grow under salinity levels as high as 0.4 mol L^{-1} NaCl (Fig. 16.1).

16.5 Mechanisms Used by *P. indica* to Alleviation of Salinity Stress in Plants

Plants are always exposed to adverse environments such as arid lands and salt stress or to pathogens, but they routinely survive. Recent advances in fungal ecology have revealed that some of this tolerance or resistance comes not from the plant itself but from the cryptic fungi residing in the plant's tissues (Yuan et al. 2010). Like AM fungi (Gamalero et al. 2009), the main mechanisms thought to be involved in salt stress alleviation by *P. indica* fungus may be summarized as follows: (1) improvement of mineral nutrition leading to plant growth promotion and (2) modification of some physiological processes and enzymatic activities involved in plant antioxidative reactions. The mechanisms by which *P. indica* fungus enhance plant tolerance to salinity are described in the following section.

16.5.1 Reactive Oxygen Species-Scavenging Enzymes

Damage to plants that are induced by salt stress may also be a consequence of the production of ROS (reactive oxygen species) (Hernandez et al. 1995). In this regard, plants with high concentrations of antioxidants or antioxidative enzymes are typically more resistant to damage by ROS (Spychalla and Desbough 1990; Dionisio-Sese and Tobita 1998; Jiang and Zhang 2002). Plants are endowed with an array of radical scavengers and antioxidant enzymes that act in concert to alleviate oxidative stress. The induction of ROS-scavenging enzymes, such as superoxide dismutase (SOD), peroxidases (POXs), and catalase (CAT), is the most common mechanism for detoxifying ROS synthesized during stress responses (Wojtaszek 1997; Mittler 2002). An imbalance between antioxidant defences and the amount of ROS results in cellular injury (Foyer and Noctor 2000). An increasing body of evidence suggests that high salinity induces oxidative stress in plants that is at least partly responsible for tissue damage (Hernández et al. 2000; Mittova et al. 2004). Several studies have demonstrated that salinity increases antioxidant activities in salt-tolerant plants above the levels found in salt-sensitive plants (Gossett et al. 1994; Gueta-Dahan et al. 1997; Mittova et al. 2004). Symbiotic *P. indica* fungus may promote the activation by plants of the biosynthesis of proline (Zarea et al. 2011b) or antioxidant enzymes to scavenge the ROS (reactive oxygen species) (Rodriguez and Redman 2005; Baltruschat et al. 2008; Kumar et al. 2009). Increased antioxidant enzyme activity, including CAT (catalase), APX (ascorbate peroxidase), DHAR (dehydroascorbate reductase), MDHAR (monodehydroascorbate reductase), and GR (glutathione reductase), plays a significant role in tolerance to abiotic stressors. Baltruschat et al. (2008) point out that these enzyme activities are maintained at a high level in *P. indica*-infected plants but decrease gradually in uninfected plants. *P. indica* stimulates antioxidant enzyme activities and prevents ROS formation by retarding the degradation of polyunsaturated lipids (Sun et al. 2010). However,

compared to APX, CAT, and DHAR antioxidant enzymes, GR activity was the least affected by *P. indica* (Baltruschat et al. 2008; Kumar et al. 2009). Several reports have demonstrated that antioxidant enzyme activities are crucial for *P. indica*-induced resistance against abiotic stress (Baltruschat et al. 2008, Vadassery et al. 2009; Sun et al. 2010).

Enzymes of the ascorbate–glutathione cycle which fund in the cytosol (Dalton et al. 1993), chloroplasts (Hossain and Asada 1984; Hossain et al. 1984), mitochondria, and peroxisomes (Jiménez et al. 1997) play a major role in the protection of the organism against reactive oxygen species because it maintains high levels of ascorbate in the different cell compartments (cf. Asada 1997). In this cycle, H_2O_2 is reduced to H_2O by ascorbate peroxidase using ascorbate, which generates monodehydroascorbate (Vadassery et al. 2009). Monodehydroascorbate is a radical and reduced back to ascorbate by monodehydroascorbate reductase (MDAR). *P. indica*-infected barley roots exposed to salt had increased activities of several antioxidant enzymes such as ascorbic acid under salt stress conditions, suggesting that these factors may be responsible for the positive effects of *P. indica* to stress. It has been previously shown that *P. indica* induces the amount of ascorbic acid, the ratio is reduced to oxidize ascorbate, and the activity of dehydroascorbate reductase was elevated in barley roots (Waller et al. 2005). The endophyte significantly elevated the amount of ascorbic acid and increased the activities of antioxidant enzymes in barley roots under salt stress conditions (Baltruschat et al. 2008). Transfer of Arabidopsis seedlings to PNM (a modified plant nutrient medium, Vadassery et al. 2009) plates with a mycelial loan of *P. indica* resulted in a ~1.5-fold increase in the total ascorbate level within 1h in roots and with ~5 h in shoots (Vadassery et al. 2009).

16.5.2 Reduction Fatty Acid Desaturation

Fatty acid desaturation is associated with salt stress in plants as well (Elkahoui et al. 2004; Liang et al. 2005). It has been shown that linolenic acid plays a pivotal role in the tolerance of tobacco plants to salt stress (Im et al. 2002). *P. indica* colonization leads to a significant reduction in the proportion of oleic acid in barley leaves. Root colonization by *P. indica* attenuated the fatty acid desaturation in leaves of the salt-sensitive barley cultivar Ingrid. The proportion of linolenic acid and the derived values for indicators of fatty acid desaturation were slightly elevated upon inoculation with the endophyte *P. indica* (Baltruschat et al. 2008).

16.5.3 P. indica-*Mediated Phytohormone*

Salinity-induced stress in plants is partly the result of ethylene production (O'Donnell et al. 1996; Cuartero and Fernandez-Munoz 1999; Blumwald 2000;

Mayak et al. 2004; Shibli et al. 2007). For instance, ethylene production was stimulated from two- to about tenfold in tomato (*Lycopersicon esculentum*) and Arabidopsis plants that were exposed to salinity stress (Richard and El-Abd 1989; Hall and Smith 1995). In this regard, in chickpea (*Cicer arietinum*), Kukreja et al. (2005) not only observed a salinity-induced increase in ethylene evolution, but also increases both in 1-aminocyclopropane-1-carboxylate (ACC) content, the immediate precursor of ethylene, and in the activity of the enzyme ACC oxidase. Furthermore, the relationship between salinity stress and ethylene production was consistent with the observation that aminoethoxyvinylglycine (AVG), a chemical inhibitor of ethylene biosynthesis, alleviated salinity-induced plant responses such as increased hook closure and thickness of seedlings (El Beltagy et al. 1979). The mechanism responsible for *P. indica*-mediated upregulation of the plant antioxidant system is not known. It has been shown recently that *P. indica* is able to produce auxin when associated with plant roots (Sirrenberg et al. 2007). Exogenous auxin has been found to transiently increase the concentration of ROS and then prevent H_2O_2 release in response to oxidative stress (caused by paraquat) and enhance APX activity, while decreasing CAT activity (Joo et al. 2001; Pasternak et al. 2007). On the other hand, *P. indica* increased the amount of methionine synthase, which plays a crucial role in the biosynthesis of polyamines and ethylene (Peškan-Berghöfer et al. 2004). Therefore if the *P. indica* synthesizes and secretes indole acetic acid (IAA), some of which is taken up by the plant. Together with the endogenous pool of plant IAA, the IAA taken up by the plant can stimulate plant cell proliferation and elongation and/or it can induce synthesis of the plant enzyme 1-aminocyclopropane-1-carboxylate (ACC) synthase which converts *S*-adenosylmethionine to ACC (Kende 1993). Some of the ACC that remains within the plant root or seed may be converted to ethylene when the enzyme ACC oxidase is present (Kende 1993).

16.5.4 Phosphate Uptake

P availability is reduced in saline soil not only because of ion competition that reduces the activity of P, but also because P concentrations in soil solution are tightly controlled by sorption processes and by the low solubility of Al–P or Fe–P precipitates (Grattan and Grieve 1992). Jakobsen et al. (1992) reported that the efficiency of P uptake by an AMF was strongly affected by the spatial distribution of its hyphae in the soil and possibly also by the differences in the capacity for uptake per unit length of hyphae. Mycorrhizal inoculation improves P nutrition of plants under salinity stress and reduces the negative effects of Na^+ by maintaining vacuolar membrane integrity, which prevents this ion from interfering in growth metabolic pathways (Rinaldelli and Mancuso 1996). Previous research has shown that the main mechanism for enhanced salinity tolerance in mycorrhizal plant was the improvement of P nutrition (Copeman et al. 1996; Al-Karaki et al. 2001). AM fungi increased P uptake, and saline stress in plants was thereby alleviated

(Tian et al. 2004). Tian et al. (2004) showed that AM fungi from saline soil promoted the growth of cotton plants under saline stress by increasing P concentrations without affecting sodium and chloride concentrations. *P. indica* increases the phosphate uptake 2–3 times higher in *Arabidopsis* seedlings. Similar findings were also observed by Yadav et al. (2010) in *Zea mays* the case of colonized with *P. indica*. However, to our knowledge, no studies have been performed on the possible enhancement of plant tolerance to salt stress by P uptake by the *P. indica* fungus.

16.5.5 Nitrate Reductase Activity

Nitrate reductase (NR) was slightly inhibited by salinity in tomato roots, while leaf NR decreased sharply (Cramer and Lips 1995). In the leaves of tomatoes and cucumbers, NR activity increased with exogenous NO_3 concentration (Maritinez and Cerda 1989). As NR is a substrate-inducible enzyme (Marschner 1995) and its decreased activity under salinization has been attributed by some researchers to decreased NO_3 uptake by plants under salt stress (Lacuesta et al. 1990; Tabatabaei 2006). The decreased of NO_3 is accompanied by a high Cl-uptake and low rate of xylem exudation in high osmotic conditions either by NaCl or other nutrients (Tabatabaei et al. 2004). Either the reduced NO_3 uptake or translocation leads to lower NO_3 concentration in the leaves, consequently reducing NR activity of leaves under salinity conditions (Tabatabaei 2006). This finding agrees with Cramer and Lips (1995), who indicated that salinity may control NR activity through NO_3 uptake since NR activity is largely determined by NO_3 flux into the metabolic pool rather than by tissue NO_3 content itself. Interaction of the endophytic fungus with Arabidopsis roots is accompanied by a considerable requisition of nitrogen from the environment (Peškan-Berghöfer et al. 2004). By analysing the interaction of *P. indica* with Arabidopsis and tobacco roots, it was found that in contrast to mycorrhizal associations, nitrate reduction in the roots is stimulated by Sherameti et al. (2005).

16.5.6 Organic Solutes Accumulation

For plants to survive under salt stress conditions, adjustment of leaf osmotic potential is very important, and it requires intracellular osmotic balance. Under salt stress, plants accumulate some organic solutes (proline, soluble sugars, and so on) and inorganic ions to maintain higher osmotic adjustment (Yang et al. 2009). Free amino acids are important osmolytes contributing to osmotic adjustment in plants (Hajlaoui et al. 2010). With increasing external salt concentration, free amino acids accumulate in the leaves and roots of maize (Abd-El Baki et al. 2000; Neto et al. 2009; Hajlaoui et al. 2010). Among free amino acids, proline is a contributor to osmotic adjustment in salt-stressed maize plants (Hajlaoui et al. 2010). It appears

that the presence of the *P. indica* fungus in the roots may modify the osmotic potential of the leaves as they have been shown to influence the composition of the level of proline (Zarea et al. 2011b). Proline accumulation is thought an adaptive feature under salinity stress in *P. indica* (Zarea et al. 2011b). Results also show that the accumulation of proline in wheat is increased by *P. indica* inoculation (Zarea et al. 2011b). The high level of proline enables the plants to maintain osmotic balance when growing under low water potentials (Stewart and Lee 1974). Proline acts as a major reservoir of energy and nitrogen for utilization by plants subjected to salinity stress (Goas et al. 1982; Ashraf and Foolad 2006). Enhanced proline accumulation in plant cells can increase plant osmotic potentials (Hajlaoui et al. 2010) and abscissic acid level (Ober and Sharp 1994), thereby improving the tolerance of mycorrhizal plants to salinity.

Salt in soil water inhibits plants' ability to take up water, and this leads to slower growth. This is the osmotic or water-deficit effect of salinity (Munns 2005). Jimenez et al. (2003) reported that salinity decreased the leaf water, osmotic, and turgor potentials in four wild and two cultivated Phaseolus species. Kumar et al. (2009) also reported that relative water content in the leaves of wheat was significantly higher in *P. indica*-inoculated than in non-inoculated wheat plants under saline conditions.

16.5.7 Chlorophyll Content

The reduction in photosynthesis in the salinity-treated plants reported by many researchers (Downton 1977; Ball and Farquhar 1984; Behboudian et al. 1986). The adverse effects of high NaCl on chlorophyll concentration have previously been shown in rice (Yeo et al. 1990), barley (Belkhodja et al. 1994), tomato (Kaya et al. 2001), and pepper (Kaya et al. 2009). In drought-induced Chinese cabbage, the presence of *P. indica* retards the decrease in the protein levels of representative components of the thylakoid membrane and of enzymes located in the plastid stroma (Sun et al. 2010). Sun et al. (2010) showed that *P. indica* retarded the drought-induced Chinese cabbage decline in the photosynthetic efficiency and the degradation of chlorophylls and thylakoid proteins. Under saline condition, *P. indica* colonization increased chlorophyll content in wheat, and the *P. indica*-inoculated plants had greener leaves than non-*P. indica*-inoculated plants under saline conditions (Zarea et al. 2011b).

16.6 Synergistic Effects with Growth-Promoting Bacteria

Several pieces of evidence suggest the possibility of additive or synergistic effects between beneficial plant growth-promoting bacteria (PGPB) or mycorrhizal fungi in supporting plant growth in saline conditions (Gamalero et al. 2009). In plants

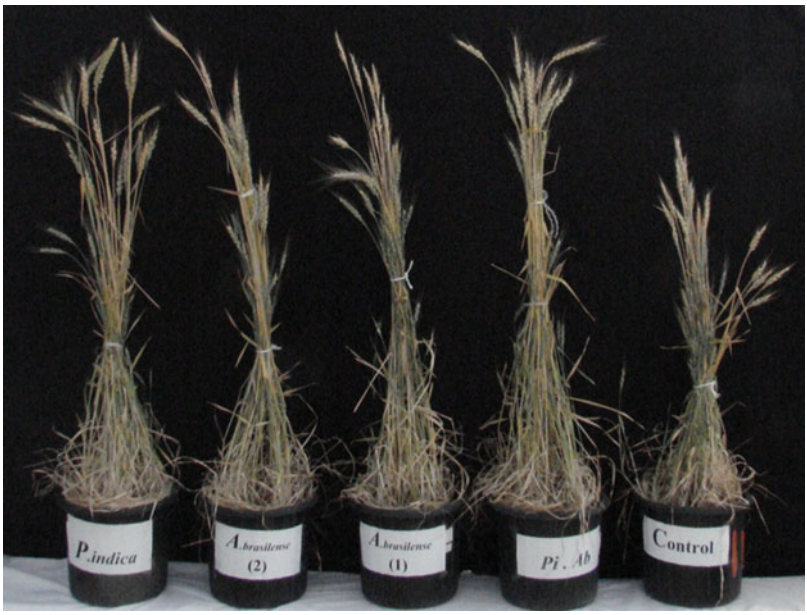

Fig. 16.2 Photographs of the effects of inoculation of wheat plants with *Piriformospora indica* (*left*), saline-adapted *Azospirillum brasilense* (2) and nonadapted *Azospirillum brasilense* (1) compared with when wheat co-inoculation by *Piriformospora indica* and both salt and non-salt *Azospirillum brasilense* (Pi + Ab) and non-inoculated (Control) at harvesting at water salinity of ECw = 4 dS m^{-1}

grown in the presence of salt and treated with both microsymbionts, the observed reduction of dry matter was lower than when plants were inoculated with any one of these organisms (Hatimi 1999; Zarea et al. 2011a, 2011b). Similar results were obtained by combining *Glomus intraradices* and *Bradyrhizobia* sp. with salt-stressed *Acacia auriculiformis* (A. Cunn. ex Benth.) or *Acacia mangium* (Willd.) seedlings (Diouf et al. 2005). The inhibitory effects of salinity on nitrogen fixation were significantly reduced by preinoculating the fava bean plants with the AM fungus than when plants were inoculated with any one of nitrogen-fixing rhizobia (Rabie and Almadini 2005). For *P. indica*, a close association to the α-proteobacterium *Rhizobium radiobacter* has been demonstrated (Molitor and Kogel 2009). Although all attempts to cure *P. indica* from these bacteria failed, it was possible to produce *R. radiobacter* in pure culture. In experiments examining the biological activity of *R. radiobacter* in barley, Sharma et al. (2008) proved the potential of the bacteria to induce growth promotion and systemic resistance to barley powdery mildew. *P. indica* interact with rhizobacteria, including *Pseudomonas florescence*, *Azotobacter chrocooccum*, *Pseudomonas putida*, *Bacillus subtilis*, *Azospirillum*, and *Bradyrhizobium*. To our knowledge, no studies have been performed on the possible enhancement of plant tolerance to salt stress by the combination of *P. indica* fungus and PGPB. Our unpublished results from a

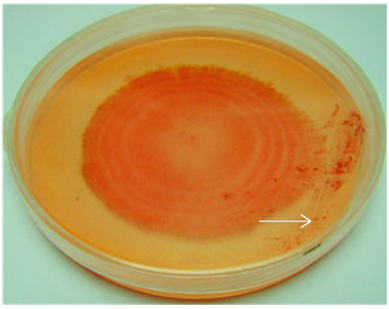

Fig. 16.3 One disc of *P. Indica* and a loop of *Azospirillum* sp. where incubation was done for 14 days on modified agar Congo red medium in the dark at 25. Centre view of the mycelia and right view of *Azospirillum* inoculums. *Open arrow* indicates the inoculation placement of *Azospirillum* sp. The *Azospirillum* was streaked on one side (*right*). The growth of *P. Indica* was promoted by *Azospirillum* sp.

greenhouse believed that study-exposed wheat to salt stress had better growth and nutrient (P and N) uptake performances with wheat co-inoculation by *Piriformospora indica* and *Azospirillum* sp. (Fig. 16.2). The growth of *P. indica* is promoted by *Azospirillum* sp. *P. Indica* was grown in the centre of plate with modified agar Congo red medium. *P. indica* and the respective bacteria *Azospirillum* were placed on medium. After 14-day incubation at 25 °C, it was found that *Azospirillum* promoted the growth of the fungus (Fig. 16.3).

16.7 Conclusions

The salinity of soils is a considerable problem in many parts of the world. This is particularly the case in regions with high rates of evaporation, where salts are easily accumulated in the topsoil (Hammer et al. 2010). One of the most widespread agricultural problems in arid and semiarid regions is soil salinity. Salt-affected lands occur in practically all climatic regions, from the humid tropics to the polar regions (Manchanda and Garg 2008). Saline soils can be found at different altitudes, from below sea level (e.g. around the Dead Sea) to mountains rising above 5,000 m, such as the Tibetan Plateau or the Rocky Mountains (Manchanda and Garg 2008). Of nearly, 160 million hectares of cultivated land under irrigation worldwide, about one-third is already affected by salt, which makes salinity a major constraint to food production (Manchanda and Garg 2008). It is the single largest soil toxicity problem in tropical Asia (Greenland 1984). Increased irrigation is needed to combat the spread of deserts and to meet the greater demand for food of a growing world population, but at the same time, inappropriate irrigation management leads to the accumulation of salt in poorly drained soils (Hammer et al. 2010). Few crop species are adapted to saline conditions (Hu and Schmidhalter 2002). Plant growth-promoting fungi/bacteria have various

mechanisms which mitigate NaCl effects and hence increase plant resistance to salt stress. From the present review, it is proposed that salt tolerance mechanisms, such as enhanced osmotic adjustment, increased phosphate uptake, stimulated nitrate reductase, and reduced oxidative damage, can explain the contribution of *P. indica* symbioses to the salinity resistance of host plants. Several pieces of evidence suggest the possibility of additive or synergistic effects between beneficial plant growth-promoting bacteria (PGPB) or mycorrhizal fungi in supporting plant growth in saline conditions (Gamalero et al. 2009). To promote plant growth under saline stress, co-inoculation by endophytic fungus *Piriformospora indica* and PGPR isolates from saline soil would have a higher capacity.

References

Abd-El Baki BGK, Siefritz F, Man HM, Weiner H, Kaldenhoff R, Kaiser WM (2000) Nitrate reductase in Zea mays L. under salinity. Plant Cell Environ 23:515–521

Al-Karaki GN, Hammad R, Rusan M (2001) Response of two tomato cultivars differing in salt tolerance to inoculation with mycorrhizal fungi under salt stress. Mycorrhiza 11:41–47

Apse MP, Aharon GS, Snedden WA, Bumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na+/H + antiport in Arabidopsis. Science 285:1256–1258

Asada K (1997) The role of ascorbate peroxidase and monodehydroascorbate reductase in H_2O_2 scavenging in plants. In: Scandalios JG (ed) Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory Press, New York, pp 715–735

Ashraf M (1989) The effect of NaCl on water relations, chlorophyll and protein and proline contents of two cultivars of blackgram (*Vigna mungo* L.). Plant Soil 129:205–210

Ashraf M, Foolad MR (2006) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206–216

Augé RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Ball MC, Farquhar GD (1984) Photosynthetic and stomatal responses of two mangrove species, Avicennia marina and *Aegiceras corniculatum*, to long term salinity and humidity conditions. Plant Physiol 1:1–6

Baltruschat H, Fodor J, Harrach BD, Niemczyk E, Barna B, Gullner G, Janeczko A, Kogel KH, Schäfer P, Schwarczinger I, Zuccaro A, Skoczowski A (2008) Salt tolerance of barley induced by the root endophyte Piriformospora indica is associated with a strong increase in antioxidants. New Phytol 180:501–510

Barassi CA, Ayrault G, Creus CM, Sueldo RJ, Sobrero MT (2006) Seed inoculation with Azospirillum mitigates NaCl effects on lettuce. Sci Hortic 109:8–14

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and Sebacina vermifera increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. Oecologia 146:234–243

Basiewicz M, Weiss M, Kogel KH, Langen G, Zorn H, Zuccaro A (2011) Molecular and phenotypic characterization of Sebacina vermifera strains associated with orchids, and the description of *Piriformospora williamsii* sp. nov. Fungal Biol 16(2):204–213. doi:10.1016/j.funbio.2011.11.003

Behboudian MH, Torokfalvy E, Walker RR (1986) Effects of salinity on ionic content, water relations and gas exchange parameters in some citrus scion rootstock combinations. Sci Hortic 28:105–116

Belkhodja R, Morales F, Abadia A, Gomez-Aparisi J, Abadia J (1994) Chlorophyll fluorescence as a possible tool for salinity tolerance screening in barley (*Hordeum vulgare* L.). Plant Physiol 104:667–673

Ben-Gal A, Borochov-Neori H, Yermiyahu U, Shani U (2009) Is osmotic potential a more appropriate property than electrical conductivity for evaluating whole-plant response to salinity? Environ Exp Bot 65:232–237

Blumwald E (2000) Sodium transport and salt tolerance in plants. Curr Opin Cell Biol 12:431–434

Cantrell IC, Linderman RG (2001) Preinoculation of lettuce and onion with VA mycorrhizal fungi reduces deleterious effects of soil salinity. Plant Soil 233:269–281

Caravaca F, Figueroa D, Barea JM, Azcón-Aguilar C, Roldán A (2004) Effect of mycorrhizal inoculation on the nutrient content, gas exchange and nitrate reductase activity of Retama sphaerocarpa and Olea europaea subsp. sylvestris under drought stress. J Plant Nutr 27:57–74

Chen N, Liu Y, Liu X, Chai J, Hu Z, Guo G, Liu H (2009) Enhanced tolerance to water deficit and salinity stress in transgenic *Lycium barbarum* L. plants ectopically expressing ATHK1, an arabidopsis thaliana histidine kinase gene. Plant Mol Biol Rep 27:321–333

Copeman RH, Martin CA, Stutz JC (1996) Tomato growth in response to salinity and mycorrhizal fungi from saline or nonsaline soils. HortSci 31:341–344

Cramer MD, Lips SH (1995) Enriched rhizosphere CO_2 concentration can ameliorate the influence of salinity on hydroponically grown tomato plants. Plant Physiol 94:425–433

Cuartero J, Fernandez-Munoz R (1999) Tomato and salinity. Sci Hortic 78:83–125

Cusido RM, Papazon J, Altabella T, Morales C (1987) Effects of salinity on soluble protein, free amino acids and nicotine contents in *Nicotiana rustica* L. Plant Soil 102:55–60

Dalton DA, Baird LM, Langeberg L, Taugher CY, Anyan WR, Vance CP et al (1993) Subcellular localization of oxygen defense enzymes in soybean (*Glycine max* [L.] Merr.) root nodules. Plant Physiol 102:481–489

Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. Plant J 4:215–223

Devitt D, Jarrell WM, Steven KL (1981) Sodium–potassium ratios in soil solution and plant response under saline conditions. Soil Sci Soc Am J 34:80–86

Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedling to salinity stress. Plant Sci 135:1–9

Diouf D, Duponnois R, Ba AT, Neyra M, Lesueur D (2005) Symbiosis of Acacia auriculiformis and Acacia mangium with mycorrhizal fungi and Bradyrhizobium spp. improves salt tolerance in greenhouse conditions. Funct Plant Biol 32:1143–1152

Downton WJS (1977) Photosynthesis in salt stressed grapevines. Aust Plant Physiol 4:183–192

Druege U, Baltruschat H, Franken P (2007) Piriformospora indica promotes adventitious root formation in cuttings. Sci Hort 112:422–426

El Beltagy A, Khalifa M, Hall M (1979) Salinity in relation to ethylene. Egypt J Hortic 6:269–271

Elkahoui S, Smaoui A, Zarrouk M, Ghrir R, Limam H (2004) Salt-induced lipid changes in *Catharanthus roseus* cultured cell suspensions. Phytochemistry 65:1911–1917

Foyer CH, Noctor G (2000) Oxygen processing in photosynthesis: regulation and signalling. New Phytol 146:359–388

Gamalero E, Berta G, Glick BR (2009) The use of microorganisms to facilitate the growth of plants in saline soils. In: Khan MS et al (eds) Microbial strategies for crop improvement. Springer, Berlin, pp 1–22

Giri B, Kapoor R, Mukerji KG (2007) Improved tolerance of *Acacia nilotica* to salt stress by arbuscular mycorrhiza, Glomus fasciculatum may be partly related to elevated K/Na ratios in root and shoot tissues. Microb Ecol 54:753–760

Glenn EP, O'Leary JW (1985) Productivity and irrigation requirements of halophytes grown with seawater in the Sonoran Desert. J Arid Environ 9:81–91

Goas G, Goas M, Larher F (1982) Accumulation of free proline and glycine betaine in Aster tripolium subjected to a saline shock: a kinetic study related to light period. Physiol Plant 55:383–388

Goicoechea N, Merino S, Sánchez-Díaz M (2005) Arbuscular mycorrhizal fungi can contribute to maintain antioxidant and carbon metabolism in nodules of *Anthyllis cytisoides* L. subjected to drought. J Plant Physiol 162:27–35

Gossett DR, Millholion EP, Lucas MC, Bands SW, Marney MM (1994) The effects of NaCl on antioxidant enzyme activities in callus tissue of salt tolerant and salt sensitive cotton cultivars. Plant Cell Rep 13:498–503

Grattan SR, Grieve CM (1992) Mineral element acquisition and growth response of plants grown in saline environments. Agric Ecosyst Environ 38:275–300

Greenland DJ (1984) Exploited plants: rice. Biologist 31:291–325

Gueta-Dahan Y, Yaniv Z, Zilinskas BA, Ben-Hayyim G (1997) Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in Citrus. Planta 203:460–469

Hajlaoui H, Ayeb NE, Garrec JP, Denden M (2010) Differential effects of salt stress on osmotic adjustment and solutes allocation on the basis of root and leaf tissue senescence of two silage maize (*Zea mays* L.) varieties. Ind Crops Prod 31:122–130

Hall MA, Smith AR (1995) Ethylene and the responses of plants to stress. Bulg J Plant Physiol 21:71–79

Hamdy A (1990) Management practices under saline water irrigation. In symposium on scheduling of irrigation for vegetable crops under field conditions. Acta Hortic 278:745–754

Hammer EC, Nasr H, Pallon J, Olsson PA, Wallander H (2010) Elemental composition of arbuscular mycorrhizal fungi at high salinity. Mycorrhiza 21:117–129

Hatimi A (1999) Effect of salinity on the association between root symbionts and Acacia cyanophylla Lind.: growth and nutrition. Plant Soil 216:93–101

Hernandez JA, Olmos E, Corpas FJ, Sevilla F, del Rio LA (1995) Salt induced oxidative stress in chloroplasts of pea plants. Plant Sci 105:151–167

Hernández JA, Jiménez A, Mullineaux PM, Sevilla F (2000) Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ 23:853–862

Hossain MA, Asada K (1984) Inactivation of ascorbate perox idase in spinach chloroplast on dark addition of hydrogen peroxide: its protection by ascorbate. Plant Cell Physiol 25:1285–1295

Hossain MA, Nakano Y, Asada K (1984) Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physiol 25:385–395

Hu Y, Schmidhalter U (2002) Limitation of salt stress to plant growth. In: Hock B, Elstner CF (eds) Plant toxicology. Dekker, New York, pp 91–224

Im YJ, Han O, Chung GC, Cho BH (2002) Antisense expression of an *Arabidopsis* omega-3 fatty acid desaturase gene reduces salt/drought tolerance in transgenic tobacco plants. Mol Cells 13:264–271

Jakobsen I, Abbott LK, Robson AD (1992) External hyphae of vesicular–arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. New Phytol 120:373–379

Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37:1–16

Jiang M, Zhang J (2002) Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. J Exp Bot 53:2401–2410

Jiménez A, Hernández JA, delRíoL A, Sevilla F (1997) Evidence for the presence of the ascorbate glutathione cycle in mitochondria and peroxisomes of pea leaves. Plant Physiol 114:275–284

Jimenez JS, Debouck DG, Lynch JP (2003) Growth, gas exchange, water relations, and ion composition of Phaseolus species grown under saline conditions. Field Crop Res 80:207–222

Joo JH, Bae YS, Lee JS (2001) Role of auxin-induced reactive oxygen species in root gravitropism. Plant Physiol 126:1055–1060

Kaya C, Kirnak H, Higgs D (2001) Enhancement of growth and normal growth parameters by foliar application of potassium and phosphorus in tomato cultivars grown at high (NaCl) salinity. J Plant Nutr 24:357–367

Kaya C, Higgs D, Sakar E (2002) Response of two leafy vegetables grown at high salinity to supplementary potassium and phosphorus during different growth stages. J Plant Nutr 25:2663–2676

Kaya C, Ashraf M, Sonmez O, Aydemir S, Levent Tuna A, Cullu AM (2009) The influence of arbuscular mycorrhizal colonisation on key growth parameters and fruit yield of pepper plants grown at high salinity. Sci Hortic 121:1–6

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 44:283–307

Kharkwal AC, Kharkwal H, Sherameti I, Oelmüller R, Varma A (2008) Novel symbiotrophic endophytes. In: Varma A (ed) Mycorrhiza. Springer, Berlin, pp 753–766

Killham K (1994) Soil ecology. Cambridge University Press, Cambridge

Kim SY, Lim JH, Park MR, Kim YJ, Park TII, Seo YW, Choi KG, Yun SJ (2005) Enhanced antioxidant enzymes are associated with reduced hydrogen peroxide in barley roots under salt stress. J Biochem Mol Biol 38:218–224

Kohler J, Hernández JA, Caravaca F, Roldána A (2009) Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. Environ Exp Bot 65:245–252

Kukreja S, Nandwal AS, Kumar N, Sharma SK, Sharma SK, Unvi V, Sharma PK (2005) Plant water status, H2O2 scavenging enzymes, ethylene evolution and membrane integrity of *Cicer arietinum* as affected by salinity. Biol Plant 49:305–308

Kumar M, Yadav V, Tuteja N, Johri AK (2009) Antioxidant enzyme activities in maize plants colonized with Piriformospora indica. Microbiology 155:780–790

Kumar V, Rajauri G, Sahai V, Bisaria VS (2011) Culture filtrate of root endophytic fungus *Piriformospora indica* promotes the growth and lignan production of *Linum album* hairy root cultures. Process Biochem 47(6):901–907

Lacuesta M, Gonzalez-Maro B, Gonzale-Murua C, Munoz-Rueda A (1990) Temporal study of the effect of phosphinothricin on the activity of glutamine synthetase, glutamate dehydrogenase and nitrate reductase in *Medicago sativa* L. Plant Physiol 136:410–414

Liang YC, Zhang WH, Chen Q, Liu YL, Ding RX (2005) Effects of silicon on H-ATPase and H-PPase activity, fatty acid composition and fluidity of tonoplast vesicles from roots of salt-stressed barley (*Hordeum vulgare* L.). Environ Exp Bot 53:29–37

Lutts S, Kinet JM, Bouharmont J (1996) NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Ann Bot 78:389–398

Manchanda G, Garg N (2008) Salinity and its effects on the functional biology of legumes. Acta Physiol Plant 30:595–618

Maritinez V, Cerda A (1989) Nitrate reductase activity in tomato and cucumber leaves as influenced by NaCl and N source. J Plant Nutr 12:1335–1350

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic, London

Martinez V, Lauchli A (1991) Phosphorus translocation in salt-stressed cotton. Physiol Plant 83:627–632

Mayak S, Tirosh T, Glick BR (2004) Plant growth-promoting bacteria that confer resistance in tomato to salt stress. Plant Physiol Biochem 42:565–572

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Mittova V, Guy M, Tal M, Volokita M (2004) Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species Lycopersicon pennellii. J Exp Bot 55:1105–1113

Molitor A, Kogel KH (2009) Induced resistance triggered by Piriformospora indica. Plant Signal Behav 4:215–216

Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol 167:645–663

Munns R, Termaat A (1986) Whole plant responses to salinity. Aust J Plant Physiol 13:143–160

Muralev E, Nazarenko PI, Poplavskij VM, Kuznetsov IA (1997) Seawater desalination. In: Nuclear desalinization of seawater. Proceedings of a symposium in Taejon, Republic of Korea, pp 355–366. International Atomic Energy Agency, Austria, Vienna

Neto ADA, Prisco JT, Gomes-Filho E (2009) Changes in soluble amino-N, soluble proteins and free amino acids in leaves and roots of salt-stressed maize genotypes. J Plant Interact 4:137–144

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ (1996) Ethylene as a signal mediating the wound response of tomato plants. Science 274:1914–1917

Ober ES, Sharp RE (1994) Proline accumulation in maize (*Zea mays* L.) primary roots at low water potentials (I. Requirement for increased levels of abscisic acid). Plant Physiol 105:981–987

Oelmüller R, Sherameti I, Tripathi S, Varma A (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Pasternak TP, Ötvös K, Domoki M, Fehér A (2007) Linked activation of cell division and oxidative stress defense in alfalfa leaf protoplast-derived cells is dependent on exogenous auxin. Plant Growth Regul 51:109–117

Peškan-Berghöfer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plant 122:465–477

Qadir M, Ghafoor A, Murtaza G (2000) Amelioration strategies for saline soils: a review. Land Degrad Dev 11:501–521

Rabie GH, Almadini AM (2005) Role of bioinoculants in development of salt-tolerance of Vicia faba under salinity stress. Afr J Biotechnol 4:210–222

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants Spilanthes calva and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128

Richard AJ, El-Abd SO (1989) Prevention of salt-induced epinasty by a-aminooxyacetic acid and cobalt. Plant Growth Regul 8:315–323

Rinaldelli E, Mancuso S (1996) Response of young mycorrhizal and non-mycorrhizal plants of olive tree (*Olea europaea* L.) to saline conditions. I. Short-term electrophysiological and longterm vegetative salt effects. Adv Hortic Sci 10:126–134

Rodriguez RJ, Redman RS (2005) Balancing the generation and elimination of reactive oxygen species. Proc Natl Acad Sci USA 102:3175–3176

Rosendahl CN, Rosendahl S (1991) Influence of vesicular arbuscular mycorrhizal fungi (Glomus spp.) on the response of cucumber (Cucumis sativus L.) to salt stress. Environ Exp Bot 31:313–318

Ruiz-Lozano JM, Azcon R (2000) Symbiotic efficiency and infectivity of an autochthonous arbuscular mycorrhizal *Glomus sp*. from saline soils and *Glomus deserticola* under salinity. Mycorrhiza 10:137–143

Sahay NS, Varma A (1999) Piriformospora indica: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Selosse M, Dubois MP, Alvarez N (2009) Do Sebacinales commonly associate with plant roots as endophytes? Mycol Res 113:1062–1069

Shannon MC (1984) Breeding, selection, and the genetics of salt tolerance. In: Staples RC, Toenniessen GH (eds) Salinity tolerance in plants: strategies for crop improvement. Wiley, New York, pp 231–254

Shannon MC (1997) Adaptation of plants to salinity. Adv Agron 60:75–120

Sharifi M, Ghorbanli M, Ebrahimzadeh H (2007) Improved growth of salinity-stressed soybean after inoculation with salt pre-treated mycorrhizal fungi. J Plant Physiol 164:1144–1151

Sharma KD, Datta KS, Verma SK (1990) Effect of chloride and sulphate type of salinity on some metabolic drifts in chickpea (*Cicer arietinum* L). Indian J Exp Biol 28:890–892

Sharma M, Schmid M, Rothballer M, Hause G, Zuccaro A, Imani J et al (2008) Detection and identification of bacteria intimately associated with fungi of the order Sebacinales. Cell Microbiol 11:2235–2246

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and Arabidopsis roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. J Biol Chem 280:2641–2647

Shibli RA, Kushad M, Yousef GG, Lila MA (2007) Physiological and biochemical responses of tomato microshoots to induced salinity stress with associated ethylene accumulation. Plant Growth Regul 51:159–169

Singh G, Jain S (1982) Effect of some growth regulators on certain biochemical parameters during seed development in chickpea under salinity. Indian J Plant Physiol 25:167–179

Sirrenberg A, Göbel C, Grond S, Czempinski N, Ratzinger A, Karlovsky P, Santos P, Feussner I, Pawlowski K (2007) *Piriformospora indica* affects plant growth by auxin production. Physiol Plant 131:581–589

Sivritepe N, Sivritepe HO, Eris A (2003) The effects of NaCl priming on salt tolerance in melon seedlings grown under saline conditions. Sci Hortic 97:229–237

Spychalla JP, Desbough SL (1990) Superoxide dismutase, catalase, and alpha-tocopherol content of stored potato tubers. Plant Physiol 94:1214–1218

Stewart CR, Lee JA (1974) The rate of proline accumulation in halophytes. Planta 120:279–289

Sudhakar C, Reddy PS, Veeranjaneyulu K (1993) Effect of salt stress on the enzymes of proline synthesis and oxidation in greengram (*Phaseolus aureus* Roxb.) seedlings. J Plant Physiol 14:621–623

Sun C, Johnson JM, Cai D, Sherameti I, Oelmuller R, Lou B (2010) *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. J Plant Physiol 167:1009–1017

Tabatabaei SJ (2006) Effects of salinity and N on the growth, photosynthesis and N status of olive (*Olea europaea* L.) trees. Sci Hortic 108:432–438

Tabatabaei SJ, Gregory P, Hadley P (2004) Uneven distribution of nutrients in the root zone affects the incidence of blossom end rot and concentration of calcium and potassium in fruits of tomato. Plant Soil 258:169–178

Tattini M (1994) Ionic relations of aeroponically-grown olive plants during salt stress. Plant Soil 161:251–256

Tian CY, Feng G, Li XL, Zhang FS (2004) Different effects of arbuscular mycorrhizal fungal isolates from saline or non-saline soil on salinity tolerance of plants. Appl Soil Ecol 26:143–148

Vadassery J, Tripathi S, Prasad R, Varma A, Oelmüller R (2009) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are rucial for a mutualistic interaction between *Piriformospora indica* and Arabidopsis. J Plant Physiol 166:1263–1274

Varma A, Verma S, Sudha Sahay NS, Bütehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sudha Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: *Mycota IX*. Springer Series, Germany, pp 123–150

Verma SA, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:898–905

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, Wettstein D et al (2005) The endophytic fungus *Piriformospora indica*

reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Wojtaszek P (1997) Oxidative burst: an early plant response to pathogen infection. Biochem J 322:681–692

Wyn Jones RG, Gorham J (1983) Osmoregulation. In: Lange OL, Noble PS, Osmond CB, Zeiger H (eds) Physiological plant ecology. III. Responses to chemical and biological environments. Springer, Berlin, pp 35–56

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. Biol Chem 285:26532–26544

Yang CW, Xu HH, Wang LL, Liu J, Shi DC, Wang GD (2009) Comparative effects of salt-stress and alkali-stress on the growth, photosynthesis, solute accumulation, and ion balance of barley plants. Photosynthetica 47:79–86

Yeo AR, Yeo ME, Flowers SA, Flowers TJ (1990) Screening of rice (*Oryza sativa* L.) genotypes for physiological characters contributing to salinity resistance, and their relationship to overall performance. Theor Appl Genet 79:377–384

Yuan ZL, Zhang CL, Lin FC (2010) Role of diverse non-systemic fungal endophytes in plant performance and response to stress: progress and approaches. J Plant Growth Regul 29:116–126

Zarea MJ (2010) Conservation tillage and sustainable agriculture in semi-arid dryland farming, pp 195–232. In: Lichtfouse E (ed) Biodiversity, biofules, agroforestry and conservation agriculture. Springer Science + Business Media, 375p

Zarea MJ, Ghalavand A, Mohammadi Goltapeh E, Rejali F, Zamaniyan M (2009) Effects of mixed cropping, earthworms (*Pheretima sp*.), and arbuscular mycorrhizal fungi (*Glomus mosseae*) on plant yield, mycorrhizal colonization rate, soil microbial biomass, and nitrogenase activity of free-living rhizosphere bacteria. Pedobiologia 52:223–235

Zarea MJ, Hajinia S, Karimi N, Mohammadi Goltapeh E, Ghalavand A (2011a) Effect of cropping systems and arbuscular mycorrhizal fungi on soil microbial activity and root nodule nitrogenase. J Saudi Soc Agric Sci 10:109–120

Zarea MJ, Hajinia S, Karimi N, Mohammadi Goltapeh E, Rejali F, Varma A (2011b) Effect of *Piriformospora indica* and *Azospirillum* strains from saline or non-saline soil on mitigation of the effects of NaCl. Soil Biol Biochem 45:139–146

Part IV
Piriformospora indica and Macronutrients for Plants

Chapter 17
Plant Nitrogen Use Efficiency May Be Improved Through Symbiosis with *Piriformospora indica*

Cristina Cruz, Zahra Fegghi, Maria Amélia Martins-Loução, and Ajit Varma

17.1 Introduction

Contemporary agriculture faces enormous challenges. Despite a 100% increase in crop productivity during the last century, globally, roughly one in seven people lacks access to food or is chronically malnourished. The situation may worsen as food prices experience shocks from market speculation, bioenergy crop expansion, and climatic disturbances (Foley et al. 2011). Even if food access problems can be solved, much higher crop productivities will probably be needed to guarantee food security. It is suggested that production would need to double to cope with demands from population growth, diet improvements (especially milk and meat consumption), and increasing bioenergy use. Agriculture must also address enormous environmental issues. In fact, agriculture is a major force driving the environment beyond the "planetary boundaries" (Rockstrom et al. 2009), and will also be one of the main sectors affected by those changes. Therefore, one of the biggest challenges for this century is to guarantee food security and at the same time minimize the environmental impacts of food production.

The present work focuses on the potential advantages of using arbuscular mycorrhizal fungi (AMF) and *Piriformospora indica* in agriculture to improve agricultural productivity and nutrient use efficiency.

The environmental impacts of agriculture include those resulting from expansion (when croplands and pastures extend into new areas, replacing natural

C. Cruz (✉) • Z. Fegghi (✉) • M.A. Martins-Loução
Faculdade de Ciências, Centro de Biologia Ambiental, Universidade de Lisboa, Bloco C2, Piso 5, Campo Grande 1749-016, Lisbon, Portugal
e-mail: ccruz@fc.ul.pt; zfeghhi@yahoo.com; maloucao@ul.pt

A. Varma
Amity Institute of Microbial Technology (AIMT), Amity University Uttar Pradesh, E-3 Block, Fourth Floor, Sector 125, Noida, UP 201303, India
e-mail: ajitvarma@amity.edu

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_17,

ecosystems) and intensification (when existing agricultural lands are managed to be more productive, often through the use of irrigation, fertilizers, biocides, and mechanization). Agricultural intensification has dramatically increased in recent decades, outstripping rates of agricultural expansion, and has been responsible for most of the yield increases obtained. Intensification has also caused water degradation, increased energy use, and widespread pollution (Galloway et al. 2009).

The intensive use of fertilizers, manure, and leguminous crops has dramatically disrupted global nitrogen and phosphorus cycles, with associated impacts on biodiversity loss and climate changes (Rockström et al. 2009).

One potential vehicle for a more sustainable agriculture is the management of the microorganisms in the plant rhizosphere. However, efforts to incorporate microbial management into mainstream agricultural practices have not been very successful.

One particularly widespread and beneficial group of rhizosphere microbes, AMF, has high potential for use in agriculture. Many studies suggest that AMF have multiple ecosystem functions and are an ideal tool in any field where plants and their communities are manipulated, including sustainable and intensive agriculture (Hart and Trevors 2005; Foley et al. 2011).

AMF are soil-dwelling fungi (phylum Glomeromycota) symbiotic with the roots of many plants (Smith and Read 1997). As a group, they may have the single largest effect on plant performance of any rhizosphere-associated microbe, functioning as an extension of the root system of the plant and increasing its absorptive area. Connections between conspecific hyphae allow plant individuals to be joined in a network with many other mycorrhizal plants; not only are more resources available to the plant but there is evidence that they are shared within the mycelial network (Simard and Read 2004). For many years, it was believed that AMF functioned primarily to improve phosphorous (P) nutrition of plants (Smith and Read 1997). However, benefits of arbuscular mycorrhizal association are more involved than simply P transfer and include improved nutrient uptake, mineralization of organic nutrients, drought resistance, seedling establishment, pathogen resistance, increased herbivore tolerance, increased pollination, heavy metal tolerance, and increased soil stability.

Since the 1970s, several attempts to use AMF as a "biofertilizer" have been made. In fact, AMF inoculum has been produced for use in agriculture and horticulture. In the early 1990s, researchers described multiple ways in which AMF management would be useful for sustainable agrosystems, including agrosystems (Bethlenfalvay and Linderman 1992; Pfleger and Linderman 1994). Despite the development of an immense amount of work, the practical application of AMF has failed to penetrate mainstream markets, and while most farmers are familiar with Rhizobium spp., AMF are only now starting to become popular among farmers.

There are several reasons for the less-than-expected use of AMF in agriculture, including the difficulty in producing AMF inoculum (DeClerk et al. 2005).

In this respect, the use of AMF-like fungi such as the endophyte *P. indica* is an alternative that may present some advantages over AMF. *P. indica* is an endophyte fungi able to be grown in pure culture (it does not need the presence of the plant; it is not an obligate biotroph); it forms a symbiosis with plant roots, imposing changes in the plant morphology very similar to those observed in mycorrhizal symbiosis (Varma et al. 2012).

In this work, we assess the potential of *G. intraradices* and *P. indica* to take up N from the medium and transport it into the plant root. The potential implications for N use efficiency and agricultural impact are discussed.

17.2 Materials and Methods

17.2.1 Root and Fungal Cultures

Experimental systems consisted of Ri T-DNA (*Agrobacterium rhizogenes*)-transformed tomato (*Lycopersicon esculentum* cv Moneymaker) roots colonized with *Glomus intraradices* Schenck and Smith (DAOM 181602) or with *P. indica* (Verma et al. 1998). Root cultures were started from previously inoculated root segments. Two-compartment petri dishes (100-mm diameter, 15-mm depth) contained 20 mL of M medium (Bécard and Fortin 1988) with normal P or ten times the normal P concentration in the root compartment; and 20 mL of the same medium without sucrose for roots incubated with *G. intraradices* or 20 ml of K medium (Cruz et al. 2007) for roots inoculated with *P. indica*. Petri dishes were incubated in the dark at 25 °C until the roots were homogeneously colonized and the Phytagel of the root-free compartment (RFC) was heavily colonized by *G. intraradices* or *P indica* (±8 weeks after the beginning of the cultures). The media of the fungal compartment was then removed and replaced by liquid M medium (14 mL) at double concentration. The mycelium was allowed to colonize this medium over the subsequent 2 weeks. Petri dishes were examined regularly, and roots were trimmed as required to prevent crossing into the RFC. Ten-week-old cultures with vigorous roots and densely colonized RFCs were selected for the experiments.

For cultures growing with normal P concentrations, after establishment of the ERM for 2 weeks in liquid M medium, the medium was replaced by fresh medium containing 5 mM $(NH_4)_2SO_4$ of 82% ^{15}N enrichment, and triplicate cultures were harvested sequentially over the subsequent 168 h. Mycelium and roots from one replicate culture were dried, and total nitrogen and ^{15}N abundance were determined on an isotope ratio mass spectrometer (Finnigan MAT Delta E; Thermo Electron) coupled to an EA 1110 elemental analyser (Thermo Electron). Four petri dishes per treatment were collected at each sampling time: 0, 1, 2, 4, 8, 24, 48, or 96 h after treatment initiation. Liquid medium was removed, and the ERM was rinsed twice with miliQ water. The mycelium was collected, blotted on absorbent paper,

weighed, and dried for ^{15}N analyses. AM roots removed from the Phytagel were rinsed twice with miliQ water, blotted, and analysed for their colonization.

Root colonization was determined on roots from 10 randomly selected cultures using a clearing and staining procedure modified after Phillips and Hayman (1970), omitting phenol from the reagents and HCl from the rinse. Two hundred intersects per sample were scored for the presence of intercellular hyphae, arbuscules, vesicles, and hyphal or arbusculate coils (McGonigle et al. 1990). Observations were performed with a microscope at 100-fold magnification.

For the root cultures established with high P concentrations in the root compartment, liquid medium in the RFC was replaced by fresh medium containing $(NH_4)2SO_4$ with 82% ^{15}N at three levels: 5, 10, and 20 mMN. Enriched cultures were harvested sequentially over 168 h using the experimental procedure described above.

17.3 Results and Discussion

Symbiotic associations with AMF enhance the acquisition of several mineral nutrients by plants. Nutrient acquisition via the fungal partner involves transfer across two interfaces: one between the soil and the extraradical mycelium (ERM) of the fungus and one between the intraradical mycelium (IRM) of the fungus and the root cortex cells. Soil-to-plant nitrogen transport by the ERM of AM fungi was first demonstrated using compartmented pots where ^{15}N-labeled nitrogen sources were applied to soil containing the ERM, but no roots (Ames et al. 1983; Tobar et al. 1994; Bago et al. 1996; Hawkins et al. 2000; Toussaint et al. 2004; Govindarajulu et al. 2005). N uptake from root-free compartments (RFCs) could account for as much as 30–80% of total plant nitrogen uptake in compartmented growth systems (Frey and Schuepp 1993; Johansen et al. 1994). The high N uptake ability of AMF was confirmed using in vitro model systems (Govindarajulu et al. 2005; Cruz et al. 2007; Tian et al. 2010). In the present study, we used transformed roots of *Solanum esculentum* (tomato), with a degree of AMF colonization varying from 36 to 48%, to assess the capacity of the AMF *G. intraradices* to take up N from the RFC and translocate it into the root compartment. The rates of ^{15}N uptake over time (Fig. 17.1) were similar to those found for *G. intraradices* in association with *Daucus carota* (Cruz et al. 2007), showing that the potential for the AMF to acquire and deliver N to the plant is not specific to one host. The N uptake rates obtained for *P. indica* in association with tomato in the same system and nutritional conditions [Plant: M medium with normal P concentrations; ERM: 5 mM $(NH_4)_2SO_4$] were higher than those obtained for *G. intraradices* (Figs. 17.1 and 17.2). However, the amount of ^{15}N transferred into the root was similar, allowing one to hypothesize that the plant controls the amount of N transferred from the fungi into the plant. The fact that the ^{15}N transferred from the fungi (either *G. intraradices* or *P. indica*) into the tomato root was independent of the N concentration in the RFC may also be evidence that the root controls the N received (Fig. 17.3).

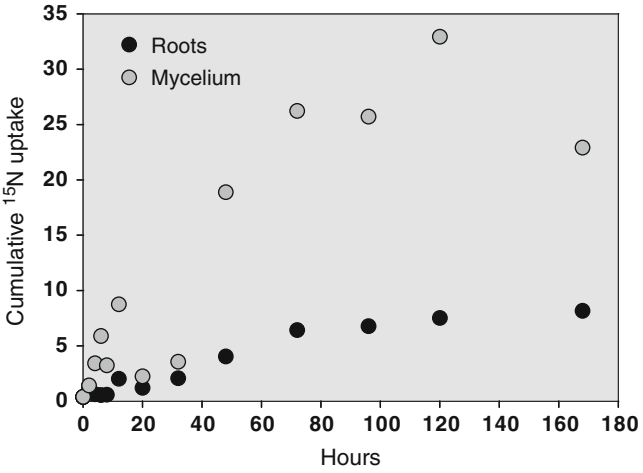

Fig. 17.1 ^{15}N uptake by the ERM of *G. intraradices* and transference into AM roots of tomato grown in an in vitro compartmented system (with normal P concentration in the M medium) after exposing the ERM to 5 mM $(^{15}NH_4)_2SO_4$ with 82% ^{15}N enrichment. N uptake was normalized against the fresh weight of the sample (n = 5–3)

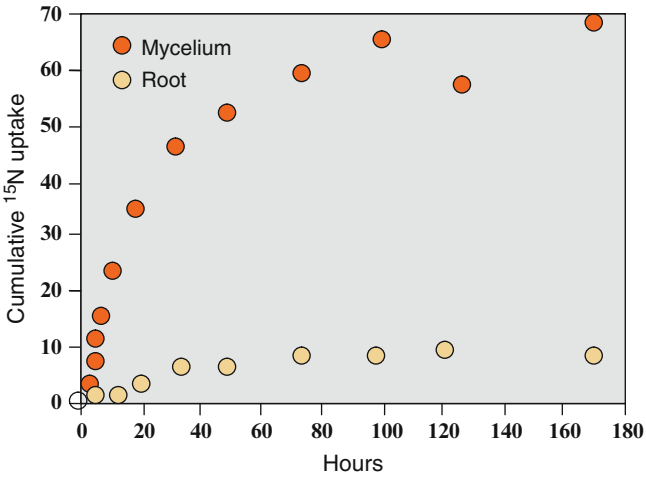

Fig. 17.2 ^{15}N uptake by the ERM of *P. indica* and transference into AM roots of tomato grown in an in vitro compartmented system (with normal P concentration in the M medium) after exposing the ERM to 5 mM $(NH_4)_2SO_4$ with 82% ^{15}N enrichment. N uptake was normalized against the fresh weight of the sample (n = 5–3)

It is clear from these results that both *G. intraradices* and *P. indica* improved N acquisition by the plants, but *P. indica* was much more efficient than *G. intraradices* (Fig. 17.4). The capacity of the *P. indica* to transfer N into the plant

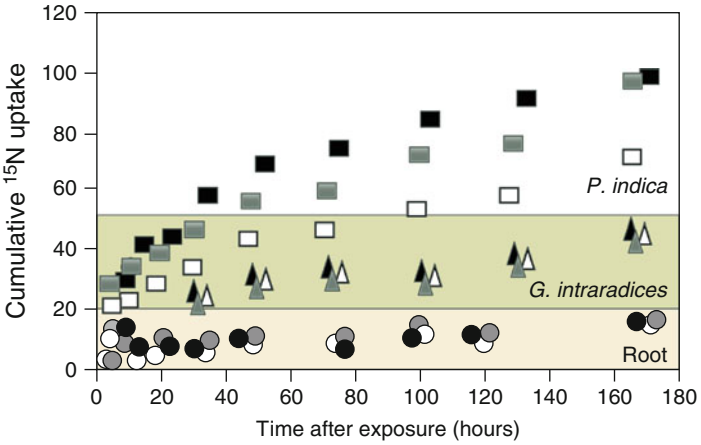

Fig. 17.3 ^{15}N uptake by the ERM of *G. intraradices* or *P. indica* and transference into AM roots of tomato grown in an in vitro compartmented system (with normal P concentration of the M medium) after exposing the ERM to 5 (white); 10 (grey); or 20 (black) mM $(NH_4)_2SO_4$ with 82% ^{15}N enrichment. N uptake was normalized against the fresh weight of the sample ($n = 5–3$)

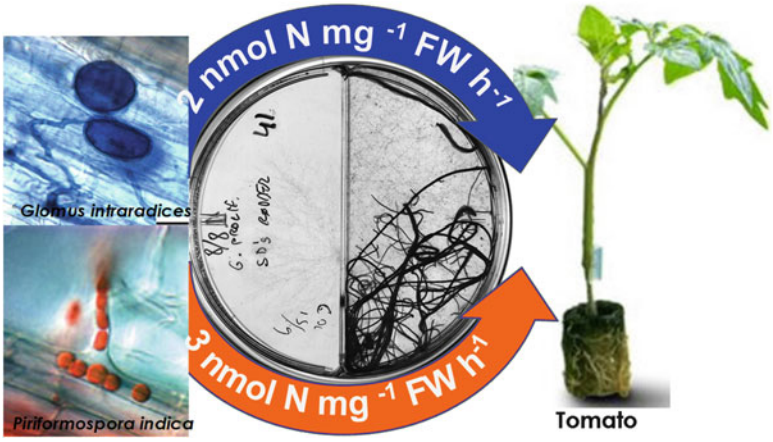

Fig. 17.4 Schematic representation of the relative efficiency of *G. intraradices* and *P.indica* in taking up^{15}N through and transfer to the tomato root independently of the N concentration in the root-free compartment, the ERM of *G. intraradices* or P

was neither affected by the increase in P level in the plant compartment (Fig. 17.5) nor was the degree of root colonization (32–45%).

In combination, these two findings—independence from P concentration in the root medium and higher capacity to take up and transfer N into the plant—indicate that *P. indica* has strong potential for use in intensive agriculture, where soils are high in phosphate. These advantages of the *P. indica* over *G. intraradices* are

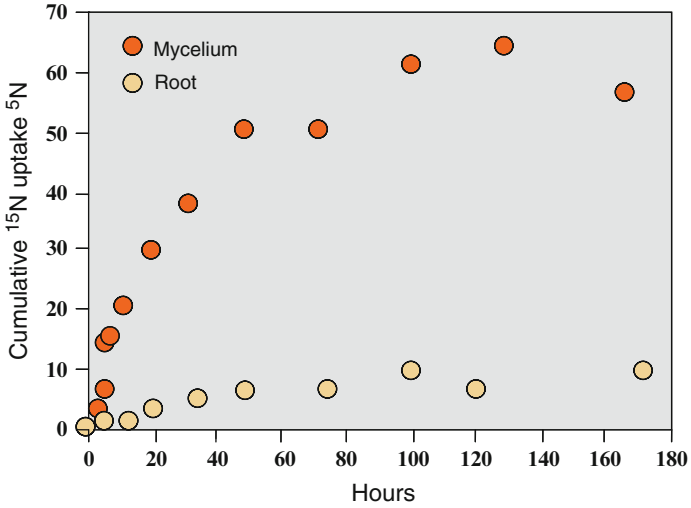

Fig. 17.5 ^{15}N uptake by the ERM of *P. indica* and transference into AM roots of tomato grown in an in vitro compartmented system (with 10 times the P concentration of the M medium) after exposing the ERM to 5 mM $(NH_4)_2SO_4$ with 82% ^{15}N enrichment. N uptake was normalized against the fresh weight of the sample ($n = 5–3$)

potentiated by the fact that production of *P. indica* inoculum is easier and cheaper than that of *G. intraradices*.

Considering the kinetics of transfer of N from the *P. indica* ERM to the host plant, it is possible that the mechanism involved is similar to that already proposed for AMF (Bago et al. 2001). However, more molecular and physiological studies are needed in order to fully understand the mechanisms involved and their regulation. A crucial step forward in this field would the establishment of field experiments in order to assess the ecological and agricultural potential of this mechanism. Indeed, several works (Varma et al. 2012) have already reported field improvement of nutrition and nutrient use efficiency of crops colonized with *P. indica* (Cruz et al. 2012), promising beneficial agricultural and environmental impacts.

References

Ames RN, Reid CPP, Porter LK, Cambardella C (1983) Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by Glomusmossae, a vesicular arbuscular mycorrhizal fungus. New Phytol 95:381–396

Bago B, Vierheilig H, Piche Y, Azcón Aguilar C (1996) Nitrate depletion and pH changes induced by the extra-radical mycelium of the arbus- cular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Bago B, Pfeffer P, Shachar-Hill Y (2001) Could the urea cycle be trans-locating nitrogen in the arbuscular mycorrhizal symbiosis? New Phytol 149:4–8

Bécard G, Fortin JA (1988) Early events of vesicular arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Bethlenfalvay GJ, Linderman RG (1992) Mycorrhizae in sustainable agriculture. American Society of Agronomy Inc., Madison, WI, USA Special publication No. 54

Cruz C, Egsgaard H, Trujillo C, Ambus P, Requena N, Martins-Loução MA, Jakobsen I (2007) Enzymatic evidence for the key role of arginine in nitrogen translocation by arbuscular mycorrhiza fungi. Plant Physiol 144:782–792

Cruz C, Martins-Loução MA, Varma A (2012) The influence of plant co-culture of tomato plants with piriformospora indica on biomass accumulation and stress tolerance. Acta Hortic 868:123–128

DeClerk S, Strullu DG, Fortin JA (2005) In vitro culture of Mycorrhiza. Springer, New york, NY

Foley JA, Ramankutty N, Brauman KA, Cassidy ES, Gerber JS, Johnston M, Mueller ND, O'Connell C, Ray DK, West PC, Balzer C, Bennett EM, Carpenter SR, Hill JL, Monfreda C, Polasky S, Rockstrom J, Sheehan J, Siebert S, Tilman D, David P, Zaks M (2011) Solutions for a cultivated planet. Nature 478:337–342

Frey B, Schuepp H (1993) Acquisition of nitrogen by external hyphae of arbuscular mycorrhizal fungi associated with *Zea mays* L. New Phytol 124:221–230

Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA (2009) Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. Science 320:889–892

Govindarajulu M, Pfeffer PE, Jin HR, Abubaker J, Douds DD, Allen JW, Bucking H, Lammers PJ, Shachar-Hill Y (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435:819–823

Hart MM, Trevors JT (2005) Microbe management: application of mycorrhizal fungi in sustainable agriculture. Ecol Soc Am 3:533–539

Hawkins HJ, Johansen A, George E (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226:275–285

Johansen A, Jakobsen I, Jensen ES (1994) Hyphal N transport by a vesicular-arbuscular mycorrhizal fungus associated with cucumber growth at three nitrogen levels. Plant Soil 160:1–9

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan SA (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–501

Pfleger FL, Linderman RG (EDS) (1994) Mycorrhizae and plant health. The American Phytopathological Society. APS Press, St Paul, MN

Phillips JM, Hayman DS (1970) Improved procedure for clearing roots and assessment of infection. Trans Brit Mycol Soc 55:158–161

Rockström J, Steffen W, Noone K, Persson A, Chapin FS III, Lambin EF, Lenton TM, Scheffer M, Folke C, Schellnhuber HJ, Nykvist B, de Wit CA, Hughes T, van der Leeuw S, Rodhe H, Sörlin S, Snyder PK, Costanza R, Svedin U, Falkenmark M, Karlberg L, Corell RW, Fabry VJ, Hansen J, Walker B, Liverman D, Richardson K, Crutzen P, Foley JA (2009) A safe operating space for humanity. Nature 461:472–475

Simard S, Read DM (2004) Mycorrhizal networks: a review of their extent, function and importance. Can J Bot 82:1140–1165

Smith S, Read D (1997) Mycirrhizal symbiosis. Academic, London

Tian C, Kasiborski B, Koul R, Lammers PJ, Bucking H, Shachar-Hill Y (2010) Regulation of the nitrogen transfer pathway in the arbuscular mycorrhizal symbiosis: gene characterization and the coordination of expression with nitrogen flux. Plant Physiol 153:1175–1187

Tobar R, Azcoón R, Barea JM (1994) Improved nitrogen uptake and transport from ^{15}N labelled nitrate by external hyphae of arbuscular mycorrhiza under water stressed conditions. New Phytol 126:119–122

Toussaint J-P, St-Arnoud M, Charest C (2004) Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck& Smith and Ri. T-DNA roots of Daucus carota L. in an in vitro compartmented system. Can J Microbiol 50:251–260

Varma A, Sherameti I, Tripathi S, Prasad R, Das A, Sharma M, Bakshi M, Johnson JM, Bhardwaj S, Arora M, Rastogi K, Agrawal A, Kharkwal AC, Talukdar S, Bagde US, Bisaria VS, Upadhyaya CP, Won PS, Chen Y, Ma J, Lou B, Adya A, Zhong L, Meghvanshi MK, Gosal SK, Srivastava RB, Johri AK, Cruz C, Oelmüller R (2012) The symbiotic fungus *Piriformospora indica*: review. In: Esser K (ed) The Mycota. Springer, Berlin

Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Butehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:896–902

Chapter 18
Role of *Piriformospora indica* in Sulfur Metabolism in *Arabidopsis thaliana*

Pyniarlang L. Nongbri and Ralf Oelmüller

18.1 Introduction

Plant performance and productivity are highly dependent on sulfur (S), which is mainly taken up from the soil as sulfate before reduction and metabolism into S-containing compounds (Leustek et al. 2000; Saito et al. 2004). Animals are unable to reduce sulfate and thus require S-containing amino acids or proteins as diet. Therefore, sulfate assimilation by plants is essential for all life on earth. Beside the presence of S in amino acids, it is found in many plant metabolites including vitamins, coenzymes, volatiles, and defense compounds (Grubb and Abel 2006; Halkier and Gershenzon 2006; Leustek et al. 2000; Saito et al. 2004). The presence of S in many redox mediators also highlights its importance for signaling processes.

Plants respond to S-limiting conditions by increasing the amount and activity of sulfate uptake and transport systems (Clarkson et al. 1983; Deane-Drummond 1987; Smith et al. 1995, 1997). High-affinity sulfate transporters located in the plasma membrane of roots (Shibagaki et al. 2002; Takahashi et al. 2000; Vidmar et al. 2000; Yoshimoto et al. 2003), transporters for vascular transport (Kataoka et al. 2004a, b; Takahashi et al. 1997; Yoshimoto et al. 2003), and the release of sulfate from the vacuole (Kataoka et al. 2004a, b) coordinate the cellular response during initial stages of S limitation. As a second strategy, synthesis of S-containing metabolites and storage compounds is downregulated, and S is released from these compounds through active breakdown processes (Hirai et al. 2004, 2005; Kutz et al. 2002). In Brassicales, up to 30 % of the S is stored in glucosinolates, S-rich metabolites that function in the defense of plants against pests and pathogens (Falk et al. 2007). Under S deficiency, there is a general downregulation of glucosinolate biosynthesis genes which accompanies an upregulation of genes

P.L. Nongbri • R. Oelmüller (✉)
Institut für Pflanzenphysiologie, Friedrich-Schiller-Universität Jena, Dornburger Str, 159, 07743 Jena, Germany
e-mail: pyndrop@gmail.com; b7oera@uni-jena.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_18,

controlling glucosinolate breakdown. Activation of sulfate acquisition and repression of glucosinolate production may occur in parallel in response to S limitation (Hirai et al. 1995, 2003, 2004, 2005, 2007; Maruyama-Nakashita et al. 2003, 2005). Thus glucosinolates may be considered a potential source of S for other metabolic processes under S limitation (Falk et al. 2007; Grubb and Abel 2006; Halkier and Gershenzon 2006). The active defense compounds are released from the glucosinolates after enzymatic cleavage. Several enzymes for these reactions play important functions in plant/microbe interactions. PEN2, for instance, a myrosinase analyzed by Bednarek et al. (2009), restricts pathogen entry into leaf cells. PEN2 exhibits striking sequence similarities to PYK10, a highly abundant enzyme in the roots that restricts root colonization by *P. indica* (Sherameti et al. 2008). We could show that PYK10 is required for the beneficial interaction between the two symbionts and speculated that Brassicaceae contain such high amounts of this enzyme in the roots to protect them against soil-borne fungi. Very recently, the importance of the glucosinolate metabolism for antifungal defense and innate immune response has been reported by two groups (Bednarek et al. 2009; Clay et al. 2009). Breakdown of indolic glucosinolates also generates auxins in roots under certain stress conditions, which may participate in the stimulation of root development for sulfate uptake. One of the central transcription factors that regulate aliphatic glucosinolate biosynthesis in *Arabidopsis* is MYB28 (Gigolashvili et al. 2008; Hirai et al. 2007; Sønderby et al. 2007). The aliphatic glucosinolates derive from methionine and are synthesized by two P450 enzymes, CYP79F1 and CYP79F2 (Chen et al. 2003). The *myb28 myb29* double mutant completely lacks aliphatic glucosinolates, and the glucosinolate biosynthesis genes are drastically downregulated (Beekwilder et al. 2008).

Processes controlling S metabolism are important for agriculture, horticulture, and medicine. The qualities and biomass production of crop plant species are severely impaired under S starvation and breakdown of endogenous S compounds such as glucosinolates affects plant fitness and reproductivity. Optimization of the volatile composition plays a major role in horticulture. Furthermore, many S-containing secondary metabolites are used as cancer preventives in diets (Talalay and Fahey 2001).

Although the network of S metabolism is coordinately regulated under S limitations, many of these processes are not fully understood yet. Several transcription factors and signaling proteins involved in indole glucosinolate biosynthesis have been characterized in *Arabidopsis* (Celenza et al. 2005; Levy et al. 2005; Skirycz et al. 2006). In 2006, Maruyama-Nakashita et al. identified sulfur limitation 1 (SLIM1) as a central regulator of plant S responses and metabolism in *Arabidopsis*. The identified mutant *slim1* was unable to induce transcripts for the high-affinity transporter SULTR1;2 under low S conditions. Sulfate uptake and plant growth under S starvation were significantly reduced in *slim1*, and SLIM1 controlled both the activation of sulfate acquisition and degradation of glucosinolates under S limitations. SLIM1 is a member of the ethylene-insensitive-like (EIL) transcription factor family and identical to EIL3 which is crucial for ethylene signal transduction (Chao et al. 1997; Guo and Ecker 2003; Solano et al. 1998). The EIL family consists

of six transcription factors (EIN3, EIL1-5; Guo and Ecker 2003). Interestingly, overexpression of *SLIM1*, but not of the other *EIL* genes from *Arabidopsis*, restored the wild-type (WT) phenotype of *slim1* mutants, suggesting uniqueness of SLIM1 in the EIL group as S response regulators. A short domain from SLIM1 (S_{162}–G_{288}) recognizes double-stranded DNA with the conserved EIL- and EIN3-binding motif AYGWAYCT (Kosugi and Ohashi 2000; Solano et al. 1998; Yamasaki et al. 2005). Furthermore, MYB72 has been identified as interaction partner of SLIM1 in the yeast-two-hybrid (Y2H) system (Van der Ent et al. 2008). MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance (ISR) in *Arabidopsis* (Van Wees et al. 2008).

Enzymes involved in S assimilation are often encoded by multigene families in *Arabidopsis*. Their members can have redundant functions (e.g., serine acetyltransferases; Watanabe et al. 2008) or are highly specialized and expressed in different tissues (e.g., branched-chain aminotransferases; Schuster et al. 2006), or they are imbedded into complex signaling pathways (e.g., adenosine 5′-phosphosulfate reductase; Koprivova et al. 2008; Vauclare et al. 2002). Several members of these gene families respond to S starvation, while others do not (Maruyama-Nakashita et al. 2003). There is increasing evidence that posttranscriptional regulatory circuits and feedback loops play important roles in the regulation of the activities of these enzymes.

We found that *Piriformospora indica* strongly affects the S metabolism in the symbiotic interaction with *Arabidopsis*. *P. indica* is an endophytic fungus of the Basidiomycetes which colonizes the roots of many plant species including mono- and dicots and mosses, many of which are of forestry, agricultural, horticultural, and medicinal importance. Root colonization is accompanied by the promotion of growth and higher seed yield, and the plants are more resistant to various biotic and abiotic stresses (Shahollari et al. 2007; Sherameti et al. 2008; Vadassery et al. 2009a, b).

S uptake and metabolism play an important role in plant/microbe interactions. However, in contrast to phosphorus and nitrogen, little is known about S regulation in beneficial interactions of roots with soilborne microorganisms. Mansouri-Bauly et al. (2006) have shown that *L. bicolor* increases sulfate supply to the plant by extended sulfate uptake and the plant provides the ectomycorrhizal fungus with reduced S. Many S-containing components play a crucial role in defense against pathogens and in biotic and abiotic stress (Hildebrandt et al. 2006; Rausch and Wachter 2005), when they are either upgraded or induced *via* jasmonic acid and/or other signals (cf. Hilpert et al. 2001; Mikkelsen et al. 2003; Nibbe et al. 2002; Xiang and Oliver 1998). While the cysteine content is tightly regulated, the cysteine precursor *O*-acetylserine (OAS) is rapidly upregulated and functions as a signaling metabolite by controlling the expression of sulfate transporters and several genes of the S-assimilation pathway (Hirai et al. 2003). Glutathione (GSH) is upgraded with increasing sulfate supply and may establish resistance to stress. GSH is a major redox buffer and protects the cell against reactive oxygen species. An *Arabidopsis* mutant lacking the gamma-glutamylcysteine ligase 1 (GSH1), the rate-limiting enzyme for GSH synthesis (Ball et al. 2004), is impaired in defense reactions

against pathogens. Waller et al. (2005) and Baltruschat et al. (2008) have shown that GSH plays a crucial role in *P. indica*-induced resistance of barley plants against pathogens. In the ascorbate–GSH cycle, the function of GSH is linked to ascorbic acid, and the electron flow from NADPH (Foyer and Noctor 2005; Rausch and Wachter 2005). Dehydroascorbate reductase (DHAR) and glutathione reductase activities are stimulated by *P. indica* in salt-stress barley (Baltruschat et al. 2008). Monodehydroascorbate reductase 2 and DHAR 5 are crucial for a mutualistic interaction between *P. indica* and *Arabidopsis* under drought stress (Vadassery et al. 2009a). Many glutathione *S*-transferases (GST) are crucial for detoxification mechanisms, and GSH is the precursor of phytochelatins, cysteine-rich peptides synthesized *via* phytochelatin synthase (Cobbett and Goldsbrough 2002). Genes for the latter examples are rapidly upregulated when *Arabidopsis* roots are exposed to *P. indica*.

GSH may also be responsible for the activation of the nonexpressor of PR genes (NPR1). NPR1 affects transcription of salicylic acid-induced genes for pathogenesis-related proteins (Dong 2004). In the oxidized, non-induced plants, NPR1 is cross-linked by intermolecular disulfide bridges and localized in aggregated form in the cytosol. Upon infection, NPR1 becomes reduced, and the monomers are translocated to the nucleus. Inactivation of *NPR1* does not affect the beneficial interaction at the seedling's stage; however, adult plants become over-colonized, and thus, the interaction shifts to parasitism (unpublished).

SLIM1/EIL3 also plays an important role in plant immune responses triggered by beneficial microbes (Van Wees et al. 2008). Plant growth-promoting rhizobacteria and mycorrhizal fungi can improve plant performance by ISR-mediated defense responses that confer resistance to pathogens and insects. Recognition of microbe-associated molecular patterns from beneficial microbes leads to the activation of the transcription factor gene *MYB72*, and the protein interacts with SLIM1/EIL3 to induce a jasmonic acid/ethylene-dependent signaling pathway which primes the aerial parts of the plant for enhanced expression of jasmonic acid/ethylene-dependent genes (Van der Ent et al. 2008).

Here, we summarize recent results which demonstrate that quite diverse aspects of the S metabolism in *Arabidopsis* are affected by the root-colonizing beneficial fungus *P. indica*.

18.2 *P. indica* Becomes More Important for Plant Performance Under S Limitations

The analysis of the interaction of *P. indica* with *Arabidopsis* roots uncovered that genes involved in S uptake and metabolism are targets of the fungus. We therefore tested how plant performance is influenced under S limitations. Reduction of the S level in the growth medium strongly enhanced the beneficial effect induced by *P. indica*, and the differences in the growth rates between untreated and treated WT

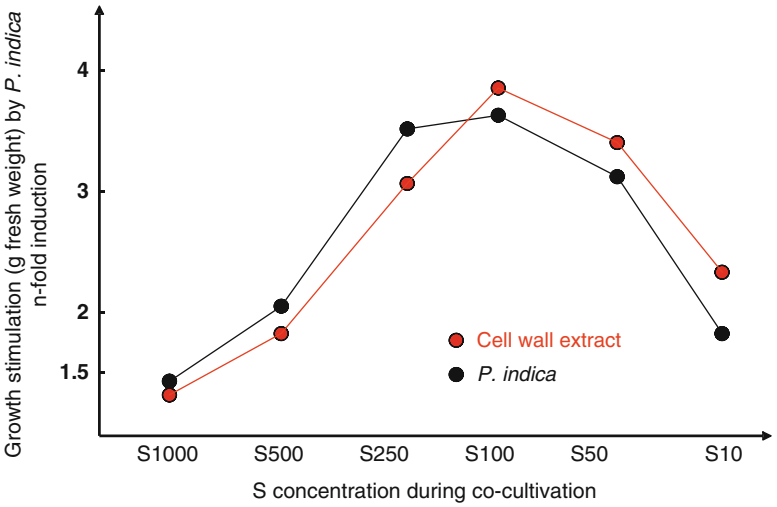

Fig. 18.1 Growth stimulation by *P. indica* (expressed as *n*-fold increase in fresh weight of *P. indica*-colonized vs. control seedlings after 10 days) on different sulfate concentration in the medium. Cocultivation occurred either with the fungus (*black*) or a cell wall extract from the fungus (*red*), cf. below

seedlings increased greater than threefold on media with 10 μM S compared to the full medium (cf. Vadassery et al. 2009a, b; Fig. 18.1). Interestingly, stimulation of plant performance under S limitations is not only promoted by the fungus itself but also observed if a cell wall extract of the fungus is applied to *Arabidopsis* roots. This suggests that microbe-associated molecular patterns present in the cell wall extract trigger processes in the roots cells which promote plant performance. Thus, better plant performance is not exclusively caused by a more efficient transfer of S nutrients *via* the hyphae to the roots. These observations in combination with the results outlined below support the idea that the S metabolism is a major target of the fungus in *Arabidopsis* and that apparently signals from the fungus trigger processes in the roots which strongly affect the S metabolism.

18.3 Transcription Factors Regulating S Metabolism and Genes Involved in S Metabolism

SLIM1 is a regulatory transcription factor controlling many downstream genes involved in S metabolism (Maruyama-Nakashita et al. 2006). Expression of *slim1* is barely regulated in Arabidopsis roots. However, we observed an approximately 50 % increase in the *SLIM1* mRNA levels in *P. indica*-colonized *Arabidopsis* roots grown under S limitations. Since this result is confirmed when a cell wall extract from the fungus was applied to the roots, we postulate that *P. indica*-derived

components trigger signaling events in the roots that activate the S metabolism by activating *slim1* gene expression.

Genes involved in S metabolism, which are regulated by SLIM1 (Maruyama-Nakashita et al. 2006), are also regulated by *P. indica* during early phases of cocultivation (i.e., within 1 h under sufficient S supply; 1,000 μM). This is particularly striking for *ASP1*, *ASP3*, and *ASP4* and demonstrates that the ATP sulfurylases are specific targets of the fungus even under sufficient S supply. However, some of them are even strongly upregulated under S starvation. Examples are *GSH1*, *GSH2*, *BCAT2*, *BCAT4*, *MAM3*, three *GST* genes, *PHYTOCHELATIN SYNTHASE*, and *NPR1*. This again confirms that *P. indica* controls S metabolism, in particular under S-limiting conditions.

18.4 Glucosinolates

The message levels for enzymes involved in glucosinolate biosynthesis and cleavage are upregulated by *P. indica* under sufficient S conditions which raises many questions. A mutant which shows a reduced response to *P. indica* has a lesion in *myb28*. We thus analyzed *myb28 myb29* seedlings in greater details and found that adult plants grow much slower compared to the uncolonized controls. These results suggest that glucosinolates are targets of the fungus and that long-term harmony between the two symbionts requires aliphatic glucosinolate biosynthesis (Fig. 18.2).

Furthermore, we have recently demonstrated that PYK10, a myrosinase abundantly expressed in roots, is required for the beneficial interaction between *P. indica* and *Arabidopsis* (Sherameti et al. 2008). Inactivation of *PYK10* results in an uncontrolled growth of fungal hyphae, presumably because the enzyme is required to release an aglycone from a so far unidentified glucosinolate substrate. PYK10 exhibits striking sequence similar to PEN2, a glycosyl hydrolase, which restricts pathogen entry of two ascomycete powdery mildew fungi into *Arabidopsis* leaf cells (Lipka et al. 2005). Like PEN2, PYK10 belongs to the class of glycosyl hydrolase family 1; both proteins are located in intracellular organellar structures (PYK10 in ER bodies and PEN2 in peroxisomes), and both proteins share a high degree of sequence similarity. The catalytic domains of both proteins contain two conserved nucleophilic glutamates. Lipka et al. (2005) have shown that glutamate^{183} is required for PEN2 function in vivo, which suggests that PEN2 catalytic activity is required for restricting pathogen entry (cf. also Bednarek et al. 2009). Thus, PYK10 might have a similar biological function in our system. This indicates that cleavage of certain glucosinolates might be important for the beneficial interaction of the two symbionts. We postulated that PYK10 as an abundant myrosinase in Arabidopsis roots controls the release of a toxic compound required to control root colonization. Partial inactivation of PYK10 results in over-colonized roots.

We established a hydroponic system in which Arabidopsis growth is strongly promoted when the roots were infected with fungal spores prior to transfer to the

Fig. 18.2 WT (*top*) and *myb28 myb29* (*bottom*) plants grown in the presence of *P. indica*. Without *P. indica*, no difference between the two genotypes can be detected

liquid medium. This allowed us to expose the roots of wild-type and mutant seedlings to liquid media with defined combinations of beneficial and pathogenic microbes. Growth of the individual microbes can be followed over time using qRT-PCR. Mutations affecting glucosinolate biosynthesis have a very strong influence on the growth rate of the individual microbes in the hydroponic media, consequently the microbial community changes. We often observe that changes in the glucosinate pattern results in one or a few very dominant microbes, while others barely grow or cannot survive at all. Interestingly, *P. indica* belongs to the class of microbes with a great potential to dominate the others, in particular in mutants impaired in the biosynthesis of certain glucosinolates. Currently, we are trying to understand how changes of the composition of the S-containing secondary metabolites can affect the microbial community in the root environment.

18.5 S as Antioxidant

S plays a crucial role as antioxidant, and ascorbate is a major antioxidant and radical scavenger. Monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) are two enzymes of the ascorbate–glutathione cycle that maintain ascorbate in its reduced state. MDAR2 (At3g09940) and DHAR5 (At1g19570) expression was upregulated in the roots and shoots of *Arabidopsis* seedlings cocultivated with *P. indica* (Vadassery et al. 2009a). Since root colonization does not only affect the redox situation in the roots but also in the leaves, an efficient information flow from the roots to the leaves has to be postulated. Furthermore,

a cell wall extract or a culture filtrate from the fungus is sufficient to establish a more reduced atmosphere in roots and leaves. This again demonstrates that *P. indica*-associated molecular patterns are sufficient to trigger signaling processes in roots, which affect the redox potential in both roots and shoots. The important role of MDAR2 and DHAR5 for the symbiotic interaction can be demonstrated by using knockout lines. While wild-type seedlings are taller and perform better in the presence of the fungus when compared to the uncolonized control, growth, plant performance, and seed production were not promoted in *mdar2* (SALK_0776335C) and *dhar5* (SALK_029966C) T-DNA insertion lines. After exposure to drought stress, the two insertion lines suffered severely in the presence of the fungus, because the roots of the drought-stressed insertion lines were colonized more heavily than were the wild-type plants. Upregulation of the message for the antimicrobial PDF1.2 protein in drought-stressed insertion lines indicated that MDAR2 and DHAR5 are crucial for producing sufficient ascorbate to maintain the interaction between *P. indica* and *Arabidopsis* in a mutualistic state. Since not only these two genes for antioxidants are upregulated in *P. indica*-colonized *Arabidopsis* roots, we hypothesize that many more proteins involved in maintaining the redox homeostasis in the cell are involved in establishing and maintaining a mutualistic interaction between the two symbionts.

18.6 Summary

Based on the preliminary results obtained so far, we propose the following hypothesis (Fig. 18.3): *P. indica* stimulates the S metabolism, in particular under S-limiting conditions, which results in better plant performance. One of the primary targets might be SLIM1/EIL3 as a central regulator of the entire S pathway. SLIM1 stimulates sulfate reduction by controlling the expression of several enzymes of the S metabolism including those that promote glucosinolate biosynthesis. The availability of higher levels of reduced S is beneficial for plant growth, whereas stimulation of glucosinolate biosynthesis provides the plant with sufficient defense compounds which are required to control the degree of root colonization. The glucosinolates are also involved in the defense against pathogens. Furthermore, the secondary metabolites function as an S storage which can be used under S-limiting conditions. The balance between these two processes might be important for a long-term harmony. The expression of genes regulating the S metabolism is not only controlled by the fungus itself but also by a cell wall extract from the fungus. Apparently *P. indica*-derived components are sufficient to trigger responses in the root cells which control S metabolism. We propose a "master switch" in the signal transduction pathway that determines whether signals from *P. indica* direct the S metabolism towards the primary S metabolism or glucosinate biosynthesis. This "master switch" should integrate biotic and abiotic signals for optimal adaption of the plant to the environment. Finally, based on two-hybrid screens, it has been proposed that SLIM1 interacts with MYB72 in the roots and induces ISR

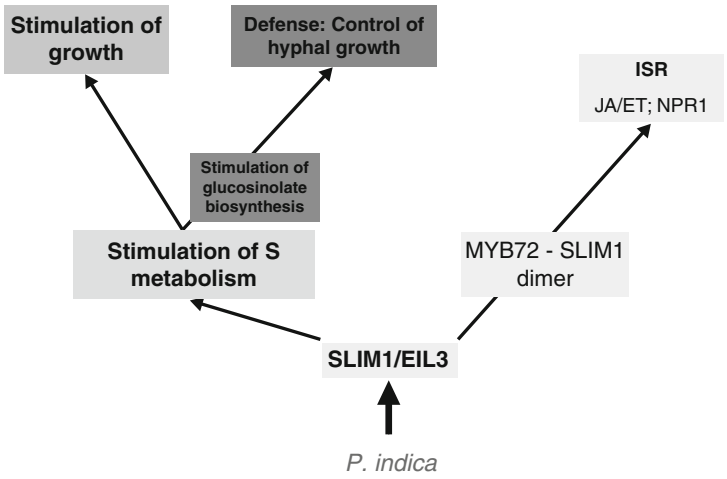

Fig. 18.3 A scheme describing the working hypothesis for this proposal. *P. indica* required SLIM1 to activate the S metabolism for the stimulation of growth and development of the plants. This also results in the stimulation of the glucosinolate metabolism to control hyphal growth and pathogen attack (under sufficient S conditions). Simultaneously, SLIM1, together with MYB72, triggers ISR.

which primes the aerial parts of the plant to respond more efficiently to pathogenic attack. If this hold true for the *in vivo* situation, ISR against pathogen attack in the leaves is also linked to the S metabolism.

References

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jimenez A, Kular B, Leyland N, Mejia-Carranza J, Reynolds H, Karpinski S, Mullineaux PM (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. Plant Cell 16:2448–2462

Baltruschat H, Fodor J, Harrach BD, Niemczyk E, Barna B, Gullner G, Janeczko A, Kogel KH, Schäfer P, Schwarczinger I, Zuccaro A, Skoczowski A (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytol 180:501–510

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A, Schulze-Lefert P (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323:101–106

Beekwilder J, van Leeuwen W, van Dam NM, Bertossi M, Grandi V, Mizzi L, Soloviev M, Szabados L, Molthoff JW, Schipper B, Verbocht H, de Vos RC, Morandini P, Aarts MG, Bovy A (2008) The impact of the absence of aliphatic glucosinolates on insect herbivory in *Arabidopsis*. PLoS One 30:e2068

Celenza JL, Quiel JA, Smolen GA, Merrikh H, Silvestro AR, Normanly J, Bender J (2005) The *Arabidopsis* ATR1 Myb transcription factor controls indolic glucosinolate homeostasis. Plant Physiol 137:253–262

Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR (1997) Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell 89:1133–1144

Chen S, Glawischnig E, Jorgensen K, Naur P, Jorgensen B, Olsen CE, Hansen CH, Rasmussen H, Pickett JA, Halkier BA (2003) CYP79F1 and CYP79F2 have distinct functions in the biosynthesis of aliphatic glucosinolates in *Arabidopsis*. Plant J 33:923–937

Clarkson DT, Smith FW, Van den Berg PJ (1983) Regulation of sulfate transport in a tropical legume. *Macroptilium atropurpureum* cv. Siratro. J Exp Bot 34:1463–1483

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an *Arabidopsis* innate immune response. Science 323:95–101

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53:159–182

Deane-Drummond CE (1987) The regulation of sulfate uptake following growth of *Pisum sativum* L. seedlings in S nutrient limiting conditions. Interaction between nitrate and sulphate transport. Plant Sci 50:27–35

Dong X (2004) NPR1, all things considered. Curr Opin Plant Biol 7:547–552

Falk KL, Tokuhisa JG, Gershenzon J (2007) The effect of sulfur nutrition on plant glucosinolate content: physiology and molecular mechanisms. Plant Biol 9:573–581

Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17:1866–1875

Gigolashvili T, Engqvist M, Yatusevich R, Müller C, Flügge UI (2008) HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana*. New Phytol 177:627–642

Grubb C, Abel S (2006) Glucosinolate metabolism and its control. Trends Plant Sci 11:89–100

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by $SCF^{EBF1/EBF2}$-dependent proteolysis of EIN3 transcription factor. Cell 115:667–677

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol 57:303–333

Hildebrandt U, Ouziad F, Marner FJ, Bothe H (2006) The bacterium *Paenibacillus validus* stimulates growth of the arbuscular mycorrhizal fungus *Glomus intraradices* up to the formation of fertile spores. FEMS Microbiol Lett 254:258–267

Hilpert B, Bohlmann H, op den Camp RO, Przybyla D, Miersch O, Buchala A, Apel K (2001) Isolation and characterization of signal transduction mutants of *Arabidopsis thaliana* that constitutively activate the octadecanoid pathway and form necrotic microlesions. Plant J 26:435–446

Hirai MY, Fujiwara T, Chino M, Naito S (1995) Effects of sulfate concentrations on the expression of a soybean seed storage protein gene and its reversibility in transgenic *Arabidopsis thaliana*. Plant Cell Physiol 36:1331–1339

Hirai MY, Fujiwara T, Awazuhara M, Kimura T, Noji M, Saito K (2003) Global expression profiling of sulfur-starved *Arabidopsis* by DNA macroarray reveals the role of *O*-acetyl-L-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J 33:651–663

Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhara M, Arita M, Fujiwara T, Saito K (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 101:10205–10210

Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, Sakurai N, Shibata D, Tokuhisa J, Reichelt M, Gershenzon J, Papenbrock J, Saito K (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in *Arabidopsis* by integration of metabolomics and transcriptomics. J Biol Chem 280:25590–25595

Hirai MY, Sugiyama K, Sawada Y, Tohge T, Obayashi T, Suzuki A, Araki R, Sakurai N, Suzuki H, Aoki K, Goda H, Nishizawa OI, Shibata D, Saito K (2007) Omics-based identification of

Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. Proc Natl Acad Sci USA 104:6478–6483

Kataoka T, Hayashi N, Yamaya T, Takahashi H (2004a) Root-to-shoot transport of sulfate in *Arabidopsis*: evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature. Plant Physiol 136:4198–4204

Kataoka T, Watanabe-Takahashi A, Hayashi N, Ohnishi M, Mimura T, Buchner P, Hawkesford MJ, Yamaya T, Takahashi H (2004b) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in *Arabidopsis*. Plant Cell 16:2693–2704

Koprivova A, North KA, Kopriva S (2008) Complex signaling network in regulation of adenosine 5′-phosphosulfate reductase by salt stress in *Arabidopsis* roots. Plant Physiol 146:1408–1420

Kosugi S, Ohashi Y (2000) Cloning and DNA binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog. Nucleic Acids Res 28:960–967

Kutz A, Müller A, Hennig P, Kaiser WM, Piotrowski M, Weiler EW (2002) A role for nitrilase 3 in the regulation of root morphology in sulphur-starving *Arabidopsis thaliana*. Plant J 30:95–106

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol 51:141–165

Levy M, Wang Q, Kaspi R, Parrella MP, Abel S (2005) *Arabidopsis* IQD1, a novel calmodulin-binding nuclear protein, stimulates glucosinolate accumulation and plant defense. Plant J 43:79–96

Lipka V, Dittgen J, Bednarek P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science 310:1180–1183

Mansouri-Bauly H, Kruse J, Sýkorová Z, Scheerer U, Kopriva S (2006) Sulfur uptake in the ectomycorrhizal fungus *Laccaria bicolor* S238N. Mycorrhiza 16:421–427

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H (2003) Transcriptome profiling of sulfur-responsive genes in *Arabidopsis* reveals global effect on sulfur nutrition on multiple metabolic pathways. Plant Physiol 132:597–605

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in *Arabidopsis* roots. Plant J 42:305–314

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) *Arabidopsis* SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell 18:3235–3251

Mikkelsen MD, Petersen BL, Glawischnig E, Jensen AB, Andreasson E, Halkier BA (2003) Modulation of CYP79 genes and glucosinolate profiles in *Arabidopsis* by defense signaling pathways. Plant Physiol 131:298–308

Nibbe M, Hilpert B, Wasternack C, Miersch O, Apel K (2002) Cell death and salicylate- and jasmonate-dependent stress responses in *Arabidopsis* are controlled by single *cet* genes. Planta 216:120–128

Rausch T, Wachter A (2005) Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci 10:503–509

Saito S, Soga T, Nishioka T, Tomita M (2004) Simultaneous determination of the main metabolites in rice leaves using capillary electrophoresis mass spectrometry and capillary electrophoresis diode array detection. Plant J 40:151–163

Schuster J, Knill T, Reichelt M, Gershenzon J, Binder S (2006) Branched-chain aminotransferase4 is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in *Arabidopsis*. Plant Cell 18:2664–2679

Shahollari B, Vadassery J, Varma A, Oelmüller R (2007) A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 50:1–13

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a

homeodomain transcription factor which binds to a conserved motif in their promoters. J Biol Chem 280:2641–2647

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmüller R (2008) PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 54:428–439

Shibagaki N, Rose A, Mcdermott JP, Fujiwara T, Hayashi H, Yoneyama T, Davies JP (2002) Selenate-resistant mutants of *Arabidopsis thaliana* identify SULTR1;2, a sulfate transporter required for efficient transport of sulfate into roots. Plant J 29:475–486

Skirycz A, Reichelt M, Burow M, Birkemeyer C, Rolcik J, Kopka J, Zanor MI, Gershenzon J, Strnad M, Szopa J, Mueller-Roeber B, Witt I (2006) DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in *Arabidopsis*. Plant J 47:10–24

Smith FW, Ealing PM, Hawkesford MJ, Clarkson DT (1995) Plant members of a family of sulfate transporters reveal functional subtypes. Proc Natl Acad Sci USA 92:9373–9377

Smith FW, Hawkesford MJ, Ealing PM, Clarkson DT, Vanden Berg PJ, Belcher AR, Warrilow AGS (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulfate transporter. Plant J 12:875–884

Solano R, Stepanova A, Chao Q, Ecker JR (1998) Nuclear events in ethylene signaling: A transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev 12:3703–3714

Sønderby IE, Hansen BG, Bjarnholt N, Ticconi C, Halkier BA, Kliebenstein DJ (2007) A systems biology approach identifies a R2R3 MYB gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. PLoS One 2:e1322

Takahashi H, Yamazaki M, Sasakura N, Watanabe A, Leustek T, Engler JA, Engler G, Van Montagu M, Saito K (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate starved roots plays a central role in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 94:11102–11107

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M, Hawkesford MJ, Saito K (2000) The role of three functional sulfate transporters involved in uptake and translocation of sulfate in *Arabidopsis thaliana*. Plant J 23:171–182

Talalay P, Fahey JW (2001) Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J Nutr 131:3027S–3033S

Vadassery J, Tripathi S, Prasad R, Varma A, Oelmüller R (2009a) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and *Arabidopsis*. J Plant Physiol 166(12):1263–1274

Vadassery J, Ranf S, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R (2009b) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J 59:193–206

Van der Ent S, Verhagen BW, Van Doorn R, Bakker D, Verlaan MG, Pel MJ, Joosten RG, Proveniers MC, Van Loon LC, Ton J, Pieterse CM (2008) MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. Plant Physiol 146:1293–1304

Van Wees SC, Van der Ent S, Pieterse CM (2008) Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol 11:443–448

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krähenbühl U, den Camp RO, Brunold C (2002) Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. Plant J 31:729–740

Vidmar JJ, Tagmount A, Cathala N, Touraine B, Davidian J-CE (2000) Cloning and characterization of a root specific high-affinity sulfate transporter from *Arabidopsis thaliana*. FEBS Lett 475:65–69

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Watanabe M, Mochida K, Kato T, Tabata S, Yoshimoto N, Noji M, Saito K (2008) Comparative genomics and reverse genetics analysis reveal indispensable functions of the serine acetyltransferase gene family in *Arabidopsis*. Plant Cell 20:2484–2496

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10:1539–1550

Yamasaki K, Kigawa T, Inoue M, Yamasaki T, Yabuki T, Aoki M, Seki E, Matsuda T, Tomo Y, Terada T, Shirouzu M, Tanaka A, Seki M, Shinozaki K, Yokoyama S (2005) Solution structure of the major DNA-binding domain of *Arabidopsis thaliana* Ethylene-insensitive3-like3. J Mol Biol 348:253–264

Yoshimoto N, Inoue E, Saito K, Yamaya T, Takahashi H (2003) Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in *Arabidopsis*. Plant Physiol 131:1511–1517

Chapter 19
"Electrotransformation" Transformation System for Root Endophytic Fungus *Piriformospora indica*

Manoj Kumar, Ruby Sharma, Meenakshi Dua, Narendra Tuteja, and Atul Kumar Johri

19.1 Introduction

Despite a worldwide intensification of agriculture and tremendous progress toward increasing yields in major crops over the last decades, the goal to reduce the problems associated with hunger is far from being reached (FAO 2004). Major causes for crop losses are abiotic and biotic stresses due to unfavorable climate and plant diseases and pests. Increased plant productivity, therefore, relies on a high chemical input and is achieved at the expense of detrimental effects on the environment (Chapin et al. 2000). Abiotic-stress tolerance can be evoked in crops by the exploitation of worldwide abundant endophytic arbuscular mycorrhiza fungi, which live in reciprocally beneficial relationships with 80 % of land plants (Newman and Reddell 1987). However, mycorrhizal plants, albeit effective against many root diseases (Azcón-Aguilar and Barea 1996; Borowicz 2001), often show enhanced susceptibility to biotrophic leaf pathogens (Gernns et al. 2001; Shaul et al. 1999). On the other hand, endophytes have been frequently reported to protect against plant pathogens and pests.

A critical review of the literature suggests that the beneficial action of the endophytes is based on direct antimicrobial and insecticidal activity due to alkaloid production. The endophyte *P. indica* serves most of the agronomically desirable traits and thus serves as the model organism for crop improvement. *P. indica* grows

M. Kumar • R. Sharma • A.K. Johri (✉)
School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India
e-mail: manojkumar.jnu@gmail.com; manikya.gem@gmail.com; akjohri14@yahoo.com

M. Dua
School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110067, India
e-mail: meenakshi72@hotmail.com

N. Tuteja
Plant Molecular Biology Group, International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India
e-mail: narendra@icgeb.res.in

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_19,

axenically and resembles arbuscular mycorrhiza (AM) fungi in several but not all functional and physiological characteristics (Schäfer et al. 2007, 2009). The fungus has attracted interest since its discovery, a decade ago, because of the beneficial effects it conveys on a broad variety of mono- and dicotyledonous hosts (Sahay and Varma 1999; Serfling et al. 2007; Varma et al. 1999; Waller et al. 2005). Along with promotion of plant biomass and increase in abiotic-stress tolerance (Baltruschat et al. 2008; Kumar et al. 2012), *P. indica* mediates disease resistance in plants to root pathogens such as *Fusarium graminearum* and *F. verticillioides* (Deshmukh and Kogel 2007; Kumar et al. 2009). By taking *P. indica* beneficial impacts on plants, it has now become mandatory to manipulate its genetic machinery. However, due to the absence of stable transformation system, *P. indica* could not be genetically manipulated to use in sustainable agriculture for crop improvement. In the present work we have tried to explore the possibility to explain how this fungus can be genetically manipulated.

19.2 Plant Mutualistic Fungus *Piriformospora indica*

The advent of techniques for manipulating and isolating genes from plant symbiotic fungus *P. indica* has lead to rapid increase in understanding how this fungus can act as a plant growth-promoting fungus. *P. indica* was discovered in the Indian Thar desert in 1997 by Verma et al. *P. indica* is shown to possess at least six chromosomes and a genome size of about 15.4–24 Mb. *P. indica* lacks host specificity and is cosmopolitan in nature. Sequence analysis of ribosomal DNA (rDNA) regions uncovered that *P. indica* belongs to the family Sebacinaceae, which is recently raised to the order Sebacinales (Weiss et al. 2004; Waller et al. 2005). This basal order of Hymenomycetes (Basidiomycetes) encompasses fungi with longitudinally septate basidia and imperforate parenthesomes (Selosse et al. 2007; Verma et al. 1998, 2001). They also lack cystidia and structures formed during cytokinesis on some basidiomycetous hyphae, the so-called clamp connections. Like other cultivable species of the Sebacinales, *P. indica* forms moniliod hyphae, which look like pearls in a chain. Based on this phenotype and rDNA sequence analyses, the endophyte is placed in the polyphyletic genus *Rhizoctonia* (Selosse et al. 2007).

19.2.1 Root Colonization

It is a wide host root-colonizing endophytic fungus, grows inter- and intracellularly, forms pear-shaped spores within the cortex and extramatrically, and does not invade the endodermis and the aerial parts of the plants. The endophyte promotes nutrient uptake; allows plants to survive under water, temperature, and salt stress; confers (systemic) resistance to toxins, heavy metal ions, and pathogenic

organisms; and stimulates growth and seed production. The fungus can be cultivated on complex and minimal substrates. The interaction of *P. indica* with the model plants *Arabidopsis thaliana* and barley (*Hordeum vulgare* L.) is used to understand the molecular basis of this beneficial plant/microbe interaction. The degree of root colonization is critical for the establishment of the beneficial interaction between symbiotic partners, and over colonization may result in a loss of the benefits. Deshmukh et al. (2006) and Schäfer et al. (2007) reported that *P. indica* requires cell death for the proliferation during mutualistic interaction with barley. The authors monitored *P. indica*-mediated local and systemic resistance against pathogenic fungi and abiotic stress. The root meristem of barley was not colonized by the fungus, and the elongation zone showed mainly intracellular colonization. In contrast, the differentiation zone was heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. The majority of the hyphae were present in dead rhizodermal and cortical cells. This suggests that *P. indica* either actively kills cells or senses cells that undergo endogenously programmed cell death. Fungal growth was strongly inhibited in transgenic barley roots expressing the BAX inhibitor-1, a highly conserved cell death regulator protein in plants. Thus, the endophyte interferes with the host cell death program to form a mutualistic interaction with the plants.

19.2.2 Nutrient Uptake

Mycorrhizal interactions are characterized by a more efficient nutrient uptake from the soil due to a better hyphal penetration into the soil compared to the penetration of the thicker root hairs. The plant delivers phosphoassimilates to the fungus.

19.2.2.1 Effect of *P. indica* on the Phosphorus (P)

Phosphorous is one of the most essential mineral nutrients for plant growth and development and constitutes up to 0.5 % of the dry weight of plant cell. It plays diverse regulatory, structural, and energy transfer roles and consequently is required in significant quantities (Bieleski and Ferguson 1983; Schachtman et al. 1998). In the soil phosphorous presents mainly in the form of sparingly soluble complexes that are not directly accessible to plants. Thus, it is the nutrient that limits crop production throughout the world (Bieleski 1973). In arbuscular mycorrhizal associations, plants acquire phosphate from the extensive network of fine extraradical hyphae of fungus, which extend beyond root depletion zones to mine new regions of the soil (Harrison and Van Buuren 1995).

Plants possess two distinct modes of phosphate uptake, direct uptake by its own transporters and indirect uptake through mycorrhizal associations. Malla et al. (2004) have shown that *P. indica* contains substantial amounts of an acid phosphatase which has the potential to solubilize phosphate in the soil and delivers it to the

host plant. Shahollari et al. (2005) could demonstrate that growth promotion of *Arabidopsis* seedlings is associated with a massive uptake of radiolabeled P from the growth medium. Although these results have to be seen with caution because it is not surprising that bigger plants recruit more P from their environment than smaller plants, this clearly indicates that the fungus can influence the P metabolism. A comprehensive microarray analysis with RNA from *Arabidopsis* roots also uncovered that several (but not all) enzymes involved in P metabolism are upregulated in colonized *Arabidopsis* roots (Oelmüller et al. 2009).

19.2.2.2 Effect of *P. indica* on Nitrogen (N)

Analysis of the N metabolism in colonized *Arabidopsis* roots leaves many questions open. The fungus stimulates the expression of one isoform of nitrate reductase (Nia2) in roots (Sherameti et al. 2005), and microarray analyses uncovered that several carriers (but not all) potentially involved in nitrate uptake are upregulated in the presence of the fungus (Oelmüller et al. 2009). Although this suggests that N uptake and metabolism is stimulated by *P. indica*, direct evidence has not yet been provided. Again, it cannot be excluded that this is a side effect due to the faster growth of colonized seedlings. Sherameti et al. (2005) identified a *cis*-regulatory element in the *Nia2* promoter that is crucial for the expression of the gene and targeted by the homeodomain transcription factor BHL1. The transcription factor forms homo- and heterodimers with various other members of the ovate family (Hackbusch et al. 2005). Ongoing studies suggest that this transcription factor in various interactions with other members of the ovate family is crucial for the beneficial interaction and does control not only the N metabolism but also the genes involved in other aspects of plant/microbe interaction.

19.2.2.3 Effect of *P. indica* on Sugar Metabolism

The supply of the fungus with carbon (C) sources and the faster growth of colonized plants require the breakdown of starch which is deposited in the root amyloplasts. Thus, it is not surprising that one of the major starch-degrading enzymes, the glucan-water dikinase, is activated by the fungus (Sherameti et al. 2005). The promoter of this gene contains the same *cis*-element that is also present in the *Nia2* promoter, and it has been hypothesized that its regulation occurs also by binding BHL1.

19.2.2.4 Effect of *P. indica* on the Sulfur (S)

Recent studies have shown that also the S metabolism is stimulated by *P. indica*. Genes which code for several plastid-localized enzymes required for sulfate reduction are upregulated by *P. indica* in *Arabidopsis* roots, and gene inactivation studies

confirmed that they are required for the beneficial effect on plants (Oelmüller et al. 2009). Taken together, the available data support the concept that *P. indica* influences the primary metabolism in the roots by delivering more nutrients for growth and development. Breakdown of starch may also be beneficial for fungal growth.

19.3 Transformation in Filamentous Fungi

Genetic manipulation using transformation and gene cloning provides the most logically directed approach to dissect and eventually alter the physiology of these filamentous fungi. Toward this end, there has been strong pressure to develop techniques of basic molecular biology suitable for these organisms. The most extensively studied fungi are the unicellular yeast *Saccharomyces cerevisiae* (Neukamm et al. 2002; Kawai et al. 2004) and the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* (Hinnen et al. 1978; Case et al. 1979). The molecular genetic systems of these organisms served as the basis for the development of similar systems in less tractable but economically important fungal species (Timberlake and Marshall 1989).

The first report of transformation of a fungal species mediated by DNA was published by Mishra and Tatum (1973). Growing cultures of inositol-requiring mutant of *N. crassa* were transformed with total DNA of the wild type together with calcium, from the conidia formed on such cultures. It was possible to select phototrophic strains. This pioneering experiment was received with skepticism, and only some years later, Hinnen et al. (1978) reported transformation of *S. cerevisiae* using protoplasts from leu2 mutant by treatment with wild-type DNA in the presence of calcium chloride. The utilization of protoplasts was immediately applied to the filamentous fungi *N. crassa* and *A. nidulans* (Case et al. 1979; Tilburn et al. 1983). Transformation is an essential part of modern fungal research. An effective way to study the fungus–plant interactions is to either disrupt or overexpress plant genes in order to determine their influence within the association. Likewise, homologous or heterologous genes are reintroduced into these organisms. The genes can be silenced or overexpressed to judge their impact. The vital fluorescent marker GFP allows the histological study of the fungal infection with higher resolutions *In Planta*.

Transformation system for model fungi such as yeast, *Neurospora crassa*, (Case et al. 1979) which involves the preparation of fungal protoplasts, delivery of the transforming DNA therein, and selection of the generated transformants is a technique being adapted for use in further filamentous fungi. Protoplast generation can be circumvented in two other methods of transformation: (1) biolistic and (2) *Agrobacterium*-mediated gene transfer. But the vast majority of transformation protocols for filamentous fungi are based on permeabilizing cell membranes with polyethylene glycol (PEG) or electroporation. PEG-mediated protoplast transformation has been the method of choice for many years, and transformation protocols

were developed in numerous species according to the original *Neurospora crassa* protocol (Case et al. 1979).

Alternative methods for direct transformation of spores or hyphae can provide efficient solution to these inherent problems. Electroporation and *Agrobacterium*-mediated transformation (AMT) are the most commonly used alternatives. Both methods can be used to transform spores or hyphae; once working they are highly reproducible, and they have been used to transform a range of fungal species. The unavailability of practical gene transfer system is the single largest obstacle preluding the use of molecular approaches for the genetic improvement of *P. indica*. The development of methods for transforming fungi opens up many possibilities of engineering suitable species for production of specific fungal traits that can be helpful in modern agriculture (Fincham 1989; Hynes 1996). Through genetic transformation systems, it is possible to manipulate the genome of organisms, correlating in vitro studies of purified DNA with biological consequences in vivo. Molecular genetic manipulation of fungi requires the development of plasmid-mediated transformation systems that include (1) introducing exogenous DNA into recipient cells, (2) expression of genes present on the transforming DNA, and (3) stable maintenance and replication of the integrated DNA, leading to expression of the desired phenotypic trait.

Obligate biotrophic AMF are recalcitrant to culture axenically. Moreover, a genetic transformation would always require the presence of their host. Hence, any genetic transformation approaches to decipher fungal genes involved in symbiosis are lacking. In contrast, *P. indica* has the advantage to be easily cultivated in axenic culture. Although *P. indica* is emerging as a model fungus for the study of mutualistic plant–fungus interactions, genetic transformation has not been accomplished yet. The transformation of *P. indica* may open up a new avenue of studying the fungal genes pivotal for the mutualistic interaction and help to understand the genome organization of this asexual endophyte. Several methods such as electroporation and PEG-mediated transformation of isolated protoplasts have been successfully employed for gene transfer into filamentous fungi, which were difficult to transform (Casas-Flores et al. 2004).

19.3.1 Selectable Markers and Vectors for P. Indica Transformation

In general, fungi can be transformed with either nutritional markers which complement an auxotrophic requirement or dominant, selectable antibiotic resistance markers. Most plant pathogens are not well characterized genetically and have few defined auxotrophic markers, or complementary genes that can be used in the former approach. This has led to the development of a variety of antibiotic resistance cassettes that can be used in most pathogens. The antibiotic resistance genes described below have been placed under the control of a variety of promoters and cloned into plasmid vectors that can be conveniently replicated in *Escherichia coli*.

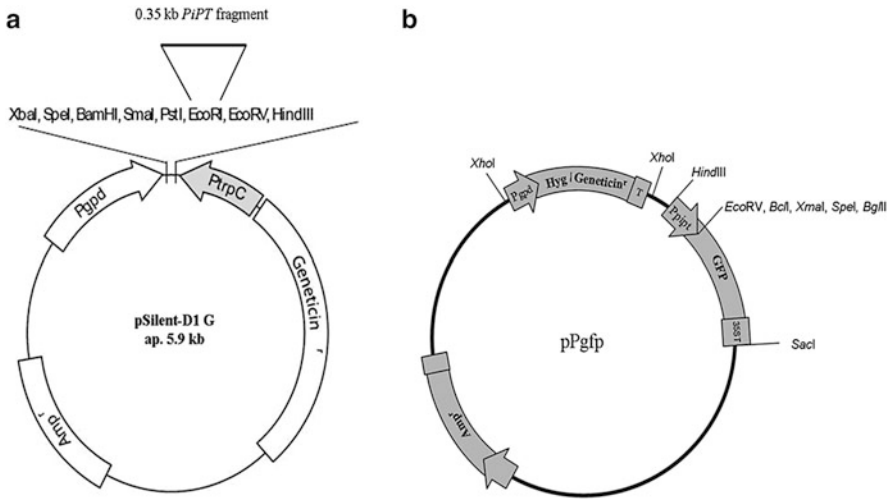

Fig. 19.1 (**a**) Restriction map of pSilent Dual-1G. A 350-bp unique fragment of PiPT gene was cloned into this vector at EcoRI site for silencing PiPT gene. (**b**) Restriction map of pPgfp vector

The most commonly used fungal promoter for transformation is *A. nidulans* gpdA (glyceraldehydes-3-phosphate dehydrogenase) promoter (Punt et al. 1987), although now it is widely accepted that use of a promoter from the organism to be transformed will aid both expression of the selectable marker gene and transformation frequency.

Hygromycin B is an aminoglycoside antibiotic, which inhibits growth of prokaryotic microorganisms (bacteria), eukaryotic microorganisms (yeasts), and mammalian cells by inhibiting protein synthesis at the translocation step, causing misreading of mRNA. Minimum inhibitory concentration (MIC) for hygromycin B is against most fungi in the range of 50–250 μg/ml, although higher amounts are needed to inhibit growth of some species. In some cases of fungal transformation, geneticin (G418) has been used successfully; however, MIC for G418 is higher in several fungal species, and selection takes about 2–3 weeks on low-carbon source. We have used both the marker for *P. indica*. MIC of both hygromycin B (pPgfp vector) and geneticin (pSD1G and pPgfp vector) for *P. indica* is 30 μg/ml and 700 μg/ml, respectively (see Fig. 19.1 for the restriction map of pPgfp and pSD1G). Both of the selection markers have been used for the transformant selection using different methods for transformation in *P. indica*, namely, electroporation.

19.3.2 Electroporation: A Common Transformation Method

Electroporation is a high-voltage electric pulse that creates a population of small, aqueous pores in the cell membrane through which DNA can enter the cell by

diffusion or electrophoretically (Weaver 1995). The technique can be used to transform protoplasts as well as mycelia or spores (Chakraborty and Kapoor 1990; Kothe and Free 1995; Kuo and Huang 2008; Richey et al. 1989). For spore transformation, the spores are usually pre-germinated or incubated with a mild concentration of cell wall degrading enzymes (Chakraborty et al. 1991; Sanchez and Aguirre 1996; Robinson and Sharon 1999). Recently, electroporation has become a valuable technique for the introduction of nucleic acids into both eukaryotic and prokaryotic cells (Miller et al. 1988; Forster and Neumann 1989). Intact cells, as wells as the cells treated with cell wall degrading enzymes, are amenable to electroporation (Fromm et al. 1985; Miller et al. 1988; Shigekawa and Dower 1988). When a cell is exposed to an electric field, the membrane components become polarized, and a potential difference develops across it. If the voltage exceeds a threshold level, the membrane breaks down in localized areas and the cell becomes permeable to exogenous molecules (Shigekawa and Dower 1988).

There are some reports about electroporation in filamentous fungi, and the method is likely to become more popular, mainly due to its simplicity and reproducibility. Ward et al. (1989) reported the transformation of protoplasts of *A. awamori* and *A. niger* by electroporation, obtained transformation frequencies similar to those obtained with PEG. Richey et al. (1989) transformed protoplasts of *Fusarium solani* and *A. nidulans*. These authors reported transformation frequencies lower than those obtained by standard transformation methods. Goldman et al. (1990) reported electroporation of *Trichoderma harzianum* using a combination of OSCs and PEG and obtained a fourfold higher transformation frequency when 1 % (w/v) PEG was present in the medium during the delivery of the electric shock. Intact cells have been used for the electroporation of filamentous fungi. Thus, it is essential to take into account the parameters like cell size, cell viability aftershock delivery, and conductivity of the electroporation media.

The electroporation-based transformation procedure, originally developed here for use with *P. indica*, is characterized with spores and filament of fungus. Use of spore for the electroporation makes the method more feasible and ensures the purity of single-spore generated transformants. Although intact spores, with thick wall, are not suitable unless spheroplasts are formed first, spores during early stages of germination are excellent recipients for exogenous DNA, following brief pretreatment with a suitable enzyme such as β-glucuronidase. Combination of several other chemicals was used to weaken the cell wall. Pretreatment with thiol compound such as DTT and DMSO alone or in combination does not result in higher transformation efficiency. However, addition of β-mercaptoethanol with β-glucuronidase enhances transformation efficiency of electroporation. All above treatments were done in medium containing different osmoprotectant such as sorbitol and mannitol alone or in combination with HEPES. Elimination of osmoprotectant from the digestive medium and electroporation buffer drastically reduces the transformation efficiency. Electroporation was done on three different volt setups which are 7,500, 10,000, and 12,500 V/cm. No transformants were observed on 7,500 and 10,000 V/cm; however, at 12,500 V/cm, several

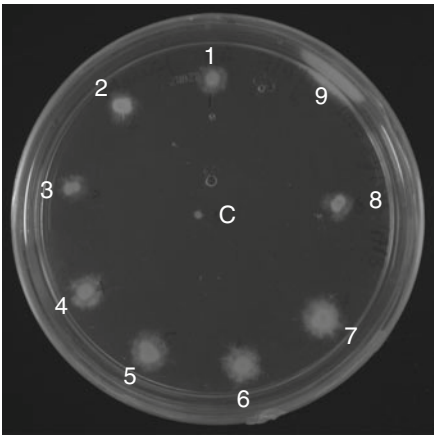

Fig. 19.2 Secondary selection of transformed *P. indica* colonies. Electroporated mycelia of *P. indica* were subjected to primary selection on KF media added with 1,000 μg/ml G418 at high concentration of phosphate (10 mM). Several colonies appeared and were again subjected to secondary selection with 1,000 μg/ml G418 at low level of carbon source (2 mM glucose) and high concentration of phosphate (10 mM). Out of nine colonies, one to eight were regenerated, while the ninth colony disappeared. In this selection, non-transformed *P. indica* colony (C) was used as a negative control

drug-resistance transformed colonies appeared. Pulse duration was kept 1, 5, and 10 ms; no transformants were observed at 1 ms, while 5 and 10 ms pulse duration was optimum.

Optimized field and pulse duration were used to analyze relation between DNA concentration and transformation efficiency. It was observed that 2–7 μg DNA gives optimum transformation. It is necessary to allow electroporated spores or mycelia to recover for efficient transformation. Addition of sorbitol at a final concentration 1.2 M, in recovery media, resulted in higher transformation efficiency. Because, *P. indica* is a slow-growing fungus, it requires incubating the electroporated spores for 6–12 h at 30 °C in the recovery medium containing 1.2 M sorbitol. The electroporated suspension was spread on MAM plate containing 1.5 % agar. After the surface has dried, overlay the plates with 5–6 mL of soft agar (0.7 % agar + MAM medium containing 1,000 μg/mL geneticin), and incubate the plates at room temperature. Geneticin-resistant colonies appeared within 2–3 weeks. No colony was observed on control electroporated mix in the absence of plasmid DNA and plated subsequently on geneticin-containing medium. Transformation efficiencies are estimated on the basis of the number of geneticin-resistant colonies recovered/μg of input plasmid DNA. A final selection of geneticin-resistant colonies was performed on MAM media plates containing 1,000 μg/mL geneticin and low-carbon source (2 g/L Glucose, Fig. 19.2); further PCR-based confirmation was done for the integrated DNA. Using this protocol, we have successfully transformed *P. indica* with RNAi construct of PiPT gene and routinely generated stable *P. indica* transformants (Yadav et al. 2010).

Table 19.1 Experimental conditions and results of electrotransformation of *P. indica* with two different dominant selective markers

Conditions	Results	
Number of Chlamydospores	$3.0–6.0 \times 10^6$	
Percent viability	30–55	
β-glucuronidase	+	
Selectable marker	Hygromycin B	Geneticin
Transformation efficiency (stable transformants/μg DNA)	4–120	10–50

19.4 Conclusion

Transformation of *P. indica* was performed using the recent technological advance of *electroporation*-mediated transformation system developed for filamentous fungi. In case of *P. indica*, protocol we have used routinely generates stable transformants (Table 19.1). We have used this system successfully in transforming plasmid containing RNAi construct of *PiPT* gene of *P. indica*, and this has resulted in the first-reported RNAi in *P. indica*. Another transformation system has also been developed (Zuccaro et al. 2009) for *P. indica* which is complicated in routine use in comparison to electroporation. We expect that electroporation-based transformation can be readily adopted to genetically manipulate *P. indica* so that its use in sustainable agriculture can be further explored for crop improvement.

Acknowledgments We are very thankful to Dr. Hitoshi Nakayashiki, Kobe University, Japan, for providing pSD-1G vector. This project was financed by Council of Scientific and Industrial Research, Government of India. MK is thankful to ICMR and JNU for providing research fellowship. AKJ and MD also acknowledge DST-Purse Grant and UGC Resource Networking grant.

References

Azcón-Aguilar C, Barea JM (1996) Arbuscular mycorrhizas and biological control of soil-born plant pathogens - an overview of the mechanisms involved. Mycorrhiza 6:457–464

Baltruschat H, Fodor J, Harrach BD, Niemczyk E, Barna B, Gullner G, Janeczko A, Kogel KH, Schäfer P, Schwarczinger I, Zuccaro A, Skoczowski A (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytol 180:501–510

Bieleski RL (1973) Phosphate pools, phosphate transport, and phosphate availability. Annu Rev Plant Physiol 24:225–252

Bieleski RL, Ferguson IB (1983) Physiology and metabolism of phosphate and its compounds. In: Bieleski AL (ed) Encyclopedia of plant physiology. Springer, New York

Borowicz VA (2001) Do arbuscular mycorrhizal fungi alter plant pathogen relations? Ecology 82:3057–3068

Casas-Flores S, Rosales-Saavedra T, Herrera-Estrella A (2004) Three decades of fungal transformation: novel technologies. Methods Mol Biol 267:315–325

Case ME, Schweizer M, Kushner SR, Giles NH (1979) Efficient transformation of Neurospora crassa by utilizing hybrid plasmid DNA. Proc Natl Acad Sci USA 76:5259–5263

Chakraborty BN, Kapoor M (1990) Transformation of filamentous fungi by electroporation. Nucleic Acids Res 18:6737

Chakraborty BN, Patterson NA, Kapoor M (1991) An electroporation based system for high-efficiency transformation of germinated conidia of filamentous fungi. Can J Microbiol 37:858–863

Chapin FS III, Zaveleta ES, Eviner VT, Naylor RL, Vitousek PM, Lavorel S, Reynolds HL, Hooper DU, Sala OE, Hobbie SE, Mack MC, Diaz S (2000) Consequences of changing biotic diversity. Nature 405:234–242

Deshmukh S, Hückelhoven R, Schäfer P, Imani J, Sharma M, Weiss M, Waller F, Kogel KH (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Deshmukh S, Kogel KH (2007) *Piriformospora indica* protects barley from root rot caused by *Fusarium graminearum*. J Plant Dis Protect 14:263–268

Fincham JR (1989) Transformation in fungi. Microbiol Rev 53:148–170

Food and Agriculture Organization of the United Nations (2004) The state of food and agriculture. http://www.fao.org/documents/show_cdr.asp?urLfile=/docrep/006/Y5160E/Y5160E00.HTM

Forster W, Neumann E (1989) Gene transfer by electroporation. In: Neumann E, Sowers AE, Jordan CA (eds) Electroporation and electrofusion in cell biology. Plenum, New York, pp 299–315

Fromm M, Taylor V, Walbot L (1985) Expression of genes transferred into monocot and, dicot plant cells by electroporation. Proc Natl Acad Sci USA 82:5824–5828

Gernns H, Von Alten H, Poehling HM (2001) Arbuscular mycorrhiza increased the activity of a biotrophic leaf pathogen is a compensation possible. Mycorrhiza 11:237–243

Goldman GH, Van Montagu M, Herrera-Estrella A (1990) Transformation of *Trichoderma harzianum* by high-voltage electric pulse. Curr Genet 17:169–174

Hackbusch J, Richter K, Müller J, Salamini F, Uhrig JF (2005) A central role of *Arabidopsis thaliana* ovate family proteins in networking and subcellular localization of 3-aa loop extension homeodomain proteins. Proc Natl Acad Sci USA 102:4908–4912

Harrison MJ, Van Buuren ML (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature 378:626–629

Hinnen A, Hicks JB, Fink GR (1978) Transformation of yeast. Proc Natl Acad Sci USA 75:1929–1933

Hynes MJ (1996) Genetic transformation of filamentous fungi. J Genet 75:297–311

Kawai S, Pham TA, Nguyen HT, Nankai H, Utsumi T, Fukuda Y (2004) Molecular insights on DNA delivery into *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 317:100–107

Kothe GO, Free SJ (1995) Protocol for the electroporation of *Neurospora* spheroplasts. Fungal Genet Newsl 43:31–32

Kumar M, Yadav V, Tuteja N, Johri AK (2009) Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. Microbiology 155:780–790

Kumar M, Sharma R, Jogawat R, Singh P, Dua M, Tuteja N, Varma AK, Oelmuller R, Johri AK (2012) Role of *Piriformospora* indica in Abiotic stress tolerance. In: Tuteja N, Gill SS, Tiburcio AF, Tuteja R (eds) Improving crop resistance to abiotic stress. Wiley Blackwell, pp 543–558

Kuo CY, Huang CT (2008) A reliable transformation method and heterologous expression of β-glucuronidase in *Lentinula edodes*. J Microbiol Methods 72:111–115

Malla R, Prasad R, Kumari R, Giang PH, Pokharel U, Oelmüller R, Varma A (2004) Phosphorus solubilizing symbiotic fungus: *Piriformospora indica*. Endocytobiosis Cell Res 15:579–600

Miller JF, Dower WJ, Tompkins LS (1988) High voltage electroporation of bacteria. Genetic transformation of Campylobacter jejuni with plasmid DNA. Proc Natl Acad Sci USA 85:856–860

Mishra NC, Tatum EL (1973) Non-mendelian inheritance of DNA induced inositol independence in *Neurospora*. Proc Natl Acad Sci USA 70:3875–3879

Neukamm B, Stahl U, Lang C (2002) Endocytosis is involved in DNA uptake in yeast. Biochem Biophys Acta 1572:67–76

Newman EI, Reddell P (1987) The distribution of mycorrhizas among families of vascular plants. New Phytol 106:745–751

Oelmüller R, Sherameti I, Tripathi S, Varma A (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CA (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117–124

Richey MG, Marek ET, Schardl CL, Smith DA (1989) Transformation of filamentous fungi with plasmid DNA by electroporation. Phytopathology 79:844–847

Robinson M, Sharon A (1999) Transformation of the bioherbicide *Colletotrichum gloeosporioides* f.sp. *aeschynomene* by electroporation of germinated conidia. Curr Genet 36:98–104

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302

Sanchez O, Aguirre J (1996) Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. Fungal Genet Newsl 43:48–51

Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant Physiol 116:447–453

Schäfer P, Khatabi B, Kogel KH (2007) Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism. FEMS Microbiol Lett 275:1–7

Schäfer P, Pfiffi S, Voll LM, Zajic D, Chandler PM, Waller F, Scholz U, Pons-Kühnemann J, Sonnewald S, Sonnewald U, Kogel KH (2009) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. Plant J 59:461–474

Selosse MA, Setaro S, Glatard F, Richard F, Urcelay C, Weiss M (2007) Sebacinales are common mycorrhizal associates of *Ericaceae*. New Phytol 174:864–878

Serfling A, Wirsel SGR, Lind V, Deising HB (2007) Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field conditions. Phytopathology 97 (4):523–531

Shahollari B, Varma A, Oelmüller R (2005) Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol 162:945–958

Shaul O, Galili S, Volpin H, Ginzberg I, Elad Y, Chet I, Kapulnik Y (1999) Mycorrhiza induced changes in disease severity and PR protein expression in tobacco leaves. Mol Plant Microbe Interact 12:1000–1007

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. J Biol Chem 280:2641–2647

Shigekawa K, Dower WJ (1988) Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. Biotechniques 6:742–751

Tilburn J, Scazzocchio C, Taylor GG, Zabicky-Zissman JH, Lockingtonm RA, Davies RW (1983) Transformation by integration in *Aspergillus nidulans*. Gene 26:205–221

Timberlake WE, Marshall MA (1989) Genetic engineering of filamentous fungi. Science 244:1313–1317

Varma A, Verma S, Sudha N, Sahay S, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sahay S, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Hock B, Maier W, Walter M,

Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) Mycota IX. Springer Series, Germany, pp 123–150

Verma SA, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:898–905

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, Von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Ward M, Kodama KH, Wilson LJ (1989) Transformation of Aspergillus awamori and A. niger by electroporation. Exp Mycol 13:289–293

Weaver JC (1995) Electroporation theory. Concepts and mechanisms. Methods Mol Biol 47:1–26

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of hetero-basidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in the phosphate transport to the host plant. J Biol Chem 285:26532–26544

Zuccaro A, Basiewicz M, Zurawska M, Biedenkopf D, Kogel KH (2009) Karyotype analysis, genome organization, and stable genetic transformation of the root colonizing fungus *Piriformospora indica*. Fungal Genet Biol 46:543–550

Part V
Experimental Protocols for *Piriformospora indica* Studies

Chapter 20
Standardized Conditions to Study Beneficial and Nonbeneficial Traits in the *Piriformospora indica*/*Arabidopsis thaliana* Interaction

Joy Michal Johnson, Irena Sherameti, Pyniarlang L. Nongbri, and Ralf Oelmüller

20.1 Introduction

The vast majority of plants live in symbiotic interaction with mycorrhizal fungi. The fungus delivers soil nutrients to the plant (Javot et al. 2007a, b; Bucher et al. 2009; Adesemoye and Kloepper 2009) while the plant is responsible for the photoassimilates to the fungus (Nehls 2008; Nehls et al. 2010). Mycorrhizal interactions are difficult to investigate at the molecular level because most of the classical model plants do not form mycorrhizal association. As an alternative, endophytic interaction of axenically cultivable *Piriformospora indica* with the model plant *Arabidopsis thaliana* might help to understand the basis of the beneficial interactions between two symbionts.

We focus on the identification of *Arabidopsis* mutants which are impaired in establishing or maintaining the interaction in a beneficial mode. For those studies, reproducible and quantitative cocultivation parameters are required. Therefore, we have developed standardized (co)cultivation conditions for the two symbionts, which are described here in details. The mode of interaction and the reproducibility of the data are highly dependent on the quality of the biological material and the cocultivation conditions. Most important parameters are the age and the density of the fungal mycelium, the age of the seedlings and plants, the ratio between the two symbiotic partners, and the cocultivation conditions like temperature, humidity, light intensity, spectral distribution, and photoperiod (Shahollari et al. 2007a; Sherameti et al. 2008, 2010; Vadassery et al. 2008, 2009a, b; Vadassery and Oelmüller 2009; Sun et al. 2010; Camehl et al. 2010, 2011; Johnson et al. 2011b; Lee et al. 2011).

J. Michal Johnson (✉) • I. Sherameti • P.L. Nongbri • R. Oelmüller
Institute of General Botany and Plant Physiology, Friedrich-Schiller University, Dornburger Str. 159, 07743 Jena, Germany
e-mail: joymkau@gmail.com; irena.sherameti@uni-jena.de; pyndrop@gmail.com; b7oera@hotmail.de

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_20,

We have standardized the growth medium, since even minor changes in the medium have severe consequences for the interaction. Cocultivation medium is designed such that both organisms can grow in harmony. On plant medium with high levels of nutrients and sucrose, the benefits for plant performance are less pronounced compared to cocultivation conditions on minimal medium (Peškan-Berghöfer et al. 2004; Shahollari et al. 2005, 2007b; Sherameti et al. 2005, 2008). None of the two symbionts should "overgrow and dominate" the partner. Furthermore, shortage in any important nutrients such as N, P, S, or Fe in the medium results initially in a better plant performance in the presence of the fungus, while during later phases of the interaction and after transfer to soil, the plants start to suffer and show growth retardation instead of growth promotion. This can be associated by a shift from mutualism to parasitism in its extreme (Kaldorf et al. 2005; Johnson and Oelmüller 2009; Oelmüller et al. 2009). Another important parameter for an optimal interaction between the two symbionts is the pH (6.5–7.0) of the medium or the soil. In pot culture and soil experiments, the amount of the inoculum and the growth conditions are crucial. Greenhouse experiments are more variable than experiments in temperature- and light-controlled growth chambers.

The standardized cocultivation conditions allow us to identify genes, proteins, and other biomolecules which participate in establishing benefits for the plants and/or maintaining the interaction in a mutually beneficial mode. We could successfully transfer these conditions to different *Arabidopsis* ecotypes and other plant species including tobacco (Johnson et al. 2011b; Sherameti et al. 2005) and Chinese cabbage (Johnson et al. 2011b; Lee et al. 2011; Sun et al. 2010). The beneficial effects of *P. indica* have also been demonstrated in maize, tobacco, *Artemisia*, parsley, *Becopa* and Poplar (Varma et al. 1999; 2001), barley (Neumann et al. 2005; Waller et al. 2005), *Coleus* (Das et al. 2012) and *Centella* (Satheesan et al. 2012).

20.2 Growth Conditions of Fungi

20.2.1 Beneficial Fungus

20.2.1.1 Growth Conditions of *P. indica*

The root-colonizing fungus *P. indica* (Fig. 20.1a, b) is cultured in a modified Kaefer Medium (KM; Table 20.1, Hill and Käfer 2001) which is a rich sugar and protein medium with slightly acidic to neutral conditions (pH 6.5–7.0). Other fungi require their own specific medium for their growth. *Trichoderma* spp., for example, a fungus widely used for biological control of fungal plant diseases, grows better in *Trichoderma* specific medium (TSM, Chet and Elad 1982).

Every 4 weeks a *P. indica* plug 5 mm in diameter is subcultured to the middle of a fresh KM Petri dish in the dark at 22–24°C in a temperature-controlled growth chamber (for details see Johnson et al. 2011b).

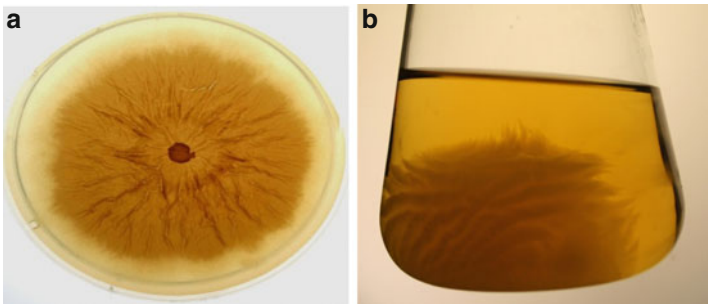

Fig. 20.1 Four-week-old *P. indica* on KM plate (**a**) and 16–18 day old in KM broth (**b**)

20.2.1.2 Maintaining "Root Colonization Efficiency" of *P. indica*

The fungus loses its "root colonization efficiency" after repeated subculturing on semisynthetic medium. To maintain this efficiency, the fungus must be periodically inoculated to the roots of host plants and re-isolated from the internally colonized roots (for details see Johnson et al. 2011b).

Cocultivation of *A. thaliana* seedlings with *P. indica* for 18–20 days is done as described in Sect. 20.4.1. After observing root colonization under the microscope the colonized roots are cut into pieces of 2–3 mm and surface-sterilized with 0.1% mercuric chloride for 1 min and washed four times with sterilized dH_2O and then the roots are treated with sterilium (for 100 ml solution: 4 ml laurylsarcosin 20%; 32 ml klorix; 64 ml dH_2O) for 1 min and then wash four times with sterilized dH_2O. In the next step the root pieces are placed on KM plates and incubated in the dark at 22–24°C in a temperature-controlled growth chamber for 5–7 days. Once the fungal growth is initiated, it should be immediately subcultured by taking single hyphal tip to fresh KM plate.

20.2.1.3 Preparation of Active Components from *P. indica*

An easily visible effect of *P. indica* on plants is the better growth of seedlings and the whole plant. This response can also be achieved with exudates from the fungus such as the biologically active cell wall extract, a water diffusible fraction and the culture filtrate (Lee et al. 2011; Vadassery et al. 2009a). This has a number of experimental advantages since it does not involve a living and growing fungus. The fungal active components are added to the medium before plating the seeds or directly to roots while performing cocultivation experiments (Fig. 20.2).

Table 20.1 Kaefer Medium for *P. indica* (Hill and Käfer 2001)

Components for 1 l	
D-glucose	0.0 g
Peptone/tryptone	2.0 g
Yeast extract	1.0 g
Casein hydrolysate	1.0 g
[1]Macronutrient mix	50.0 ml
[2]Micronutrient mix	10.0 ml
[3]Fe-EDTA	1.0 ml
Agar	10.0 g
• Adjust pH to 6.5 with 10N KOH	
• Autoclave at 121°C for 20 min	
• Add 1 ml/l filter-sterilized [4]Vitamin mix (at temperature 45–50°C)	
[1]Macronutrient mix/liter stock solution	
$NaNO_3$	12.0 g
KCl	10.4 g
$MgSO_4 \cdot 7H_2O$	10.4 g
KH_2PO_4	30.4 g
All components are dissolved in dH_2O and then stored at 4°C	
[2]Micronutrient mix/liter stock solution	
$ZnSO_4 \cdot 7H_2O$	2.2 g
H_3BO_3	1.1 g
$MnSO_4 \cdot 4H_2O$	0.5 g
$CoCl_2 \cdot 5H_2O$	0.16 g
$CuSO_4 \cdot 5H_2O$	0.16 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.11 g
All components are dissolved in dH_2O and then stored at 4°C	
[3]Fe-EDTA	
• Add 2.5 g $FeSO_4 \cdot 7H_2O$ in 400 ml dH_2O	
• Add 3.36 g $Na_2EDTA \cdot 2H_2O$	
• Heat to boil in the microwave	
• Stir for about 30 min while cooling	
• Bring to the final volume of 450 ml	
Vitamin mix/liter stock solution	
Thiamin	0.1 g
Glycin	0.04 g
Nicotinic acid	0.01 g
Pyridoxine	0.01 g
$CuSO_4 \cdot 5H_2O$	0.16 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.11 g
All components are dissolved in dH_2O, filter-sterilized, and then stored as aliquots of 1 ml at −20°C	

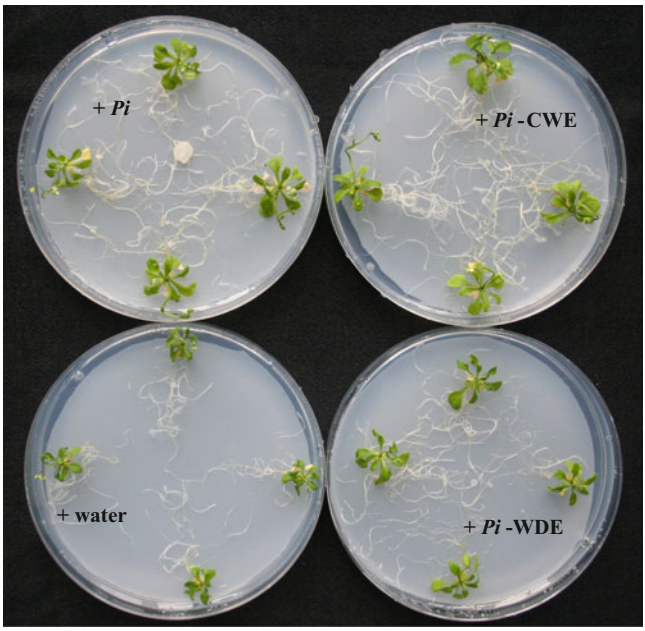

Fig. 20.2 *A. thaliana* seedlings after 14 days cocultivation on PNM medium with the *P. indica* plug, the crude cell wall extract (*Pi*-CWE) and water diffusible extract (*Pi*-WDE). *Pi*-CWE and *Pi*-WDE promote growth of *A. thaliana* seedlings as the fungus does. Water is used as control

Preparation of Cell Wall Extract (CWE) from *P. indica*

The fungal cell wall extract is prepared as described by Anderson-Prouty and Albersheim (1975) and Johnson et al. (2011a). Fungal mycelia from 16–18-day-old liquid culture (Fig. 20.1b) are harvested and filtered through four layers of nylon membrane (Sefar AG, Heiden, Switzerland, mesh size 70 μm) and washed five times with sterilized dH_2O. Homogenization is done with sterilized dH_2O (w/v 1:5) and the homogenate is filtered through four layers of nylon membrane. The collected residue is re-homogenized (three times with sterilized dH_2O, twice with chloroform/methanol 1:1 and finally twice in acetone). In the next step, the residue is air dried for 2 h (the mycelial cell wall is recovered). 1 g cell wall is suspended in 100 ml dH_2O and autoclaved for 30 min at 121 °C. The active fungal fraction which is highly thermostable is released. After cooling, the extract is filtered through four layers of nylon membrane, then two layers of filter paper, and finally filter-sterilized (pore size: 0.22 μm). To get the active fraction, the crude CWE is further purified by passing through a Reverse Phase Supelclean LC-18 Cartidges (Sigma-Aldrich, Germany).

Preparation of Water-Diffusible Extract (WDE) from *P. indica*

P. indica mycelium propagated in liquid KM medium for 16–18 days is filtered through four layers of sterilized nylon membrane, collected in a funnel and washed intensively seven times with sterilized dH_2O. After air drying, the mycelium is resuspended in sterilized dH_2O and incubated at 22°C in a horizontal rotating shaker at 60 rpm. After 48 h, the mycelium is filtered through four layers of nylon membrane, then two layers of filter paper, and finally filter-sterilized (pore size: 0.22 μm). The crude water-diffusible fraction is further purified by passing through a Reverse Phase Supelclean LC-18 Cartidges (Sigma-Aldrich, Germany).

Culture Filtrate

The culture filtrate is obtained from a 20-day-old liquid culture. The fungal material is filtered through four layers of sterilized nylon membrane and the culture filtrate is then spun down for 50 min at 40,000 × *g* to remove all fungal hyphae and spores. The filter-sterilized supernatant is used for the biological assays.

20.3 Growth Conditions of *Arabidopsis thaliana*

A. thaliana wild type seeds (ecotype Columbia 0) are surface-sterilized with sterilium for 8 min followed by a series of washing with sterilized dH_2O for 7–8 times and placed on Petri dishes containing MS medium (0.3% gelrite, pH 5.6–5.8, Murashige and Skoog 1962). After cold treatment at 4°C for 48 h, plates are incubated for about 10 days at 22°C under continuous illumination (100 μmol m^{-2} s^{-1}). For cocultivation experiments, only the seedlings which are equally grown are used.

20.4 Cocultivation of *A. thaliana* and *P. indica*

20.4.1 Short Term Cocultivation

Cocultivation of *Arabidopsis* seedlings with *P. indica* is done as described by Johnson et al. (2011b). For cocultivation experiments we use modified PNM medium (Table 20.2) which allows appropriate growth of both organisms (Peškan-Berghöfer et al. 2004; Shahollari et al. 2005, 2007b; Sherameti et al. 2005, 2008).

Table 20.2 Modified PNM medium used for cocultivation of *A. thaliana* with *P. indica*

Components for 1 l	
KNO_3	5.0 mM
$MgSO_4 \cdot 7H_2O$	2.0 mM
$Ca(NO_3)_2$	2.0 mM
[1]Fe-EDTA/liter	2.5 ml
[2]Micronutrient-mix/liter	1.0 ml
Agar (Serva)/liter	10.0 g
• Sterilize at 121°C for 20 min	
• Adjust pH (under sterile conditions) to 5.6 by adding	
2.5 ml filter-sterilized 1M KH_2PO_4	
(Use sterile filter 0.22 μM)	
[1]Fe-EDTA	
• Add 2.5 g $FeSO_4 \cdot 7H_2O$ in 400 ml dH_2O	
• Add 3.36 g $Na_2EDTA \cdot 2H_2O$	
• Heat to boil in the microwave	
• Stir for about 30 min while cooling	
• Bring to the final volume of 450 ml	
[2]Micronutrient mix	
H_3BO_3	70.0 mM
$MnCl_2 \cdot 4H_2O$	14.0 mM
$CuSO_4 \cdot 5H_2O$	0.5 mM
$ZnSO_4 \cdot 7H_2O$	1.0 mM
$Na_2MoO_4 \cdot 2H_2O$	0.2 mM
NaCl	10.0 mM
$CoCl_2 \cdot 6H_2O$	0.01 mM

Before cocultivation the following steps are needed:

Step I *A. thaliana* seedlings are grown on MS medium for 12 days (4°C for 48 h followed by 10 days under continuous illumination at 100 μmol m^{-2} s^{-1} in a temperature-controlled room).

Step II A *P. indica* plug from a 3–4-week-old fungal lawn is grown for 7 days on a sterile membrane on the top of PNM medium. The membrane (Sefar nitex 03-70/33; pore size 65–70 μm, mesh count 81 cm^{-1}; Sefar GmbH, Switzerland) was soaked in dH_2O, heated in the microwave for about 3 min, spreaded without folding in aluminum sheet, and sterilized in the autoclave before transferring on the top of the Petri dish. The fungal plug without medium is placed on the center of the PNM plate. The plates are incubated for 7 days at 22°C in a temperature-controlled Biotron under 12/12 h light/dark illumination. By this time the mycelia of the fungus grow about 4 cm in diameter.

Cocultivation: 4–8 twelve day-old *A. thaliana* seedlings which were grown on MS medium (*Step I*) are transferred to one Petri dish with PNM medium on which a *P. indica* fungal plug was grown for 7 days (*Step II*). Plates are sealed with parafilm and incubated at 22°C in a temperature-controlled room for 6–14 days. One plug from the uninoculated KM plate serves as the control. The plates are incubated at different light intensities viz., low light (30 μmol m^{-2} s^{-1}), normal light

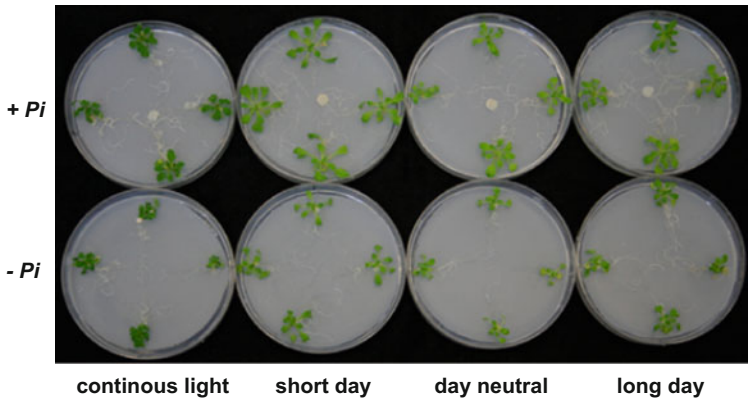

Fig. 20.3 *A. thaliana* seedlings after 10 days cocultivation with *P. indica* on PNM medium under different light/dark conditions

(80 µmol m^{-2} s^{-1}), high light (250 µmol m^{-2} s^{-1}), photosystem I, photosystem II, short day (8 h light/16 h dark), long day (16 h light/8 h dark), and day neutral (12 h light/12 h dark) with illumination from the top (Fig. 20.3). Growth promotion after *in vitro* cocultivation with *P. indica* is observed for different plants such as *A. thaliana*, Chinese cabbage, *Phleum pretense* and *Nicotiana tabaccum* (Johnson et al. 2011b; Lee et al. 2011; Sun et al. 2010). Fresh weights are determined for shoots and roots at 6, 10, and 14 day after cocultivation. For each treatment at least 24 seedlings per line are used and 5–7 independent biological experiments are performed.

Figure 20.4 describes the cocultivation steps between *A. thaliana* seedlings and *P. indica* on PNM medium.

20.4.2 Long Term Cocultivation

Sterilized garden soil–vermiculite mix (9:1), vermiculite, and expanded clay are used for the long term cocultivation of *A. thaliana* with *P. indica*. Seedlings are cocultivated for 15–20 days with/without *P. indica* on Petri dishes with PNM medium as described above (Sect. 20.4.1). Before transferring to soil, the roots are examined under the microscope to check the fungal colonization. Colonized plants and uncolonized controls are transferred to pots with soil–vermiculite mix

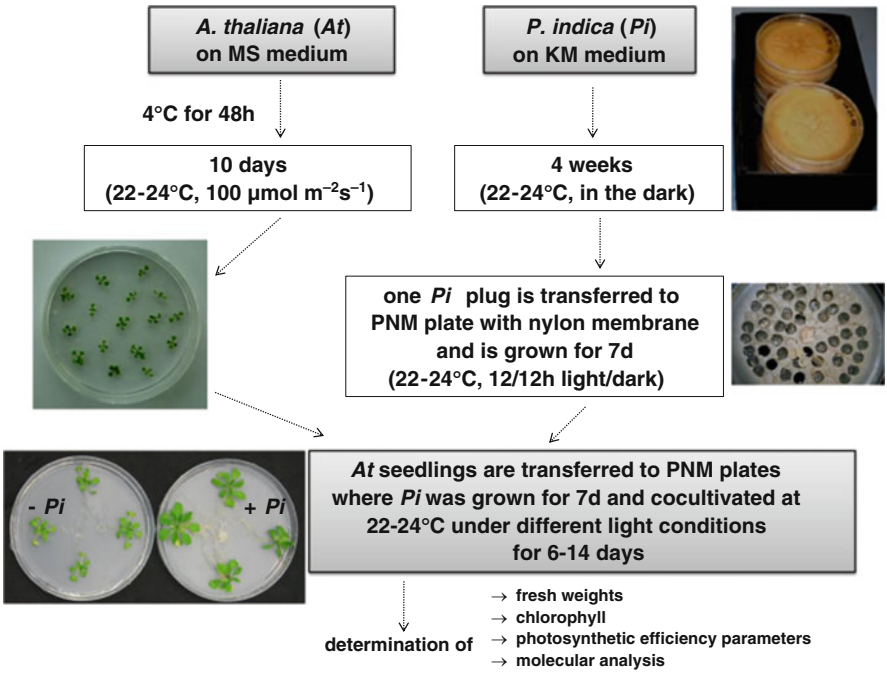

Fig. 20.4 A scheme describing cocultivation steps between *A. thaliana* seedlings and *P. indica*

(9:1) or vermiculite and kept in separate trays at 22°C in a temperature-controlled growth chamber, light intensity 80 µmol m^{-2} s^{1}, under short day conditions (8 h light/16 h dark) until flowering (Fig. 20.5a) followed by long day conditions (16 h light/8 h dark, Fig. 20.5b). Plants are watered weekly.

The size of the plants, the number and size of leaves, flowering, number of pods/plant, and number of seeds/pods are monitored after 2, 4, 6, and 8 weeks. Seeds are collected in the Aracon tubes with tripod and quantified as gram seed/plant. Various chlorophyll fluorescence parameters viz., maximum quantum yield of photosystem II, effective quantum yield of photosystem II, proportion of open photosystem II, and non photochemical quenching using a Fluorocam are measured (Oelmüller and Briggs 1990; Maxwell and Johnson 2000).

Long term cocultivation of *P. indica* with *A. thaliana*, Chinese cabbage and *Nicotiana tabaccum* is done in pots with soil, soil–vermiculite mix, and expanded clay induces growth promotion which is demonstrated by Johnson et al. (2011b); Lee et al. (2011); Sun et al. (2010) and Sherameti et al. (2005).

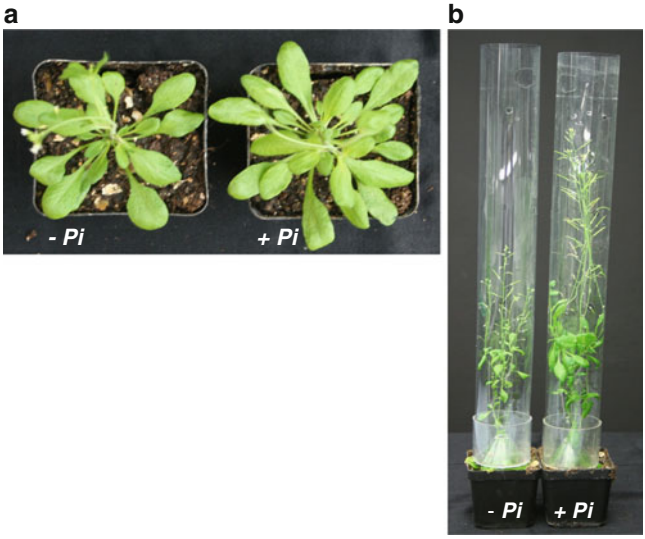

Fig. 20.5 *A. thaliana* plants after 4 (**a**) and 8 weeks (**b**) cocultivation with *P. indica* in soil. Before transferred to pots *A. thaliana* seedlings were grown with/without *P. indica* for 2 weeks on PNM medium

Table 20.3 Potato Dextrose Agar (PDA) medium for *Alternaria brassicae* and dual culture experiment (Bains and Tewari 1987; Dungia and Sinclair 1995)

Components for 1 l	Amounts
Peeled and sliced potato	200.0 g
Dextrose (D-glucose)	20.0 g
Agar	10.0 g

20.5 Pathogenic Fungus

P. indica protects *Arabidopsis* against pathogens by conferring resistance to host plant or inhibiting the growth of the pathogenic fungi (Fakhro et al. 2010; Knecht et al. 2010). We use as pathogen the necrotrophic fungus *Alternaria brassicae* because it can infect both roots and leaves of the plants (Thomma et al. 1999; Dungia and Sinclair 1995; Bains and Tewari 1987).

20.5.1 Growth Conditions of Alternaria brassicae

20.5.1.1 Culturing *A. brassicae*

Many pathogenic fungi including *A. brassicae* grow well in Potato Dextrose Agar medium [PDA (Table 20.3)] at pH 6.5–7.0 (Bains and Tewari 1987; Dungia and

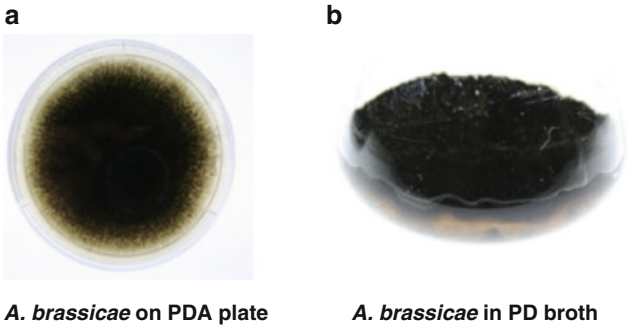

Fig. 20.6 Two-week-old *A. brassicae* on PDA plate (**a**) and PD broth (**b**)

Sinclair 1995). For preparing the medium, the peeled and sliced potatoes are boiled for 20 min in dH_2O and the extract is filtered through two layers of nylon membranes. After cooling, dextrose is added to potato extracts and the pH is adjusted to 6.5–6.8. After adding agar, the medium is sterilized at 121°C for 20 min. The sterilized medium is distributed to sterile Petri plates (in a sterile bench). The fungal plug of 5 mm diameter is placed on the center of a PDA plate and the Petri dish is incubated at 22–24°C in a temperature-controlled chamber under 12/12 h light/dark illumination and 75% relative humidity for a period of 2 weeks. Two-week-old fungus (Fig. 20.6a) is used for root and leaf infection.

20.5.1.2 Maintaining Virulence of *A. brassicae*

To maintain the virulence of *A. brassicae*, the fungus should be inoculated and reisolated from the infected tissues periodically. 12–14-day-old *Arabidopsis* seedlings are placed on a PNM plate with nylon membrane on the top. Leaves are inoculated with 5 µl of *A. brassicae* spore suspension (which contains 10^5–10^6 spores per ml) directly on to leaves and the plates are incubated at 22–24°C under 12/12 h light/dark illumination and 75% relative humidity for 4–5 days. This period is needed to see the symptom development. Once symptoms are observed on leaves, the fungus should be re-isolated from the infected tissues. For this, the infected leaf tissues are cut into small pieces of 2–3 mm and surface-sterilized with 0.1% mercuric chloride or 70% methanol for 1–2 min followed by a series of washing in sterilized dH_2O (five times). The surface-sterilized infected leaf pieces are placed on the PDA plates and plates are incubated as described above and fungal growth has to be observed. The fungus is transferred to the fresh PDA plates by taking single hyphal tip and incubated for 2 weeks. Every 2 weeks, the fungus is subcultured to new PDA plates.

20.5.2 Inoculation of A. thaliana *with* A. brassicae

20.5.2.1 Preparation of Spores

Many pathogenic fungi sporulate in Potato Dextrose broth (PDA without agar). Spores are generally used for leaf and root infection assays. 100 ml of Potato dextrose broth are distributed to 250 ml conical flasks and autoclaved at 121°C for 20 min. One fungal plug 5 mm in diameter from a 2-week-old culture is inoculated to the flask and incubated at 22–24°C under 12/12 h light/dark illumination and 75% relative humidity for 2 weeks. By this time the fungus grows completely and sporulates heavily (Fig. 20.6b). To harvest the spores, the medium is drained out by filtering through four layers of sterilized nylon membrane and the mycelium with spores is washed three times with sterilized dH_2O. The spores and mycelia are gently homogenized with 50–100 ml of sterilized dH_2O and filtered through four layers of sterilized nylon membrane. The spore count is determined by serial dilution or by using a Haemocytometer and is adjusted to 10^5–10^6/ml. For getting a uniform dispersion of spores, 1–2 drops of Tween-20 per 100 ml spore suspension are added.

20.5.2.2 Root Infection

Root infection with *A. brassicae* is done as described above for *P. indica*; in this case instead of *P. indica* plugs we use *A. brassicae* plugs. After 2, 5, 7, 10, and 14 days of inoculation, the disease intensity/severity is recorded as Percentage Disease Index (PDI). Root infection can be also performed by adding 10–20 µl spore suspension directly to the roots (Fig. 20.7a).

20.5.2.3 Leaf Infection

Leaf infection is done by adding the spore suspension of the pathogenic fungus directly to the plant leaves (Fig. 20.7b). For this we use two methods:

Method 1—for *A. thaliana* seedlings grown on plates
12–14-day-old *A. thaliana* seedlings are transferred to PNM plates with nylon membrane and 5 µl spore suspension containing 10^5–10^6 spores per ml is inoculated directly to the middle whorl of the leaves (usually we use 4–5 leaves/seedling). Plates are sealed with parafilm and incubated at 22–24°C in a temperature-controlled growth room under 12/12 h dark/light illumination. After 5–7 days, symptoms can be seen on leaves (Fig. 20.7b) and the disease intensity/severity is recorded as Percentage Disease Index (PDI).

Method 2—for adult *A. thaliana* plants
Leaf infection can also be performed with detached leaf infection. For this we use 4–5-week-old plants grown under short day condition. Sterile Whatmann filter

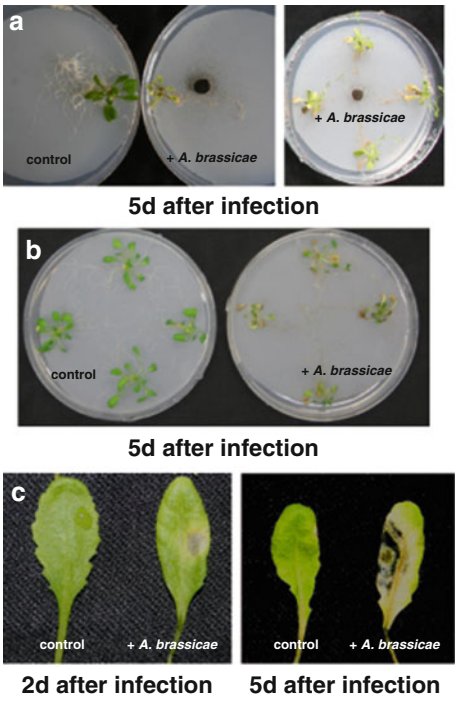

Fig. 20.7 *A. thaliana* seedlings on PNM medium after root and leaf infection with *A. brassicae* (**a**) after 5 days root infection with *A. brassicae* plug; (**b**) after 5 days leaf infection with *A. brassicae* spores; (**c**) after 2 and 5 days detached leaf infection with *A. brassicae* spores

paper is placed on Petri dish and dH_2O is added just enough to soak the filter paper. The detached leaves (3–5) are kept on the soaked filter paper and inoculated with 10 µl spore suspension containing 10^5–10^6 spores per ml directly on to leaves. The plates are sealed with parafilm and incubated at 22–24°C in a temperature-controlled growth room under 12/12 h light/dark illumination. After 5–7 days symptoms can be seen on leaves (Fig. 20.7c) and the disease intensity/severity is recorded as Percentage Disease Index (PDI).

20.6 Interaction of *P. indica* with Pathogenic Fungi

Dual culture experiment is generally used to test the effect of one microbe on another microbe. This effect can be antagonistic—where one microbe inhibits the growth of another by producing antimicrobial compounds (antibiosis) and lytic enzymes (inhibition zone), coiling around the mycelium and choking, overgrowing and sporulating as parasitism, or mutualism-where both partners do not harm each other and mutually benefit. For the dual culture experiment, we use PDA medium because many fungi can grow on it.

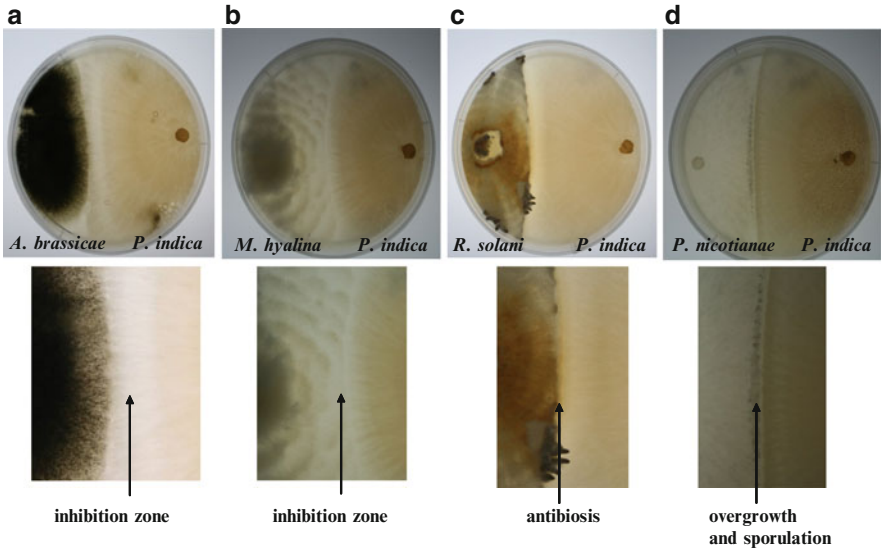

Fig. 20.8 *In vitro* interaction of *P. indica* with different fungi in dual culture experiment. (**a**) *Alternaria brassicae* (left) and *P. indica* (right); (**b**) *Mortierella hyalina* (left) and *P. indica* (right); (**c**) *Rhizoctonia solani* (left) and *P. indica* (right); (**d**) *Phytophthora nicotianae* (left) and *P. indica* (right)

20.6.1 Dual Culture Experiment

In dual culture experiment two interacting microbes are grown together on the same PDA p1ate. One *P. indica* plug 5 mm in diameter is placed at one end of the PDA plate and the *A. brassicae* plug (or any other fungi) at the other end. The plates are sealed with parafilm and incubated at 22–24°C under 12/12 h light/dark illumination and 75% relative humidity. The plates are observed for the inhibition zone and/or antibiosis at 3, 5, 7, 10, and 15 days of inoculation (Fig. 20.8).

20.7 Interaction of Both Beneficial and Pathogenic Fungi with *Arabidopsis*

Many endophytic root colonizing fungi can be used as bioprotectant against various biotic and abiotic stress. *P. indica* endophytically colonizes the roots and does not migrate into the foliage of plants because the colonization is restricted only in epidermis and cortical tissues of roots. Priming of roots with *P. indica* and subsequent challenge-inoculation of biotrophic and necrotrophic fungi on leaves significantly suppressed the disease in terms of number and size of lesions and rate of the disease development.

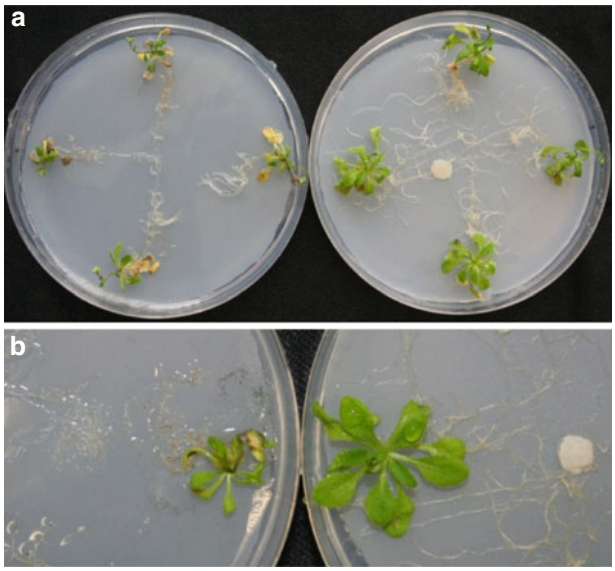

Fig. 20.9 Activation of induced systemic resistance in *A. thaliana* by *P. indica* (**a**, **b**). *Arabidopsis* roots were primed with *P. indica* for 48 h and then leaves were infected with *A. brassicae* spores; infected unprimed plants (left) and healthy primed plants (right) (**b**) Closer view

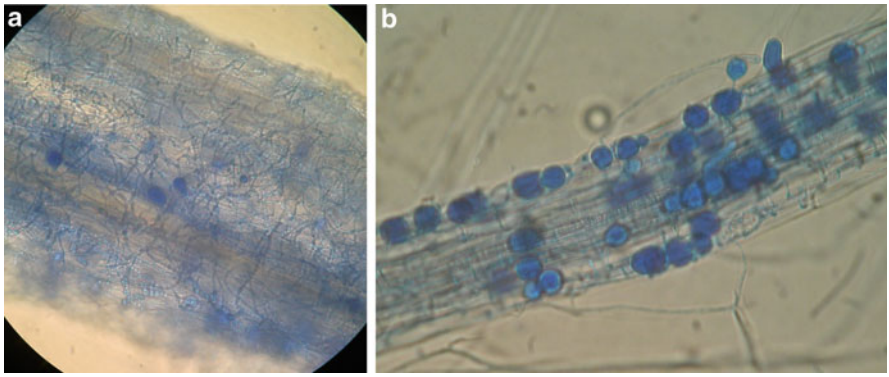

Fig. 20.10 Root colonization of *A. thaliana* after18 days cocultivation with *P. indica* on PNM medium (**a**) hyphae and young chlamydospores; (**b**) mature chlamydospores inside the root cells

20.7.1 Induction of Systemic Resistance by P. indica

Priming of *A. thaliana* roots with *P. indica* confers resistance or tolerance against various foliar pathogenic fungi including *A. brassicae*. *A. thaliana* and *P. indica* are cocultivated for 48 h as described in Sect. 20.4.1 so that the roots are initially

colonized with the fungus and the shoots are primed which induces resistance against pathogenic fungi whenever it attacks the aboveground shoot. After 48 h cocultivation, the leaves are inoculated with 5 µl *A. brassicae* spore suspension containing 10^5–10^6 spores per ml. Plates are sealed with parafilm and incubated at 22–24°C in a temperature-controlled growth room under 12/12 h light/dark illumination. The seedlings which are not primed with *P. indica* and are inoculated with *A. brassicae* serve as control. After 3, 5, 7, 10, and 14 days of incubation, the disease development is observed on leaves (Fig. 20.9) and the disease intensity/severity is recorded as Percentage Disease Index (PDI).

20.8 Colonization of *P. indica*

The internal colonization of *P. indica* in *Arabidopsis* root is monitored by taking periodically ten small root samples from the seedlings cocultivated with *P. indica*. The colonized and uncolonized roots are washed thoroughly in running deionized water and cut into 1 cm long pieces. These roots are treated with 10 % KOH and incubated overnight at room temperature in a sterile bench. After washing the root pieces five times with dH_2O, the roots are incubated with 1% HCl for 3 min. The treated roots are mounted in 0.05% trypan blue in lactophenol and examined under the microscope (Fig. 20.10). The roots can also be quickly examined under microscope by adding 0.05% cotton blue to them.

20.9 Conclusions

The endophytic interaction of the fungus *P. indica* with the model plant *A. thaliana* might help to understand the basis of the beneficial interactions between these two symbionts. We have established standardized cocultivation conditions for the two symbionts that allow to monitor beneficial and nonbeneficial traits during the whole life of the plant and which are described in this chapter.

Acknowledgements We acknowledge Sarah Mußbach and Claudia Röppischer for their excellent technical help.

References

Adesemoye AO, Kloepper JW (2009) Plant-microbes interactions in enhanced fertilizer use efficiency. Appl Microbiol Biotechnol 85:1–12

Anderson-Prouty AJ, Albersheim P (1975) Host-pathogen interactions. VIII. Isolation of a pathogen-synthesized fraction rich in glucan that elicits a defense response in the pathogens host. Plant Physiol 56:286–291

Bains PS, Tewari JP (1987) Purification, chemical characterization and host specificity of the toxin produced by *Alternaria brassicae*. Physiol Mol Plant Pathol 30:259–271

Bucher M, Wegmüller S, Drissner D (2009) Chasing the structures of small molecules in arbuscular mycorrhizal signalling: a review. Curr Opin Plant Biol 2:500–507

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Oelmüller R (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and non-beneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol 185:1062–1073

Camehl I, Drzewiecki C, Vadassery J, Shahollari B, Sherameti I, Forzani C, Munnik T, Hirt H, Oelmüller R (2011) The OXI1 kinase pathway mediates *Piriformospora indica* - induced growth promotion in *Arabidopsis*. PLoS Pathog 7:e1002051

Chet I, Elad Y (1982) Prevention of plant infection by biological means. In: La Selection des Plantes, vol 11. Colloq. l'INRA, Bordeaux (France), pp 195–204

Das A, Kamal S, Shakil NA, Sherameti I, Oelmüller R, Dua M, Tuteja N, Johri AK, Varma A (2012) The root endophyte fungus *Piriformospora indica* leads to early flowering, higher biomass and altered secondary metabolites of the medicinal plant, *Coleus forskohlii*. Plant Signal Behav 7:103–112

Dungia OD, Sinclair JB (1995) Basic plant pathology methods (2nd edn). CRC Press, Inc., Boca Raton, FL, p 18

Fakhro A, Andrade-Linares DR, von Bargen S, Bandte M, Büttner C, Grosch R, Schwarz D, Franken P (2010) Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens. Mycorrhiza 20:191–200

Hill TW, Käfer E (2001) Improved protocols for Aspergillus medium: trace elements and minimum medium salt stock solutions. Fungal Genet Newsl 48:20–21

Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ (2007a) A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 104:1720–1725

Javot H, Pumplin N, Harrison MJ (2007b) Phosphate in the arbuscular mycorrhizal symbiosis: transport properties and regulatory roles: a review. Plant Cell Environ 30:310–322

Johnson JM, Nongbri PL, Sherameti I, Oelmüller R (2011a) Calcium signaling and cytosolic calcium measurements in plants. Endocyt Cell Res: 21:64–76. http://zs.thulb.unijena.de/receive/jportal_jparticle_00231945

Johnson JM, Sherameti I, Ludwig A, Nongbri PL, Sun C, Lou B, Varma A, Oelmüller R (2011b) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* co-cultivation - a model system to study plant beneficial traits. Endocyt Cell Res: 21:101–113. http://zs.thulb.uni-jena.de/receive/jportal_jparticle_00247947

Johnson JM, Oelmüller R (2009) Mutualism or parasitism: life in an unstable continuum. What can we learn from the mutualistic interaction between *Piri-formospora indica* and *Arabidopsis thaliana?* A review. Endocyt Cell Res: 19:81–111

Kaldorf M, Koch B, Rexer KH, Kost G, Varma A (2005) Patterns of interaction between *Populus Esch5* and *Piriformospora indica*: a transition from mutualism to antagonism. Plant Biol 7:210–218

Knecht K, Seyffarth M, Desel C, Thurau T, Sherameti I, Lou B, Oelmüller R, Cai D (2010) Expression of BvGLP-1 encoding a germin-like protein from sugar beet in *Arabidopsis thaliana* leads to resistance against phytopathogenic fungi. Mol Plant Microbe Interact 23:446–457

Lee YC, Johnson JM, Chien CT, Sun C, Cai D, Lou B, Oelmüller R, Yeh KW (2011) Growth promotion of Chinese cabbage and *Arabidopsis* by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin. Mol Plant Microbe Interact 24:421–431

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence - a practical guide: a review. J Exp Bot 51:659–68

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco cultures. Physiol Plant 15:473–497

Nehls U (2008) Mastering ectomycorrhizal symbiosis: the impact of carbohydrates: a review. J Exp Bot 59:1097–1108

Nehls U, Göhringer F, Wittulsky S, Dietz S (2010) Fungal carbohydrate support in the ectomycorrhizal symbiosis: a review. Plant Biol 12:292–301

Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Oelmüller R, Briggs WR (1990) Intact plastids are required for nitrate- and light-induced accumulation of nitrate reductase activity and mRNA in squash cotyledons. Plant Physiol 92:434–439

Oelmüller R, Sherameti I, Tripathy S, Varma A (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Peškan-Berghöfer T, Markert C, Varma A, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. Physiol Plant 122:465–477

Satheesan J, Narayanan AK, Sakunthala M (2012) Induction of root colonization by *Piriformospora indica* leads to inhanced asiaticoside production in *Centella asiatica*. Mycorrhiza 22:195–202

Shahollari B, Varma A, Oelmüller R (2005) Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol 162:945–958

Shahollari B, Vadassery J, Varma A, Oelmüller R (2007a) A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 50:1–13

Shahollari B, Bhatnagar K, Sherameti I, Varma A, Oelmüller R (2007b) Molecular symbiotic analysis between *Arabidopsis thaliana* and *Piriformospora indica*. In: Varma A, Oelmüller R (eds) Advanced techniques in soil microbiology. Springer, Berlin, pp 307–318

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. J Biol Chem 280:2641–2647

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmüller R (2008) PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 54:428–439

Sherameti I, Johnson JM, Nongbri P, Oelmüller R (2010) The central roles of iron and calcium for plant/microbe interaction and shaping microbial communities in the soil: a review. Albanian J Agric Sci 3:1–24

Sun C, Johnson JM, Cai D, Sherameti I, Oelmüller R, Lou B (2010) *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. J Plant Physiol 167:1009–1017

Thomma BPHJ, Nelissen I, Eggermont K, Broekaert WF (1999) Defciency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J 19:163–171

Vadassery J, Oelmüller R (2009) Calcium signaling in pathogenic and beneficial plant microbe interactions What can we learn from the interaction between *Piriformospora indica* and *Arabidopsis thaliana*. Plant Signal Behav 4:1024–1027

Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B, Novák O, Strnad M, Ludwig-Müller J, Oelmüller R (2008) The role of auxins and cytokinins in the mutualistic interaction between *Arabidopsis* and *Piriformospora indica*. Mol Plant Microbe Interact 21:1371–83

Vadassery J, Ranf S, Drzewiecki C, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R (2009a) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J 59:193–206

Vadassery J, Tripathi S, Prasad R, Varma A, Oelmüller R (2009b) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and *Arabidopsis*. J Plant Physiol 166:1263–1274

Varma A, Verma S, Sudha, Sahay N, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sudha S, Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Bock B (ed) Mycota IX. Springer Series, Springer, Berlin, pp 123–150

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Chapter 21
Characterization of *Piriformospora indica* Culture Filtrate

Ashok K. Adya, Anju Gautam, Lixin Zhang, and Ajit Varma

21.1 Introduction

Piriformospora indica tremendously improves the growth and overall biomass production of diverse hosts, including legumes, medicinal, and economically important plants (Varma et al. 1999, 2000). It provides a promising model organism for the investigation of beneficial plant–microbe interactions. It also enables the availability of active compounds, which if identified and characterized properly may help improve plant growth and productivity and maintain soil productivity. This would open up numerous opportunities for the optimization of plant productivity in both managed and natural ecosystems while minimizing the risk of environmental damage. The various multifunctional roles of *P. indica* are outlined in Fig. 21.1.

P. indica with its identified and recognized multifunctional roles is thus a potential candidate to serve as a biofertilizer, a bioprotector, a bioregulator, a bioherbicide, and an excellent source for the hardening of tissue culture-raised plants (Chauhan et al. 2006). It promises to be a boon for the plant industries and

A.K. Adya (✉)
Division of Biotechnology & Forensic Sciences, BIONTHE (Bio- and Nano-technology for Health & Environment) Centre, School of Contemporary Sciences, University of Abertay Dundee, Bell Street, Dundee DD1 1HG, United Kingdom
e-mail: ashok.adya@abertay.ac.uk

A. Gautam • L. Zhang
CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, 8 Bei'ertiao, Zhongguancun 100190, Beijing, People's Republic of China
e-mail: anjela628723@gmail.com; lzhang03@gmail.com

A. Varma
Amity Institute of Microbial Technology, Amity University Uttar Pradesh, E-3 Block, IV Floor, Amity University Campus, Sector -125, Noida 201303, U.P, India
e-mail: ajitvarma@amity.edu

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_21,

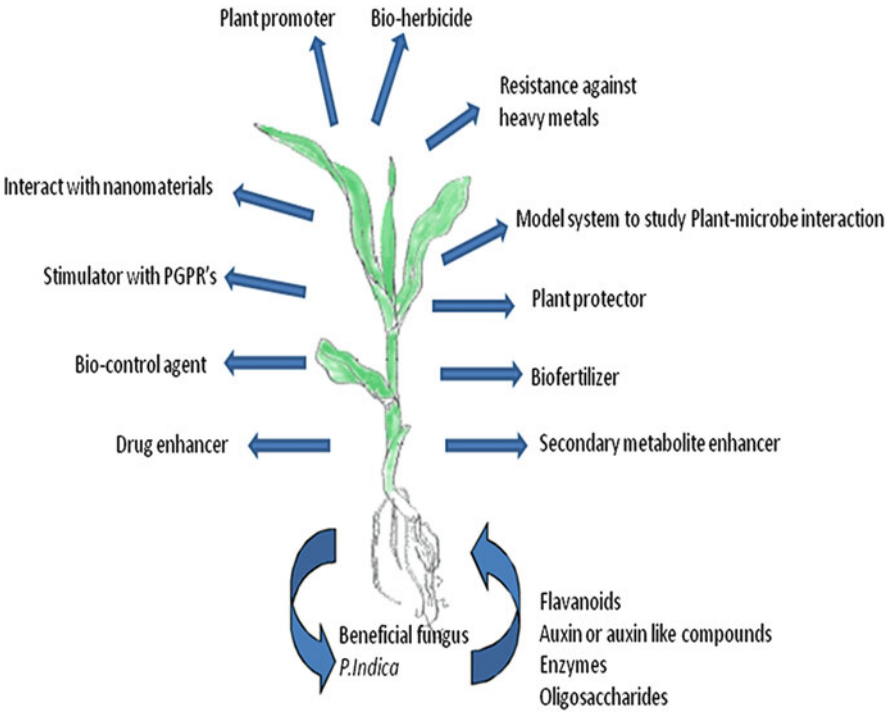

Fig. 21.1 Multifunctional roles of *P. indica*

agricultural science. However, the mechanism by which *P. indica* or its culture filtrate acts as a plant growth promoter/stimulator is totally unclear at present, although some studies have identified a variety of factors, originating either directly from *P. indica* or those induced by it, to be responsible for promoting/stimulating the growth of plants. Some of these factors include IAA (indoleacetic acid) or auxin production by *P. indica* (Sirrenberg et al. 2007) or *P. indica*-induced changes in phytohormone synthesis in plants (Barazani et al. 2005; Oelmüller et al. 2009; Schafer et al. 2007). The complexity of the underlying responsible mechanism can be appreciated from the fact that it may involve a single or a multiple phytohormone activity or it may be due to agglomerative effects resulting from small molecules and enzymes and root proliferation. Additionally, it could be that no single but multiple mechanisms are responsible to effect plant growth, and the effect may be cumulative (with related or unrelated mechanisms) and vary according to the plant species. To understand these mechanisms, it is an essential first step that a protocol be designed to properly characterize *P. indica* culture filtrate.

With the above aims in mind, we provide in the following sections the details of isolation, purification, and characterization (including instrumentation and methodologies) of *P. indica* culture filtrate with the hope that these would open up further avenues of application of *P. indica* resulting from a wide range of

beneficial interactions of *P. indica* culture filtrate with a broad spectrum of plants for improving plant productivity.

In particular, Sect. 21.2 discusses the impact of *P. indica* culture filtrate on cocultivation with plants. The effect of nanoparticles on the growth enhancement potential of *P. indica* culture filtrate is also discussed in this section with the hope to find efficient and eco-friendly nanoparticles. In Sect. 21.3 we provide a detailed protocol, involving application of multifarious techniques, for the characterization and identification of *P. indica* culture filtrate.

21.2 Impact of *P. indica* Culture Filtrate on Cocultivation with Plants

21.2.1 Primary and Secondary Metabolites

Plants produce an amazing diversity of low-molecular-weight compounds. There are probably hundreds of thousands of such compounds in existence. Although the structures of close to 50,000 have already been elucidated (De Luca and St Pierre 2000), only a few of these are part of "primary" metabolic pathways, i.e., those common to all organisms. The rest are termed "secondary" metabolites (Pichersky and Gang 2000). Plants' secondary metabolites can be defined as those compounds that have no recognized role in the fundamental life processes in the plants that synthesize them. They, however, have an important role to play in the interaction of plants with their environment. Almost 100,000 secondary metabolites have been discovered from the plant kingdom, but the structures of only about half of them have been fully elucidated (Verpoorte 1998; Dixon 2001) so far. This is because only small amounts of these compounds are found in whole plants and cell culture systems. A combined application of biotic elicitation along with other yield-enhancement strategies can develop techniques for large-scale production of commercially important compounds by plant cell cultures (Baldi et al. 2009). Since plants are adapted to various functions in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes. Endophytic fungi inhabit such a biotope (Schulz et al. 2002). Endophytic fungi are one source for intelligent screening of secondary metabolites from plants. They grow within their plant hosts without causing apparent disease symptoms, and growth in this habitat involves continual metabolic interaction between fungus and host.

21.2.2 Potential of P. indica Culture Filtrate

The culture filtrate of *P. indica* containing fungal exudates has significant potential to produce stimulatory effects and disease control in various plants. *P. indica*

culture filtrate is known to enhance seed germination and growth of several plants (Oelmüller et al. 2009). It is observed that among the compounds released in root exudates infected with *P. indica*, flavonoids are present. Flavonoids have been suggested to be involved in stimulation of precontact hyphal growth and branching (Gianinazzi-Pearson et al. 1989; Siqueira et al. 1991), which is consistent with their role as signaling molecules in other plant–microbe interactions (Giovannetti and Sbrana 1998). Cell wall degrading enzymes like cellulase, polygalacturonase, and xylanase are also found in significant quantities both in the culture filtrate and in the root exudates colonized by *P. indica* fungus. Mechanism by which *P. indica* promotes the growth of plants is not yet known (Sirrenberg et al. 2007). Some studies have implicated various ingredients originating from *P. indica* or those induced by it in plants for its positive effects. Examples include indole-3-acetic acid (IAA) production in culture filtrate of *P. indica* or induction of IAA by *P. indica* in plants, proteins in *P. indica* showing similarity to myrosinase binding, and myrosinase-associated proteins raising IAA that trigger growth promotion in plants. Improvement in the growth of the plant may also occur due to induction of systemic resistance by *P. indica* against diseases in the plants, as has been reported by many researchers (see, e.g., Bagde et al. 2010b). To sum up, culture filtrate acts as bioenhancing, antiviral, antioxidant, and anti-inflammatory and has shown the presence of flavonoids.

HPLC traces of methanolic extract from 10-day-old fungal biomass and 6-week-old infected roots of maize with *P. indica* revealed the presence of at least eight prominent peaks as compared to five peaks in uncolonized roots. With spectroscopic methods, the structure of the main component was identified as benzoic acid (BA). However, eluates did not promote plant growth. In another independent HPLC experiment, four broadband prominent peaks were observed as compared to narrow peaks in control. It seems that the detected compounds may be carbohydrates and/or saponins (Varma et al. 2001).

21.2.3 Cocultivation of P. indica Culture Filtrate with Plants

In a recent investigation, Bagde et al. (2011) studied the impact of *P. indica* culture filtrate on *Helianthus annuus* Sungold and *H. annuus* Japanese gold varieties in the greenhouse. Their results convincingly demonstrated the positive effects of *P. indica* on the growth of *Helianthus annuus* Sungold, as evidenced by the following increases: 52.70 % (root-collar diameter), 69.0 % (root number), 35.85 % (root length), 24.27 % (dry root weight), 39.24 % (stem diameter), 19.86 % (stem height), 33.33 % (number of leaves), 12.2 % (leaf length), 17.82 % (leaf width), 43.3 % (flower diameter), 188.42 % (flower dry weight), 9.12 % (seed number), 45.89 % (dry seed weight), and 51.13 % (seed oil content) when plants were treated with *P. indica* culture filtrate as compared to the untreated control plants. For instance, the seed oil content of treated plants ranged from 3.03 to 6.28 % as compared to 2.2 to 3.55 % in untreated plants, and seed number

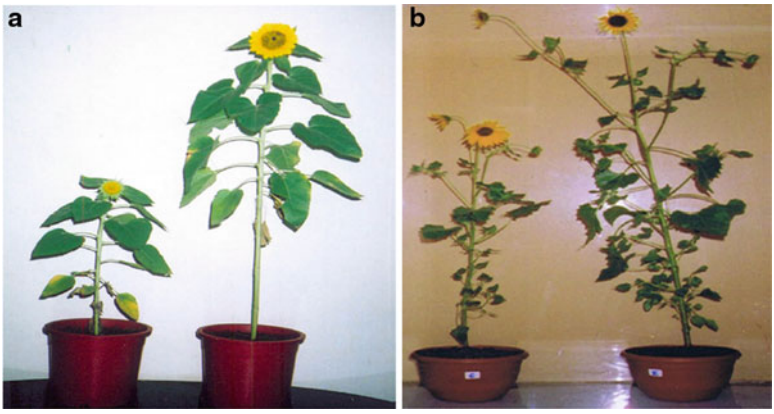

Fig. 21.2 The effect of *P. indica* culture filtrate inoculation on the growth of plant *Helianthus annuus* (*left*: control, *right* with *P. indica*). (**a**) Sungold. (**b**) Japanese gold

ranged from 141 to 153 in untreated plants, while it ranged between 158 and 167 in treated plants. There was an increase in total biomass content of 36.70 % (roots, stems, leaves, flowers, seeds) of the plants over the untreated ones. Treatment with *P. indica* culture filtrate thus led to an undoubted overall growth of the *H. annuus* Sungold plants (Fig. 21.2).

The mechanism involved behind this novel quality of axenically grown *P. indica* culture filtrate to promote plant growth, though still unknown, is found to be linked to the presence of IAA in it. In addition, it is conjectured that the fungus may induce auxin production in the host plant (Peškan-Berghöfer et al. 2004).

21.2.4 Effect of Nanoparticles on Growth Enhancement Potential of P. indica Culture Filtrate

Suman et al. (2010) investigated the growth enhancement potential of nanomaterial-treated *P. indica* fungal broth on the seeds of broccoli. Their results indicate enhanced stimulation of seed germination in the presence of all the nanomaterials studied, namely, titanium dioxide nanoparticles (TNPs), carbon nanotubes (CNTs), and silver nanoparticles (AgNPs), in the order TNPs > CNTs > AgNPs. The nanomaterial-treated *P. indica* culture filtrate thus further promotes seed germination and growth of seedlings in plants.

Their results on the interaction between nanomaterials and *P. indica* showed two- to threefold increase in the fungal biomass (see Fig. 21.3) in the presence of almost all the nanomaterials used compared to the negative controls for which they used activated charcoal and control (without charcoal). Fresh fungal biomass was found to be largest in the case of TNPs followed by CNTs. The colony morphology

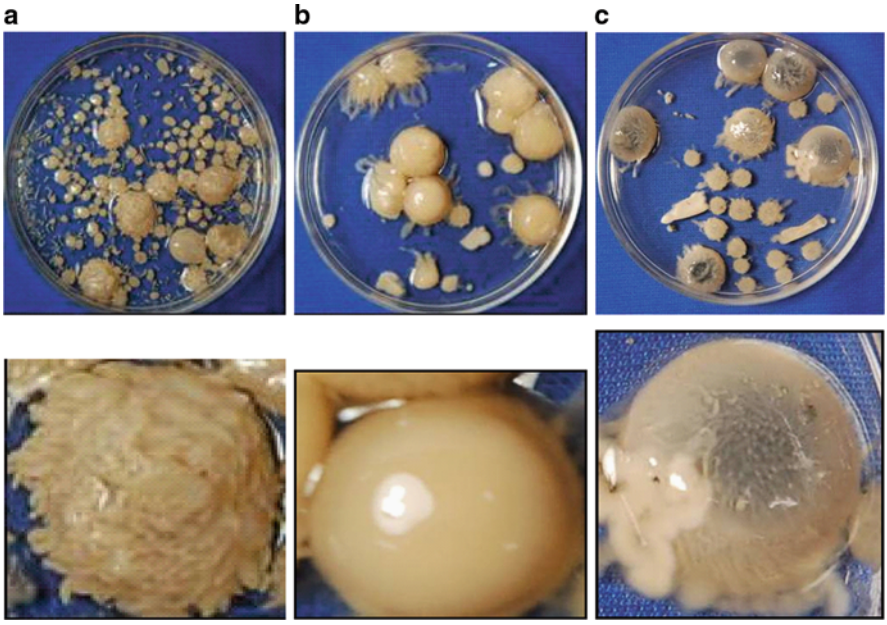

Fig. 21.3 The fungus *Piriformospora indica* interacting with the nanomaterials: control, titanium dioxide nanoparticles (TNP), carbon nanotubes (CNT), and activated charcoal. (**a**) Control without nanoparticles—fungal surface is rough and overall size of the colonies is not very large. (**b**) TNP environment—colonies are larger in size, more smooth, and spherical. (**c**) CNT environment—colonies are bigger, and morphologically it is not smooth but bulging outwards

of *P. indica* also differed with the addition of NPs. In particular, the colonies were found to be larger in size, more smooth, and round in TNP-infused medium. Similar observations were made in the CNT-infused medium except that the colonies in this case were irregular with long and short protrusions. The SEM (scanning electron microscopy) results indicated the stimulating effect on the size of the chlamydospores.

The culture filtrate retrieved after nanomaterial-embedded incubation also promoted the early germination and growth of broccoli (*Brassica oleracea*) (Fig. 21.4).

21.2.5 P. indica Culture Filtrate Causes Root and Shoot Growth in a Wide Spectrum of Plants

The culture filtrate of *P. indica* mycelium contains fungal exudates, hormones, enzymes, proteins, etc. A very small amount (50 µl) is sufficient to promote root as well as shoot growth. Bagde et al. (2010b), however, reported that 15 ml of freshly

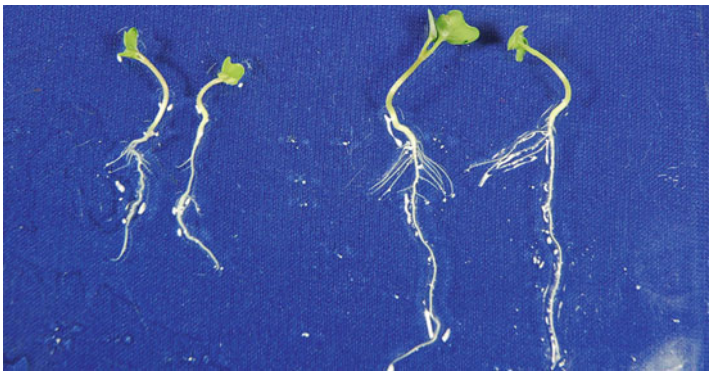

Fig. 21.4 Influence of 7-day-old *P. indica* culture filtrate on broccoli. 50 µl was paced in the center of the agar well and young seedlings at a distance of 4 cm. Culture filtrate is diffusible and thermostable

eluted culture filtrate of *P. indica* when used for application to the pot experiment resulted in increase of root length, shoot length, and plant biomass in *P. indica*-treated host plants, maize, Bacopa, and tobacco. Similar observations were also reported in culture tube experiments with the induction of secondary roots (Varma et al. 2001). This remains to be the first report of its kind showing positive impact of *P. indica* culture filtrate on the promotion of plant growth. These findings were further corroborated by Singh et al. (2003) who observed that culture filtrate of *P. indica* significantly increased the neem and maize plant growth and development over the control.

Supplementation of *P. indica* culture filtrate in the substratum growing *A. elegans* mart promoted overall biomass, rendered it greener, and improved both the leaf texture and active ingredient—aristolochic acid. In contrast, in unsupplemented control, the plant growth was stunted and foliage mass was reduced. Treatment of *A. elegans* plants with the culture filtrate of *P. indica* exhibited significant increase in growth with respect to the plant height, leaf diameter, length and dry weight, total biomass, and aristolochic acid contents of the plants than the untreated plants.

Interestingly, *P. indica* has evolved to elevate cytosolic Ca^{2+} in the roots of *Arabidopsis* and tobacco (Vadassery and Oelmüller 2009). Fungus-derived signals are secreted into the medium or rhizosphere and activate a plant receptor at the root plasma membrane. Early signaling events in the root cells include phospholipids and phosphorylation and an increase in cytoplasmic and nuclear calcium levels (Oelmüller et al. 2009; Vadassery et al. 2009). Besides having access to the reduced carbon, it appears that the plant provides a shelter for the fungus. Considering that *P. indica* was isolated from a desert region, living in harmony with a host and strengthening its performance under stress conditions also ensure better propagation of the fungus (Oelmüller et al. 2011). Similarly in *Arabidopsis*, *P. indica* requires jasmonic acids signaling and cytoplasmic function of NRR1 to confer systemic resistance (Stein et al. 2009).

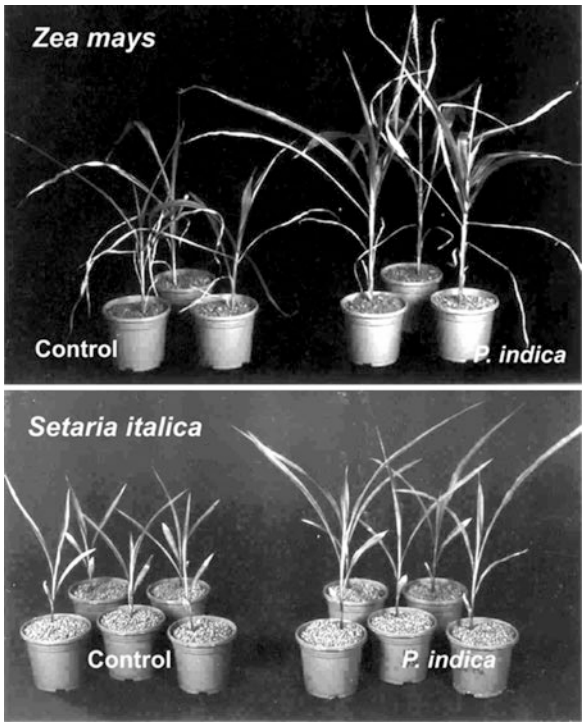

Fig. 21.5 Interaction of *P. indica* with *Zea mays* and *Setaria italica*

Induced systemic resistance (ISR) response is further observed in fungus *P. indica* (Molitor and Koegel 2009). *P. indica* is found to trigger systemic resistance based on two factors, plant hormone ethylene and proteobacterium *Rhizobium radiobacter*, to colonized plants. The fungus, *P. indica*, induces fast root surface pH signaling and prime systemic alkalinization of the key apoplast powdery mildew infection found in *Hordeum vulgare* L. It is speculated that the primed pH increase is indicative of and supports the potential systemic response to *B. graminis* f. sp. *hordei* induced by *P. indica* (Felle et al. 2009).

Recently, cloning and the functional analysis of gene encoding phosphate transporter (PiPT) from *P. indica* were performed (Yadav et al. 2010), where the expression of PiPT was found to be localized to the external hyphae of *P. indica* colonized with maize plant root (see Fig. 21.5). These findings suggest that external hyphae are the initial site of phosphate uptake from the soil.

PiPT is essential for phosphate transport to the host plant, and this is demonstrated by using electroporation for transformation and an RNAi approach, both being powerful tools for gene silencing in fungi. Yadav et al. (2010) suggest that exploitation of *P. indica* and its PiPT is actively involved in the phosphate transformation and, in turn, *P. indica* helps to improve the nutritional status of the host plant.

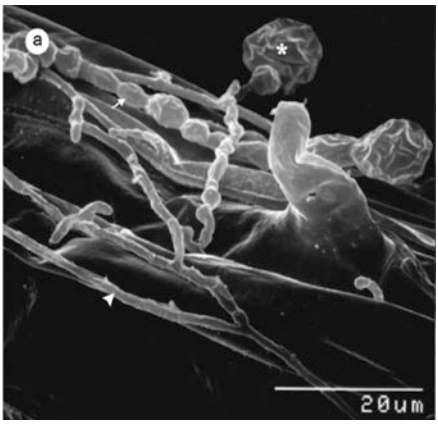

Fig. 21.6 Interaction of *P. indica* with *Arabidopsis thaliana*. An SEM picture of hyphae closely attached to the root surface. Besides normal hyphae (*arrowhead*), *P. indica* also forms coralloid hyphae (*arrow*) and chlamydospores

It is already documented that *P. indica* produces indoleacetic acid (IAA) in the culture filtrate as a diffusible factor. It is also suggested that auxin production affecting root growth is responsible for or at least contributes to the beneficial effect of *P. indica* on its host plants. Sufficient quantities of IAA in culture filtrate of axenically grown *P. indica* were detected (Peškan-Berghöfer et al. 2004). It was also observed that the culture filtrate caused the described root phenotype implicate production of auxin by the fungus. In addition, it has been suggested that the fungus may induce auxin production in the plant (Peškan-Berghöfer et al. 2004). Sirrenberg et al. (2007) examined the auxin-producing capacity in the culture filtrate of *P. indica* by GC–MS technique. Furthermore, by using HPLC–ESI–MS/MS technique, they confirmed that the culture filtrate of *P. indica* produces auxin during its interaction with *Arabidopsis* (see Fig. 21.6).

During culture in a rich medium (M1), approximately 0.16 mmol (28 mg) of IAA was produced within 4.5 weeks of culture, leading to a final concentration of 1.36 mM IAA in the growth medium. Growth temperature did not significantly influence the IAA production. Two mechanisms are found to be at play, which is evident from the observations that fungus (*P. indica*) could control plant growth. First, a diffusible factor caused changes in the root architecture. Second, *P. indica* formed structures in root epidermal cells (Sirrenberg et al. 2007).

21.2.6 Necessity of P. indica Culture Filtrate Characterization

It is clear from the above discussion of various published literature results that the plant growth promotional effect of *P. indica* culture filtrate is due to the production or addition of known and unknown compounds from the culture filtrate. It contains many known (IAA, flavonoids, cell wall degrading enzymes such as cellulose and xylanase) and unknown constituents, including hormones and enzymes. Thus, precise chemical identification/characterization is required for further work on the

characterization of novel compounds present in the culture filtrate of *P. indica* fungus. Such chemical characterization may be performed by thin-layer chromatography (TLC), column chromatography, high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), mass spectroscopy (MS), Raman spectroscopy (RS), proteomics, bioinformatics, atomic force microscopy (AFM), and scanning electron microscopy (SEM). The next section of this chapter concentrates on the development of a protocol for characterization of *P. indica* culture filtrate by using these techniques.

21.3 Protocol for Characterization and Identification of *P. indica* Culture Filtrate

21.3.1 Equipment and Laboratory Material

21.3.1.1 Equipment

Laminar flow bench or clean area for sterile work, pipettes 20–200 ml, incubator, autoclave, sonicator, rotary evaporator, round-bottom flasks, vacuum pump, UV detector, fermentor, shaker, Erlenmeyer flask, HPLC–ESI–MS system, column (silica gel/Sephadex), mass spectrometer, medium-pressure liquid chromatography, nuclear magnetic resonance spectrometer (proton, ^{13}C), Fourier transform infrared (FTIR) spectrometer, pH meter, centrifuge, analytical balance, and TLC plates.

21.3.1.2 Laboratory Material

Hill and Kafer medium, inoculating loop, cultures of fungi to be tested, Petri dishes (preferably square, 10 × 10 cm), autoclaved calibrated cylinder or sterile plastic tube (50 ml), methanol, dryer, capillary tube, sterile aluminum foil, tweezers, sterile demineralized water, sterile filter paper, sterile toothpicks, syringe, syringe filter with 0.45 μm pore size, scalpel (optional), spatula (optional), ethyl acetate, methanol, water, acetonitrile, and acetic acid (for HPLC/MS analysis).

21.3.2 Inoculation of the Fungus

Circular agar disks (~4 mm dia) infested with spores and actively growing hyphae of *P. indica* are placed onto Petri dishes containing solidified Hill and Kafer medium. Inoculated Petri dishes are subsequently incubated in an inverted position for 7 days at 28 ± 2 °C in the dark.

21.3.2.1 Protocol

(a) Hold the mother culture of *P. indica* grown on Hill and Kafer medium inside a laminar flow hood.
(b) Make the disks by using the bottom of a sterile glass Pasteur pipette measuring about 4 mm in diameter.
(c) Inoculate one disk per Petri plate fortified with Hill and Kafer medium containing 1 % agar.
(d) Wrap the Petri plates with paraffin tape to avoid any contamination.
(e) Incubate the Petri plates at 28 ± 2 °C for 7 days in inverted position.

21.3.3 Fungal Fermentation

The seed culture of *P. indica*—each fully grown fungus agar disk (4 mm dia)—is inoculated in 40 ml of production media (Hill and Käfer 2001) contained in a 250-ml Erlenmeyer flask. For large-scale fermentation, usually 4–5 (or even more) disks may be used with each disk inoculated in a separate 250-ml flask containing 40 ml of production media. The flasks containing the media and seed culture are then incubated at 28 ± 2 °C with constant agitation at 220 rpm on a rotary shaker and harvested for 6–7 days of incubation.

21.3.3.1 Protocol

(a) Autoclave 40 ml of production media in 250-ml flask at 121 °C and 15 lbs pressure for 15 min.
(b) Inoculate four or five fully grown fungus agar disks (4 mm dia) into the production media.
(c) Incubate the flasks at 28 ± 2 °C with constant shaking at 220 rpm on a rotary shaker (Fig. 21.7) for 6–7 days.

21.3.4 Isolation of Bioactive Metabolites

The harvested fermentation broth is centrifuged at 3,000 rpm for 20 min. The supernatant is fractionated with ethyl acetate to get the total extract that subsequently undergoes biological and chemical testing.

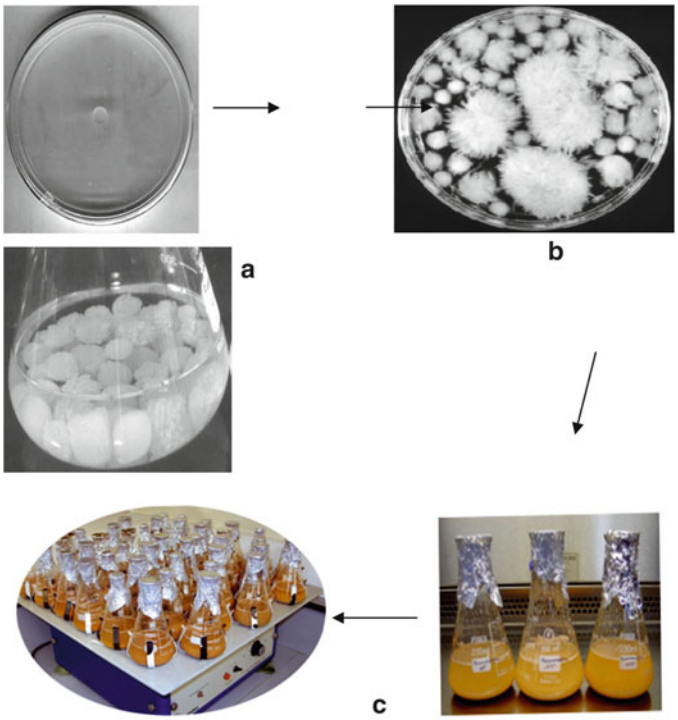

Fig. 21.7 (**a**) Circular agar disks (about 4 mm in diameter) infested with spores and actively growing hyphae of *P. indica* inoculated onto Petri dishes containing solidified Hill and Käfer medium, (**b**) growth of *P. indica* on Käfer liquid medium, and (**c**) fungal fermentation process

21.3.4.1 Protocol

(a) Centrifuge the fermentation broth for separating the biomass and supernatant at 3,000 rpm for 20 min.
(b) Treat the supernatant with 1:1 ratio of ethyl acetate with stirring.
(c) Dry the separated organic layer with a Rotavapor.
(d) Repeat the process three times or until the organic solvent becomes colorless.
(e) Weigh the crude extract obtained after drying.
(f) Dissolve the crude extract in methanol by using least amount of the solvent.
(g) Perform various chromatographic techniques on crude extract to check its chemical and biological profiling.

21.3.5 Chromatography

21.3.5.1 Thin-Layer Chromatography

In thin-layer chromatography (TLC), the stationary phase is a polar adsorbent, usually finely ground alumina or silica particles. This adsorbent is coated on a glass slide or plastic sheet creating a thin layer of the particular stationary phase. Almost all mixtures of solvents can be used as the mobile phase. By manipulating the mobile phase, organic compounds can be separated.

TLC is performed on precoated TLC plates with silica gel 60 F254 (layer thickness 0.2 mm) for none or semipolar compounds and on reversed phase RP-18 F254 (layer thickness 0.25 mm, Merck, Darmstadt, Germany) for polar compounds. The compounds are then detected from their UV absorbance at 254 and 366 nm.

TLC is also used to (1) optimize the solvent system for column chromatography, (2) monitor the identity of each of the yielded fractions, and (3) check the qualitative purity of the isolated compounds.

Protocol

(a) Cut a TLC plate, 5 cm tall and 0.2 mm by width.
(b) Spot a small amount of crude extract dissolved in methanol solvent [see Sect. 21.3.4.1 (f)] 0.5 cm from bottom of the TLC plate using a capillary tube. The spot should have as small a diameter as possible.
(c) Let the solvent evaporate.
(d) Make the TLC solvent and place it in a TLC chamber. Wait for 1–5 min to equilibrate the atmosphere in the chamber.
(e) Place the TLC plate in the chamber and let the solvent front run up the plate. When the plate has run far enough (solvent front approx. 1–0.5 cm from top of the plate), remove the plate from the TLC chamber.
(f) Let the TLC plate dry and then visualize under a UV lamp.

21.3.5.2 Column Chromatography

Column chromatography is the benchmark method for the isolation of natural products, with silica gel being the most widely used adsorbent (Mitsuhashi et al. 1960). In order to collect the separated materials and components, visualized by TLC, column chromatography is used. In this, the stationary phase (a solid adsorbent) is placed in a vertical glass column, and the mobile phase (a liquid) is added to the top and flows down under gravity (gravity column chromatography). Column chromatography allows us to separate/isolate and individually collect the desired compounds present in the crude extract of *P. indica* fungus which contains a

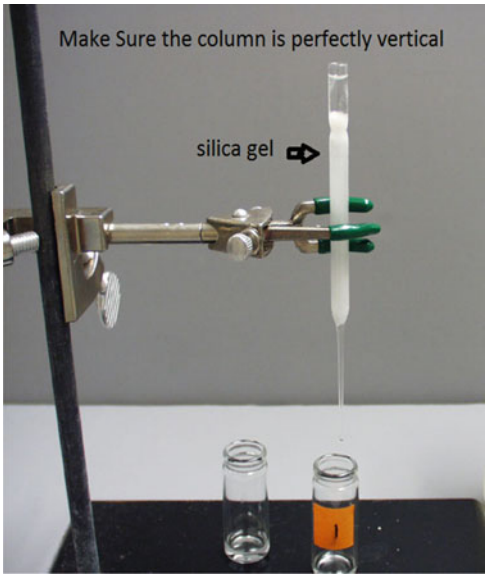

Fig. 21.8 Packaging for column chromatography

complex mixture of different compounds. Column chromatography is performed by packing a glass tube with an adsorbent using silica gel (SiO_2) or Sephadex as shown in Fig. 21.8. A common nonpolar solvent for column chromatography is hexane. It can be used with a variety of polar solvents, e.g., chloroform, ethyl acetate, methylene chloride, and methanol in order of increasing polarity. The column is packed "wet" by pouring solvent adsorbent slurry into the tube. The crude extract to be purified is then dissolved in a small amount of an appropriate solvent and added carefully to the top of the column so as not to disturb the packing. The column is developed by adding more solvent at the top and collecting the fractions of eluent that come out of the bottom in separate test tubes by using an automatic fraction collector.

Protocol

(a) Suspend the gel (silica gel or Sephadex) in a large volume of elution buffer until the gel is fully swollen via continuous stirring with a glass rod.
(b) Attach the column to a ring stand and make sure that the column is securely fastened in a vertical position, thereby avoiding air bubbles and cracks.
(c) Place a small paper or cotton plug loosely at the bottom of the column and close the clamp.
(d) After proper column packaging is achieved, allow a buffer of choice to run through the gel to saturate it. The flow rate of the buffer can be adjusted with a tap.
(e) Load the crude extract directly at the top of the column.

(f) Adjust the flow rate so that only a few drops enter the column in a given time interval without disrupting the flat top surface of the column.
(g) Collect the eluted fractions with a fraction collector.
(h) Monitor the column throughout (if the compounds need to be separated are colored, the colored bands will move down the column along with the solvent as they approach the end of the column).
(i) Collect the fractions (if the fractions are colored, separate the fractions in individual containers; otherwise collect in the same container). Check the collected fractions by spotting each on thin-layer chromatography (TLC).
(j) Develop the plate and use the observed spot(s) to determine which compound is present in each of the collected fractions.

21.3.5.3 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) uses the same principles as in TLC or column chromatography to detect the presence of interesting/active compounds in the extracts and fractions and also to evaluate the purity of the isolated compounds. In HPLC, rather than a solvent being allowed to drip through under gravity, it is forced through the column under high pressures (up to ~400 atm) which makes this technique much faster. In addition, it also allows us to use much smaller particle size for the column packing material that gives a much greater surface area for interactions between the stationary phase and the molecules running past it, thereby allowing a much better separation of the components of the mixture.

HPLC provides necessary information about the nature or the class of the detected compounds through the UV absorbance of each molecule. Additionally, it is used for the isolation of pure compounds from fractions previously separated using gravity column chromatography. The solvent system is composed of methanol-distilled water or acetonitrile–water. The eluted peaks are observed on the online UV detector.

In normal-phase HPLC or adsorption chromatography, the separation of compounds occurs due to their relative difference in mobility through the column under application of pressure. In this process, the column packing or stationary phase is polar (e.g., silica gel), and the mobile phase is nonpolar (e.g., hexane, benzene, toluene). The individual components in the mixture of compounds present in the crude extract travel with different speeds due to their relative affinities with the solvent and stationary phase. If the polar compounds or molecules in the crude extract under analysis have higher affinity to the stationary phase, they are retained longer in the column than nonpolar ones. Hence nonpolar compounds get eluted first due to their stronger affinity to nonpolar mobile phase, while polar molecules are eluted later.

Both normal (silica is polar and solvent is nonpolar)-phase HPLC and reverse-phase (silica is made nonpolar and solvent polar, e.g., methanol/acetonitrile/water) HPLC can be employed (Gangadevi and Muthumary 2008).

21.3.6 Spectroscopic and Diffraction Techniques

For the identification of the main constituents of the crude extract, nuclear magnetic resonance spectrometry (NMR), high-resolution mass spectrometry (MS), and LC–UV have been employed. These different hyphenated techniques have been used either separately or in partial combination beforehand (Queiroz et al. 2002). The use of all these hyphenated techniques allows the rapid structural determination of known constituents with only a minute amount of crude extract. With such an approach, the time-consuming isolation of common natural products is avoided, and an efficient targeted isolation of compounds presenting interesting spectroscopic or biological features can be performed (Wolfender et al. 1998). The availability of the above-mentioned hyphenated techniques has made possible the preisolation and analyses of the crude extracts from fungi and other natural sources, isolation and detection of various compounds, chemotaxonomic studies (Frisvad et al. 2008), chemical fingerprinting (Schaneberg et al. 2003), dereplication of products (Cordell and Shin 1999), and metabolomic studies (Pauli et al. 2005; Rochfort 2005).

21.3.6.1 Ultraviolet (UV) Spectroscopy

UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules present in the crude extract. Many molecules absorb ultraviolet or visible light. The absorbance of a test analyte increases as attenuation of the beam increases. The theory of UV spectrophotometer states that the absorbance of a test analyte is directly proportional to the concentration of the absorbing species in the analyte, which can be used to determine the concentration of the absorber. It is necessary to know how quickly the absorbance changes with concentration. This can be taken from the molar absorptivity of the chemical compound.

A UV/Vis spectrophotometer can be used as a detector for HPLC. The presence of an analyte gives a response assumed to be proportional to the concentration. The instrument's response to the analyte in the unknown can be compared with the response to a standard (Gangadevi and Muthumary 2008). The LC–UV technique can be used in a complementary manner for metabolite profiling of the specimen. It often gives an unambiguous assignment for known products and permits the partial structure determination of unknown constituents. The LC–UV method is also very efficient for recording of spectra in a crude reaction mixture at microgram level (Wolfender et al. 2008). The wavelengths of absorption peaks can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups within a molecule.

21.3.6.2 Mass Spectroscopy

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining the masses of particles and the elemental composition of a crude extract and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. Mass spectrometry (MS) can also be used to confirm the chromatographic profile obtained by UV detection (Cao et al. 2008).

A crude extract is a very complex mixture containing sometimes hundreds or thousands of different metabolites (Hamburger and Hostettmann 1991). The chemical nature of these constituents differs considerably within a given extract, and the variability of the physicochemical as well as the spectroscopic parameters of these compounds causes numerous detection problems. As every compound possesses a given molecular weight, detection of all these compounds using mass spectroscopy (MS) can be ideally considered as a universal detection technique (Niessen and van der Greef 1992). At present, MS is the most sensitive method of molecular analysis and has the potential to yield information on the molecular weight as well as the structure of the analytes. Furthermore, due to its high power of mass separation, very good selectivities can be obtained (Wolfender et al. 1998).

21.3.6.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool available for determining the structure of organic compounds. NMR spectroscopy is one of the principal techniques used to obtain physical, chemical, electronic, and structural information about molecules. It can provide detailed information on the three-dimensional structure, dynamics, and chemical environment of molecules in both the liquid and solid states. NMR is a physical phenomenon in which the magnetic nuclei in a magnetic field absorb and reemit electromagnetic radiation. This energy is at a specific resonance frequency which depends on the strength of the magnetic field and the magnetic properties of the isotope of the atoms. The energy or frequency at which this occurs can be measured and is displayed as an NMR spectrum (Kim et al. 1999). All isotopes that contain an odd number of protons and/or neutrons have an intrinsic magnetic moment and angular momentum, in other words a nonzero spin, while all nuclides with even numbers of both protons and neutrons have a total spin of zero. The most commonly studied nuclei are ^{1}H and ^{13}C, although nuclei from isotopes of many other elements have been studied by high-field NMR spectroscopy as well.

^{1}H NMR spectrum also known as proton NMR is the application of nuclear magnetic resonance with respect to hydrogen-1 nuclei within the molecules of a substance. ^{1}H NMR identifies the hydrogen atoms, and practically all of the hydrogen consists of the isotope ^{1}H (hydrogen-1, i.e., having one proton in its nucleus). Deuterated solvents are especially preferred for use, e.g., deuterated water

D_2O, deuterated acetone $(CD_3)_2CO$, deuterated methanol CD_3OD, deuterated dimethyl sulfoxide $(CD_3)_2SO$, and deuterated chloroform $CDCl_3$. However, a solvent without hydrogen, such as carbon tetrachloride CCl_4 or carbon disulfide CS_2, may also be used. Since the resonant frequency depends on the strength of the magnetic field, the shift in frequency is converted into a field-independent dimensionless value called the chemical shift. Proton NMR spectra of most organic compounds are characterized by chemical shifts in the range +14 to −4 ppm (proton precession magnetometers) and by spin–spin coupling between protons. The integration curve for each proton reflects the abundance of the individual protons. Together with carbon-13 NMR, proton NMR is a powerful tool for molecular structure characterization.

Carbon-13 NMR is the application of nuclear magnetic resonance (NMR) spectroscopy to carbon. It is analogous to proton NMR (^{1}H NMR) and allows the identification of carbon atoms in an organic molecule, just as proton NMR identifies hydrogen atoms. As such ^{13}C NMR is an important tool in chemical structure elucidation in organic chemistry. ^{13}C NMR detects only the ^{13}C isotope of carbon, whose natural abundance is only 1.1 %, because the main carbon isotope, ^{12}C, is not detectable by NMR due to its zero net spin.

Liquid-State NMR: Liquid-state NMR has become recognized as an important tool for chemical structural analysis of various compounds (Bubb 2003). The concepts of liquid-state NMR spectroscopy are largely built on the two isotropic interactions, namely, the isotropic chemical shifts, which provide the spectral resolution, and the indirect spin–spin (or J-) couplings, which reflect the network of chemical bonds (Levitt 2001). In principle, these two interactions do not hamper spectral resolution because they lead to sharp resonance lines. The normal approaches to analyze the chemical structures of various compounds are through 1D and 2D NMR spectroscopy. 1D and 2D homo- or heteronuclear experiments include technologies, such as DQF-COSY (double-quantum-filtered correlation spectroscopy), HSQC (heteronuclear single-quantum coherence), HMBC (heteronuclear multiple-bond coherence), and NOESY (nuclear Overhauser enhancement spectroscopy). These are widely used to analyze the chemical structures and configurations of various bioactive compounds (Yang and Zhang 2009). The fine chemical structures such as chemical compositions, configurations, and types of glycosidic linkages may be analyzed using two-dimensional (2D) NMR technologies. 3D or higher multiple-dimensional NMR experiments can be carried out by the combination of 2D experiments with multiple evolution times (Duus et al. 2000). Three-dimensional (3D) NMR correlates the interactions of different nuclei along the x, y, and z dimensions. The main advantage of using high-dimension NMR experiments is to increase the separation of resonances in the ^{13}C dimension, resulting in improving the resolution of the spectra (Allerdings et al. 2005; Xu and Bush 1996).

Solid-State NMR: Solid-state NMR spectroscopy is a kind of nuclear magnetic resonance spectroscopy characterized by the presence of anisotropic (directionally dependent) interactions. Solid-state NMR is used to analyze the chemical structures in order to overcome the solubility problem because the samples can be measured in a solid and dehydrated form (Spevacek and Brus 2008). The basis of this

technology is called magic-angle spinning (MAS). By spinning the sample (usually at a frequency of 1–70 kHz) at the magic angle, θ_m with respect to the direction of the magnetic field, the normally broad lines become narrower, thereby increasing the resolution for better identification and analysis of the spectrum. This technique of MAS is very essential to achieve high-resolution ^{13}C solid-state NMR spectra (Mcbrierty and Packer 1993). The intensity of the solid ^{13}C signals can be enhanced by using cross polarization (CP) technology in which the polarization transfers from ^{1}H to ^{13}C (Mcbrierty and Packer 1993).

21.3.6.4 X-Ray Diffraction

X-ray crystallography of an organic compound is a method of determining the precise arrangement of atoms within a crystal. It is an experimental technique in which X-rays after impinging on crystals get diffracted by them into many specific directions. X-ray crystallography has the advantage of defining ligand-binding sites with high certainty. It can also be used as a rapid technique to guide the elaboration of the fragments into larger molecular weight compounds (Blundell and Patel 2004).

From the angles and intensities of the diffracted beams, a three-dimensional picture of the density of electrons within the crystal can be produced. This information about the electron density within the crystal provides valuable information about the mean positions of the atoms in the crystal, the nature of the chemical bonds, their disorder, and a variety of other information. In order to measure the X-ray diffraction, the crystal is mounted on a goniometer and gradually rotated while being bombarded with X-rays, producing a diffraction pattern of regularly spaced spots known as reflections. The two-dimensional images taken at different rotations are converted into a three-dimensional (3D) model of the density of electrons within the crystal using the mathematical method of Fourier transforms, combined with chemical data known for the test sample.

In recent years, the advent of powerful synchrotron radiation sources, such as DIAMOND light source in UK, ESRF (European Synchrotron Radiation Facility) in France, SPring-8 in Japan—the 2nd- and 3rd-generation synchrotron sources—has led to production of light that is one billion times more brilliant than conventional X-ray sources. The availability of area detector-based data collection instruments (diffractometers) built on these central facilities, high-speed computers, and advanced data analysis routines has further enabled a dramatic enhancement in the efficiency of precise crystallographic structure determination.

Recently, X-ray diffraction patterns for the structures of biologically active natural products isolated from fungi Aphyllophorales, known as polypores in the phylum *Basidiomycota*, revealed that the isolated compounds contain ß-glycans having a secondary structure of a right triple standard helix (Zjawiony 2004). Similarly, two new cyclohexadepsipeptides from the fungus *Isaria* have been isolated by reverse-phase HPLC that were subsequently characterized by ESI–MS and ^{1}H-NMR, and their 3D structures elucidated by X-ray diffraction.

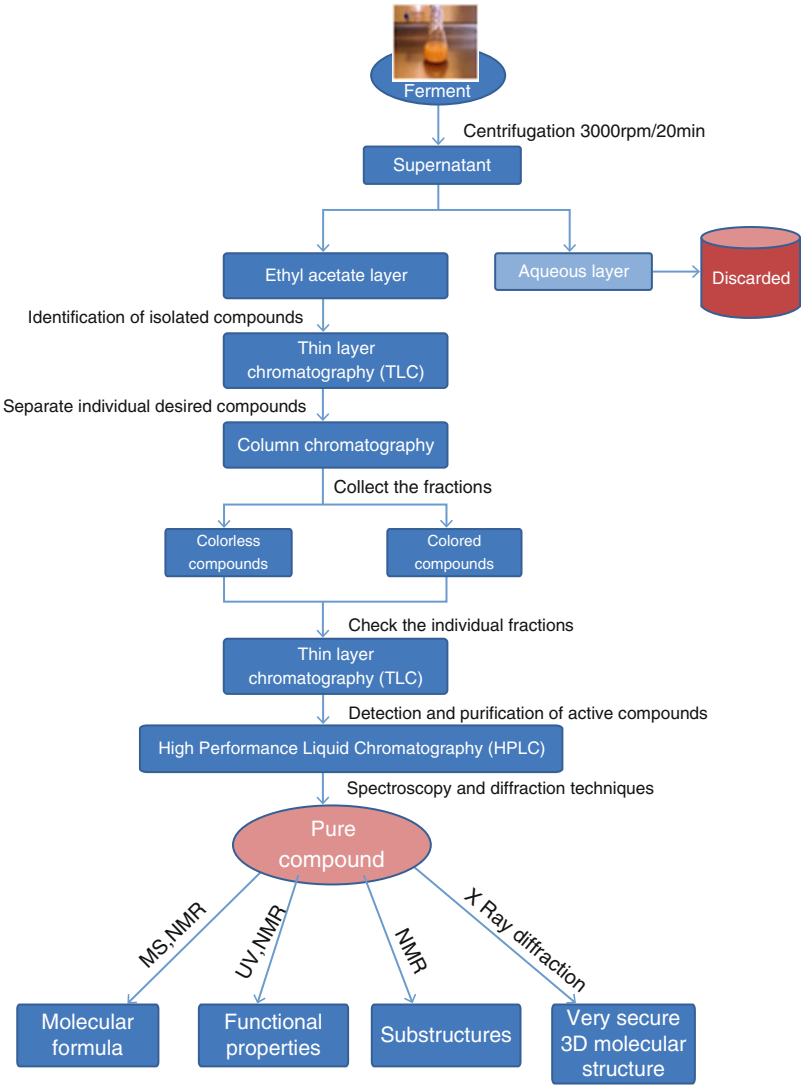

Fig. 21.9 Isolation of bioactive compound(s) from *P. indica* culture filtrate and their structural characterization

All the techniques described above in Sects. 21.3.1–21.3.6 for the isolation, identification, detection, and purification of bioactive compounds from *P. indica* culture filtrate and their structural characterization are summarized in Fig. 21.9.

21.3.7 Modern Detection Techniques

21.3.7.1 SDS–PAGE and Proteomics

After fungal components are isolated from the culture filtrate, preparative liquid-phase isoelectric focusing is used for the separation of culture filtrate proteins (CFP) that are purified by 2D gel electrophoresis (2DE). For characterization of culture filtrate proteins, aliquots of harvested proteins are screened on analytical SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Molecular mass is determined by comparing the migration distance of the protein with molecular mass standards. Exact molecular weight is determined by an electrospray ionization mass spectroscopy.

In order to fully characterize fungal plant-growth-promoting and/or virulence factors present in fungal culture filtrate, proteomic research involving a high-throughput technology can be undertaken in this "post-genomic era." Proteomic techniques (Gonzalez-Fernandez and Jorrin-Novo 2010) can make invaluable contribution in identifying and characterizing the proteins present in culture filtrate, the proteins that fungi synthesize and secrete to the environment to promote plant growth or infection.

A good experimental design is crucial to the success of any MS-based proteomic experiment. It should include all or most of the following steps:

- Proper sampling—sample collection/concentration/preparation depending on whether it is a solid or liquid material
- Protein extraction/fractionation/purification
- Protein separation
  - Gel-based (2D polyacrylamide gel electrophoresis, 2DE)
  - Gel-free (2D chromatography—coupled chromatography columns)
- MS or MS/MS analysis
  - MALDI TOF—matrix-assisted laser desorption ionization time of flight or MALDI TOF/TOF for gel-based proteomic systems
  - MudPIT—multidimensional protein identification technology/Orbitrap mass analyzer/SILAC protocol for gel-free proteomic systems
- Protein identification (identification only or identification with quantification)
- Replicate experiments
- Statistical analysis
- Validation of identification

The result of a proteomic experiment is a long list of identified proteins, and researchers have to deal with the sheer quantity of proteomic-generated data. While data analysis and bioinformatics, requiring special skills and tools, are absolutely essential for this type of experiment, statistical tools immensely help in validating the identification.

With *P. indica* fungal genome having been sequenced (Zuccaro et al. 2011) as recently as in October 2011, bioinformatic approaches can also be applied in conjunction with the genomic data, as explained in Sect. 21.3.7.2, to characterize proteins in the fungal culture filtrate.

21.3.7.2 Bioinformatics

Zuccaro et al. (2011) reported the first in-depth genomic study describing a mutualistic symbiont *P. indica* with a biphasic (live and dead) plant root colonization lifestyle strategy. With fungal genome now sequenced, bioinformatic approaches along with other tools can be used in conjunction with the genomic data on *P. indica* as described below to characterize nucleic acids and proteins present in the fungal culture filtrate.

Nucleic Acids

To investigate whether any genomic DNA from *P. indica* fungus is present in the culture filtrate, the first step is to design species-specific PCR primers using multiple DNA sequence alignments of genes from the chosen (*P. indica*) fungus and other fungal/yeast relatives, whose genome sequence is known, e.g., baker's yeast *Saccharomyces cerevisiae*. For this analysis, a multiple sequence alignment (MSA) program "MUSCLE" (Edgar 2004), offering a range of options that provide improved speed, computational complexity, and alignment accuracy, can be used. MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) is freely available. The second step then involves extracting nucleic acid using a suitable cell genomic extraction kit and carrying out PCR analysis using the species-specific primers. Any products generated should be sized and separated, and if they are of the predicted size, this would be good evidence that genomic DNA from *P. indica* fungus is present in the filtrate. For further evidence, these PCR products could be purified and sequenced, and the results used to BLAST (Camacho et al. 2009) search the DNA databases to check for exact homology to the *P. indica* or a related fungus.

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences and also helps identify members of gene families. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) is freely available.

Proteins

To investigate whether any particular proteins from *P. indica* fungus are present in the culture filtrate, the first step is to carry out a protein purification process on the filtrate. Denature the protein using beta mercaptoethanol and run on SDS

polyacrylamide gels (SDS–PAGE). In the second step, any bands present are then cut out, purified, and sent for sequencing by mass spectroscopy. Finally, the generated sequence of these proteins can then similarly be BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) searched against the protein databases for matches to the *P. indica* or a related fungus.

21.3.7.3 Raman Spectroscopy and Microscopy

Raman spectroscopy (RS), named after Sir Prof. C. V. Raman, is used as an analytical tool because it can probe the chemical composition and molecular structure of any complex matrix in any state of matter. It can yield the vibrational spectrum of an analyte (fungus—*P. indica* culture filtrate in this case) that can be regarded as its "chemical fingerprint," allowing easy identification and characterization (Baena and Lendl 2004) of the various compounds, such as hormones, proteins, enzymes, and fungal exudates that may be present in it. Recent revolutionary developments in instrumentation, lasers, and data processing methods have overcome problems, such as poor sensitivity, reproducibility, and fluorescence reduction of weak Raman signals. These have also led to the development of several advanced Raman techniques (Das and Agrawal 2011), such as Fourier transform (FT)—Raman, confocal Raman microscopy, resonance Raman spectroscopy (RRS), surface-enhanced Raman spectroscopy (SERS), surface-enhanced resonance Raman spectroscopy (SERRS), modulated Raman (MR) spectroscopy, coherent anti-stoked Raman scattering (CARS), and Raman microspectroscopy (Krafft et al. 2003).

A sketch of a modulated Raman (MR) experimental setup is shown in Fig. 21.10. RS relies on inelastic or Raman scattering of monochromatic light usually from a laser in the visible, near-infrared, or near-ultraviolet range. Photons of the laser light are absorbed by the sample and then reemitted. This results in the energy of the photons being shifted up or down (Raman shift) in comparison with the original monochromatic frequency, and this effect is called the Raman effect. One has to realize, however, that majority (~99.99 %) of the photons get scattered elastically (Rayleigh scattering) and these are of no use for molecular characterization purposes. RS is thus very weak, and special measures are adopted by using optical instruments, such as holographic gratings, notch filters, band-pass filters, and long-pass filters (as shown in Fig. 21.10) to reduce Rayleigh scattering and obtain high-quality Raman spectra.

For instance, indole-3-acetic acid (IAA)—a phytohormone of the auxin series, if present in *P. indica* culture filtrate—should give characteristic Raman peaks (Kamnev et al. 2001) corresponding to this compound. Raman spectroscopy can be used further to study the changes occurring in conjugates of auxins (Bajguz and Piotrowska 2009) in various plant species. Similarly, characterization of proteins

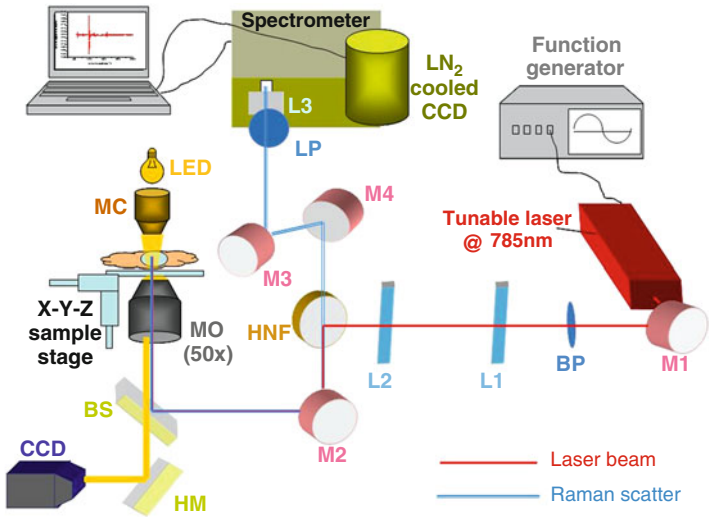

Fig. 21.10 A sketch of a modulated Raman spectroscopy (MRS) experimental setup. Abbreviations are as follows: *M* mirror, *BP* band-pass filter, *L* lens, *HNF* holographic notch filter, *HM* hot mirror, *BS* beam splitter, *MO* microscope objective, *MC* microscope condenser, *LP* long-pass filter. The setup is identical to the standard Raman (RS) but for the tunable diode laser shown here operating at 785 nm. An external waveform/function generator is connected to the tunable laser to modulate the wavelength at frequencies as low as a few mHz

present in the culture filtrate through a simple band fitting of the amide I region can be undertaken.

Raman spectroscopy can also be coupled with many hyphenated analytical techniques, such as high-performance liquid chromatography (HPLC), microchromatography, atomic force microscopy (AFM), and tip-enhanced Raman spectroscopy (TERS), which is the same as SERS.

21.3.7.4 Atomic Force Microscopy and Integrated (Colocalized) AFM + Raman

Atomic force microscopy (AFM) is a surface characterization technique in which a fine probe/tip (Si_3N_4) microfabricated at the end of a cantilever is scanned over the sample surface or the sample surface is scanned under the probe. It can be operated in either imaging or force spectroscopy (AFM–FS) mode. In imaging mode, it can generate 3D topographic and phase images of native live fungal cells (in their culture medium) and fixed cells/air-dried cells (in air) in contact or tapping mode with nanometer-scale resolution.

This information can reveal the cell wall surface architecture (Dufrene 2010; Ma et al. 2006; Holder et al. 2007; Wu et al. 2008) which is important because fungal activities are well known to be mediated through their cell walls. Fungal cells can

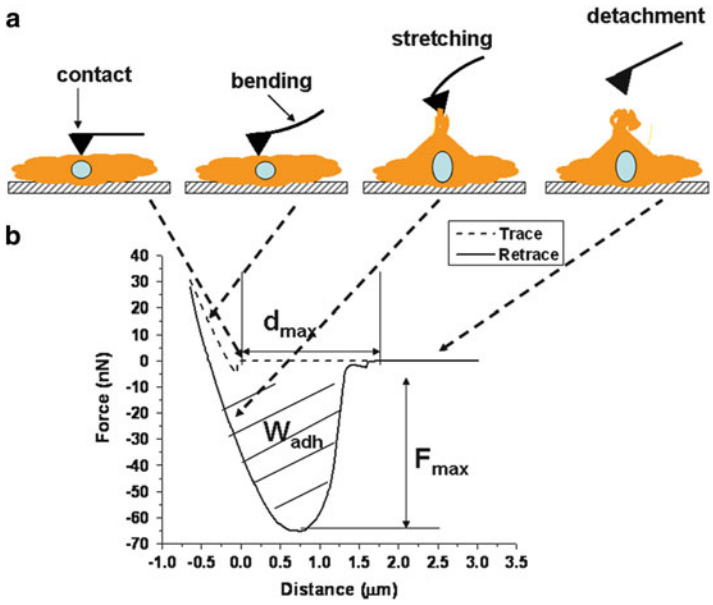

Fig. 21.11 Sketch of the stages of an AFM force spectroscopy (AFM–FS) experiment [adapted from Canetta and Adya 2011]. (**a**) Contact between the AFM probe and the sample surface, and cantilever bending with AFM probe indentation occurs during the trace (approach) cycle of the AFM experiment. On the retrace (retract) cycle, the sample surface is stretched until the point of detachment. (**b**) An example of a typical experimental *F–d* curve for a trace and retrace cycle is also presented; the stages are identified by the *arrows*. The meanings of F_{max}, d_{max}, and W_{adh} (work or energy of adhesion) are also identified

be immobilized on glass slides/cover slips/Labteks/Petri dishes precoated with poly-L-lysine, or the cells can be placed on appropriate pore-size Millipore filters. AFM–FS measures the force between the tip and the sample as a function of distance as the tip approaches the surface, jumps into contact with it, and is then retracted from the surface. The result of an FS experiment is a force–distance (*F–d*) curve (see Fig. 21.11) which can yield important information on nanomechanical/physical parameters, such as adhesion, stiffness, and elasticity of the cell wall. Force mapping (AFM–FM) generates an array of *F–d* curves over the scanned sample area from which 2D property maps can be constructed. AFM–FM can thus yield information on how the physical parameters change across a given fungal cell, thereby reflecting structural heterogeneities across nuclei, pear-shaped spores, and filamentous meshwork of interwoven hyphae. AFM–FS with biologically modified/functionalized tips can, in addition, be used to study specific (receptor–ligand) interactions with the surface-associated molecules.

The single-cell AFM approach, as discussed above, is uniquely beneficial to probe the surface of fungal cells down to molecular resolution by yielding the surface ultrastructure, its heterogeneity and associated nanophysical properties, and specific biological interactions with the surface molecules. AFM can be similarly

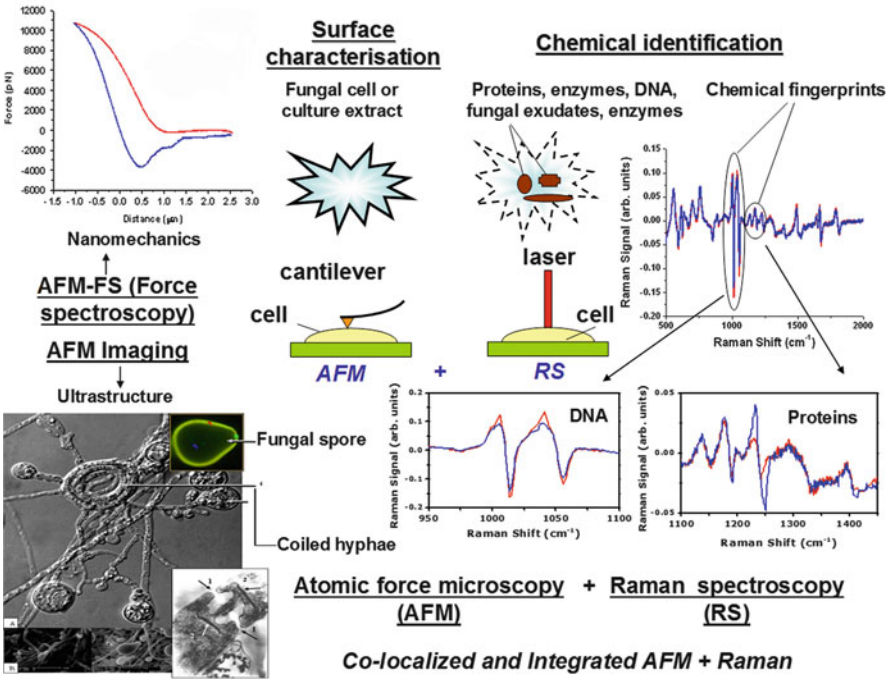

Fig. 21.12 A sketch showing the outcome from colocalized AFM + Raman experiments. Surface (AFM) + chemical (RS) characterization reveals a *complete map* of nanomechanical and biochemical fungal features. These experiments can provide sample topography and mechanical properties of the heterogeneous surface at the nanometer scale while yielding the chemical fingerprints (identification) of the same sample surface area, may it be a single fungal cell or culture extract

used to characterize the culture filtrate. The fungal culture filtrate and the control (medium without fungus) can be dried on glass slides into powdered form (under identical conditions by optimizing various parameters) to study their morphology and surface characteristics.

To reiterate, Raman microspectroscopy (RM) is a noninvasive, label-free, molecular spectroscopy method that can be used to record the vibrational spectroscopic fingerprints based on molecular bonds. It has been applied to characterize living cells. AFM is a high-resolution form of scanning probe microscopy (SPM) that can provide information, including surface topography, cell adhesion, elasticity, and stiffness of a single cell. However, the integrated AFM/RM technique can investigate both biomechanical and biochemical properties of living cells in near physiological conditions. Comprehensive sample characterization can thus be achieved by using integrated (colocalized) Raman–AFM system because it can provide (see Fig. 21.12) sample topography and mechanical properties of its heterogeneous surface at the nanoscale while yielding the chemical fingerprints (identification) of the same sample surface area, may it be a single fungal cell or dried culture filtrate.

21.3.7.5 Scanning Electron Microscopy (SEM), Environmental SEM (ESEM) + Energy Dispersive X-Rays (EDX)

SEM and AFM are complementary techniques that can be used to characterize the surface architecture of fungal spores and cells at high resolution and thus clarify our understanding of fungal biology. While AFM imaging can generate images of living and/or fixed cells with nanometer scale resolution and AFM force spectroscopy (AFM–FS) can probe their nanomechanical properties/physical parameters, SEM preparation works only under high vacuum and with fixed/frozen/dehydrated specimens. CryoSEM can offer high resolution for frozen fungal samples. Environmental SEM (ESEM), however, allows the analysis of fungal samples, including spores, under ambient conditions. Dual-beam SEM allows elemental detection via energy dispersive X-rays (EDX). AFM has similar lateral, but better, depth resolution compared to SEM and can image live cells, including growing fungal hyphae. Nevertheless, ESEM can provide complementary information to AFM on a single fungal cell or culture filtrate for their characterization.

21.4 Conclusion and Future Prospective

Several terrestrial fungi are recognized to colonize the plant root but do not cause disease. These include Mycorrhizae, dark septate Rhizoctonia, *P. indica*, *P. williamsii*, various plant-growth-promoting rhizobacteria, and *Trichoderma* spp. Many of these organisms are known for decades as agents that biocontrol plant disease, but recent studies have demonstrated that they have many other useful attributes. Typically, these fungi penetrate the outer layers of the epidermis and plant cortex and establish intense chemical and molecular communication with the plant. The chemical communicants (effectors/elicitors) released by the fungi are well documented; these include small protein, peptide, and other metabolites including volatile ones. These have been recently reviewed as has the signal transduction in plants.

We have reported that culture filtrate of plant-growth-promoting fungi also promotes early seed germination, thereby breaking the seed dormancy, causing early flowering and plant promotion. Likewise the culture filtrate obtained as a result of cocultivation with nanomaterials further enhanced the plant growth. It also is remarkable that qualitatively similar effects are induced in plants by a variety of plant-associated root-colonizing microbes (symbiotic and asymbiotic). This is, apparently, an example of convergent evolution by very dissimilar organisms. It is therefore imperative to characterize the culture filtrate for its biotechnological applications.

Acknowledgments We are grateful to our respective institutions for providing necessary resources to help us complete this work. We thank our many colleagues and collaborators, in particular Dr. Elisabetta Canetta (Cardiff University), Dr. Douglas Lester (University of Abertay

Dundee), and Dr. Krishna Bolla (Institute of Microbiology, Chinese Academy of Sciences) for fruitful discussions on several occasions. One of us (AKA) gratefully acknowledges the kind hospitality extended to him by Amity University and the sponsorship of the Royal Society of Edinburgh (RSE, UK) and Indian National Science Academy (INSA) to facilitate his recent visit to India when this work really took off. Ashok Adya is also grateful to Amity University for the kind offer of an honorary adjunct professorship. Ajit Varma is thankful to Department of Biotechnology, ICAR and DRDO, for partial financial support.

References

Allerdings E, Ralph J, Schatz PF, Gniechwitz D, Steinhart H, Bunzel M (2005) Isolation and structural identification of diarabinosyl 8-O-4-dehydrodiferulate from maize bran insoluble fibre. Phytochemistry 66:113–124

Baena JR, Lendl B (2004) Raman spectroscopy in chemical bioanalysis. Curr Opin Chem Biol 8:534–539

Bagde US, Prasad R, Varma A (2010b) Interaction of mycobiont: *Piriformospora Indica* with medicinal plants and plants of economic importance. Afr J Biotechnol 9:9214–9226

Bagde US, Prasad R, Varma A (2011) Influence of culture filtrate of *Piriformospora indica* on growth and yield of seed oil in *Helianthus annuus*. Symbiosis 53:83–88

Bajguz A, Piotrowska A (2009) Conjugates of auxin and cytokinin. Phytochemistry 70:957–969

Baldi A, Jain A, Gupta N, Srivastava AK, Bisaria VS (2009) Co-culture of *Linum album* cells and *Piriformospora indica* for improved production of phytopharmaceuticals. In: Varma A, Kharkwal AC (eds) Soil biology, symbiotic fungi, edn 18. Springer, Berlin, Heidelberg, p 361

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuate*. Oecologia 146:234–43

Blundell TL, Patel S (2004) High-throughput X-ray crystallography for drug discovery. Curr Opin Pharmacol 4:490–496

Bubb WA (2003) NMR spectroscopy in the study of carbohydrates: characterizing the structural complexity. Concepts Magn Reson Part A 19A:1–19

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) Blast+: Architecture and applications. BMC Bioinformatics 10:421, Open access: http://www.biomedcentral.com/1471-2105/10/421

Canetta E, Adya AK (2011) Atomic force microscopic investigation of commercial pressure sensitive adhesives for forensic analysis. Forensic Sci Int 210:16–25

Cao J, Wei Y-J, Qi L-W, Li P, Qian Z-M, Luo H-W, Chen J, Zhao J (2008) Determination of fifteen bioactive components in Radix et Rhizoma Salviae Miltiorrhizae by high-performance liquid chromatography with ultraviolet and mass spectrometric detection. Biomed Chromatogr 22:164–172

Chauhan AK, Das A, Kharkwal AC, Varma A (2006) Impact of micro-organisms on environment and health. In: Chauhan AK, Varma A (eds) Microbiology series—microbes: health and environment. IK International Publishing House Pvt. Ltd., New Delhi, pp 1–12

Cordell GA, Shin YG (1999) Finding the needle in the haystack. The dereplication of natural product extracts. Pure Appl Chem 71:1089–1094

Das RS, Agrawal YK (2011) Raman spectroscopy: recent advancements, techniques and applications. Vib Spectroscopy 57:163–176

De Luca V, St Pierre B (2000) The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci 5:168–173

Dixon RA (2001) Natural products and plant disease resistance. Nature 411:843–847

Dufrene YF (2010) Atomic force microscopy of fungal cell walls: an update. Yeast 27:465–471

Duus J, Gotfredsen CH, Bock K, Seetharaman K (2000) Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations. Chem Rev 100:4589–4614

Edgar RC (2004) Muscle: a multiple sequence alignment method with reduced time and space complexity. BMS Bioinform 5:113

Felle HH, Waller F, Molitor A, Kogel KH (2009) The mycorrhiza fungus *Piriformospora indica* induces fast root-surface pH signalling and primes systemic alkalinisation of the leaf apoplast upon powdery mildew infection. Mol Plant Microbe Interact 22:1179

Frisvad JC, Andersen B, Thrane U (2008) The use of secondary metabolite profiling in chemotaxonomy of fiin chemot fungi. Mycol Res 112:231–240

Gangadevi V, Muthumary J (2008) Isolation of *Colletotrichum gloeosporioides*, a novel endophytic taxol-producing fungus from the leaves of a medicinal plant, *Justicia gendarussa*. Mycologia Balcanica 5:1–4

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243–255

Giovannetti M, Sbrana C (1998) Meeting a non-host: the behaviour of AM fungi. Mycorrhiza 8:123–130

Gonzalez-Fernandez R, Jorrin-Novo JV (2010) Proteomics of plant pathogenic fungi. J Biomed Biotechnol 2010:1–36

Hamburger M, Hostettmann K (1991) Bioactivity in plants: the link between phytochemistry and medicine. Phytochemistry 30:3864–3874

Hill TW, Kafer E (2001) Improved protocols for Aspergillus minimal medium: trace element and minimal medium salt stock solutions. Fungal Genet Newsletter 48:20–21

Holder DJ, Kirkland BH, Lewis MW, Keyhani NO (2007) Surface characteristics of the entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana*. Microbiology 153:3448–3457

Kamnev AA, Shchelochkov AG, Perfiliev YD, Tarantilis PA, Polissiou MG (2001) Spectroscopic investigation of indole-3-acetic acid interaction with iron(III). J Mol Struct 563–564:565–572

Kim BK, Moon SS, Hwang BK (1999) Isolation, identification, and antifungal activity of a macrolide antibiotic, oligomycin A, produced by *Streptomyces libani*. Can J Bot 77:850–858

Krafft C, Knetschke T, Siegner A, Funk RHW, Salzer R (2003) Mapping of single cells by near infrared Raman microspectroscopy. Vib Spectroscopy 32:75–83

Levitt MH (2001) Spin dynamics: basics of NMR. Wiley, NY

Ma H, Snook LA, Tian C, Kaminsky SG, Dahms TE (2006) Fungal surface remodeling visualized by atomic force microscopy. Mycol Res 110:879–886

Mcbrierty VJ, Packer KJ (1993) Nuclear magnetic resonance in solid polymers. Cambridge University Press, New York, pp 37–103

Mitsuhashi H, Nagai U, Muramatsu T, Tashiro H (1960) Chem Pharm Bull 8:243–245

Molitor A, Koegel KH (2009) Induced resistance triggered by *Piriformospora indica*. Plant Signal Behav 4:215–216

Niessen WMA, van der Greef J (1992) Liquid chromatography–mass spectrometry, principles and applications. Marcel Dekker, New York

Oelmüller R, Sherameti I, Tripathi S, Varma A (2009) Piriformospora indica, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Oelmüller R, Shrivastava N, Pohani M, Upadhyaya KC, Sherameti I, Murugan P, Singh S, Varma A (2011) Microbe–microbe, microbe–plant biocommunication. In: Witzany G (ed) Biocommunication in soil microorganisms, Soil biology 23. Springer, Berlin, Heidelberg, 10.1007/978-3-642-14512-4_18

Pauli GF, Jaki BU, Lankin DC (2005) Quantitative ^{1}H NMR: development and potential of a method for natural products analysis. J Nat Prod 68:133–149

Peškan-Berghöfer T, Shahollari B, Pham HG, Hehl S, Markent C, Blanke V, Kost G, Varma A, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represent a novel system to study beneficial plant–microbe interactions and involve in early

plant protein modifications in the endocytoplasmic reticulum and at the plasma membrane. Physiol Plant 122:465–471

Pichersky E, Gang DR (2000) Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci 5(10):439–445

Queiroz EF, Wolfender JL, Atindehou KK, Traore D, Hostettmann K (2002) On-line identification of the antifungal constituents of Erythrina vogelii by liquid chromatography with tandem mass spectrometry, ultraviolet absorbance detection and nuclear magnetic resonance spectrometry combined with liquid chromatographic micro-fractionation. J Chromatogr A974:123–134

Rochfort S (2005) Metabolomics reviewed: a new "omics" platform technology for systems biology and implications for natural products. Res J Nat Prod 68:1813–1820

Schafer P, Khatabi B, Kogel KH (2007) Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism. FEMS Microbiol Lett 275:1–7

Schaneberg BT, Crockett S, Bedir E, Khan IA (2003) The role of chemical fingerprinting: application to *Ephedra*. Phytochemistry 62:911–918

Schulz B, Boyle C, Draeger S, Rommert AK, Krohn K (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol Res 106:996–1004

Singh HP, Batish DR, Kaur S, Kohil RK (2003) Phytoxic interference of *Ageratum conyzoides* with Wheat (*Triticus aestivation*). J Agron Crop Sci 189:341–346

Siqueira JO, Safir GR, Nair MG (1991) Stimulation of vesicular arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. New Phytol 118:87–93

Sirrenberg A, Göbel C, Grond S, Czempinski N, Ratzinger A, Karlovsky P, Santos P, Feussner I, Pawlowski K (2007) *Piriformospora indica* affects plant growth by auxin production. Physiol Plantarum 131:581–589

Spevacek J, Brus J (2008) Solid-state NMR studies of polysaccharide systems. Macromol Symposia 265:69–76

Stein E, Molitor A, Kogel KH, Waller F (2009) Systemic resistance in Arabidopsis conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signalling and the cytoplasmic function of NPR1. Plant Cell Physiol 49:1747–51

Suman PR, Jain VK, Varma A (2010) Role of nanomaterials in symbiotic fungus growth enhancement. Curr Sci 99:1189–1191

Vadassery J, Oelmüller R (2009) Calcium signaling in pathogenic and beneficial plant microbe interactions: what can we learn from the interaction between *Piriformospora indica* and *Arabidopsis thaliana*. Plant Signaling Behav 12:4–11

Vadassery J, Ranf S, Drzewiecki C, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R (2009) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. Plant J 59: 193–206

Varma A, Verma S, Sudha A, Sahay N, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:2741–2744

Varma A, Rai MK, Sudha A, Sahay N (2000) Microbial biotechnology: new paradigms and role in sustainable agriculture. In: Rajak RC (ed) Microbial biotechnology for sustainable development and productivity. Scientific Publishers, New Delhi, pp 22–37

Varma A, Singh A, Sudha Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: an axenically culturable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) The mycota IX. Springer, Berlin, Heidelberg, pp 125–150

Verpoorte R (1998) Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. Drug Dev Trends 3:232–238

Wolfender JL, Rodriguez S, Hostettmann K (1998) Liquid chromatography coupled to mass spectrometry and nuclear magnetic resonance spectroscopy for the screening of plant constituents. J Chromatogr A 794:299–316

Wolfender JL, Ndjoko K, Hostettmann K (2008) Liquid chromatography with ultraviolet absorbance–mass spectrometric detection and with nuclear magnetic resonance spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites. J Chromatogr A 1000:437–455

Wu J-Z, Lin Y, Zhang X-L, Pang D-W, Zhao J (2008) IAA stimulates pollen tube growth and mediates the modification of its wall composition and structure in *Torenia fournieri*. J Exp Bot 59:2529–2543

Xu Q, Bush CA (1996) Molecular modeling of the flexible cell wall polysaccharide of Streptococcus mitis J22 on the basis of heteronuclear NMR Coupling Constants. Biochemistry 35:14521–14529

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T, Tuteja N, Saxena AK, Johri AK (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in the phosphate transport to the host. Plant J Biochem Mol Biol 285:26532–26544

Yang L, Zhang LM (2009) Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. Carbohydr Polym 76:349–361

Zjawiony JK (2004) Biologically active compounds from aphyllophorales (Polypore) fungi. J Nat Prod 67:300–310

Zuccaro A, Lahrmann U, Guldener U, Langen G, Pfiffi S, Biedenkopf D, Wong P, Samans B, Grimm C, Basiewicz M, Murat C, Martin F, Kogel K-H (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. PLoS Pathog 7:1–26

Chapter 22
Mass Cultivation of *Piriformospora indica* and Sebacina Species

Anjana Singh, Kartikeya Rajpal, Mahip Singh, Amit C. Kharkwal, Monika Arora, and Ajit Varma

22.1 Introduction

Most bacteria and fungi can be cultivated in various types of media, but very little literature is available for cultivation of symbiotic fungi. The development of method, which would enable the mass culture of symbiotic fungi, may be conveniently applied to studies from practical and theoretical standpoints.

22.2 Arbuscular Mycorrhizal Fungi

Arbuscular Mycorrhizal Fungi (AMF) are obligate symbiotic fungi that can grow only in the presence of a living plant, which means that their propagation requires the use of pot cultures, either in growth chambers or in greenhouses. Therefore there are few types of cultures that can be used for the mass cultivation of Sebacinales for its use and study. Some cultures are follows:

1. Trap cultures
2. Monospecific cultures
3. Pot cultures
4. Root Organ culture

A. Singh (✉)
Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
e-mail: anjanas67@yahoo.com

K. Rajpal • M. Singh • A.C. Kharkwal • M. Arora • A. Varma
Amity Science and Technology Foundation, Amity Institute of Microbial Technology (AIMT), Block 'A', Amity Campus Sector 125, Noida 201303, UP, India
e-mail: kartikeya.rajpal@yahoo.com; mksingh@amity.edu; ackharkwal@amity.edu; marora4@amity.edu; ajitvarma@amity.edu

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1_22,

22.2.1 Trap Cultures

Trapping helps to obtain healthy spores of colonizing fungi for identification and as inoculum to establish monospecific cultures. Field soil bear problems like (a) soil fungi might appear healthy but are not viable (some persisting as dead husks for years or possibly decades), (b) they lose or change appearance of their structural characters in response to root pigments, soil chemistry, temperature, moisture, and microbial activity, and (c) they represent only those colonizing arbuscular fungi with enough activity and biomass to trigger sporulation. Trap cultures depend on the biotic and abiotic factors that go into growth, reproduction, and helps in screening of colonization and sporulation bringing up good results. When species richness is high and colonization by all members of the assemblage is relatively low (e.g., arid environments), most if not all-indigenous fungi will sporulate after 2–3 successive propagation cycles. But when species richness and colonization are both moderate to high, often only the most aggressive species will sporulate and 2–3 successive propagation cycles could have the opposite effect of yielding just 1–2 sporulating species. In the following circumstances trap cultures are important.

- If the mycorrhizae are present in the roots of a plant community, but little or no sporulation occurs (a common condition in many arid and hydric sites).
- Soil that is high in microbial activity, especially those in tropical environments (high temp, moisture) and containing high organic matter. In these environments, a high proportion of spores undergo so much structural changes or degradation that discovery and identification of species is difficult to impossible.

Helps to obtain abundant healthy spores of different species and establish monospecific cultures for various research problems or to develop "custom inoculum" consisting of well-defined species mixtures.

22.2.2 Monospecific Cultures

Trap cultures are stored for at least 30 days before spores are taken out for inoculation onto seedlings. Some species spores (particularly those in *Acaulospora* and *Entrophospora*) appear to require a dormancy period before they become infective; however, this can vary with habitat. For trap cultures that are to be propagated for 1–2 additional cycles, the bagged material is used as inoculum (undiluted) and set up within 30 days of harvest. In extremely low sporulation shoots are removed and the pot contents are reseeded.

22.2.2.1 Spore Collection

A single species cultures are started from carefully selected spores. The healthy and infective spores can be isolated from a field soil, but probability of success varies

greatly with soil type, amount of organic matter, and sometimes environmental conditions or seasons. Success appears great when sandy low organic matter soils are used. Consistent results are obtained using spores collected from pot cultures for following reasons: (1) many spores are newly formed and thus are healthy and of the same age and (2) morphological distinctions between species are easier to detect (minimizing contamination).

Spores are extracted from the source material (field or culture) 2–3 days before inoculation onto plant seedlings using sucrose density-gradient centrifugation. After repeated washing, the spores of each target species are collected manually with a pasteur pipette and stored in a watchglass (or sealed in petri dish) at 4 °C. Spores are examined carefully till the day of inoculation. Any changes in morphology (e.g., loss of contents, collapse, color change) or parasitism are removed. Before inoculation, water is added to the spore preparation, the watch glass is agitated, and any particles, hyphal fragments, or atypical spores are removed. Containers (presterilized in 10 % bleach) are first filled to the top with sterile sand:soil mix, labels applied, and 6–7 cm deep holes made in centers with a sterile glass rod. They are arranged in the same pattern as spores are distributed in microtiter wells to avoid any confusion at the time of inoculation. An alcohol lamp and a beaker of 95 % ethanol are set up for flaming the glass rod after each transplant operation.

22.2.2.2 Seedling Preparation

Approximately 12 days before a monospecific culture is planned on being set up, approximately 40–50 sorghum seeds are evenly spaced in a 15-cm diameter pot using our standard sand–soil mix. This seeding rate provides enough seedlings for a given "run," and they are far enough apart to separate without much damage to root systems. Sorghum is used in place of sudangrass because it has a harder root system that buffers the seedling against transplant shock.

Pot contents can be removed intact by 12 days. The mass is placed in a large glass bowl filled with water so that it is completely immersed. Roots are gently teased from the growth medium and left undisturbed for at least 30 min.

22.2.3 Pot Cultures

Glomalean are not cultivable on the synthetic medium. AMF are usually propagated by growing them on a living host plant in soil pot cultures. Mother soil cultures from vermiculite balls or soil, are all micropropagated in the sterile substratum. The plastic or earthenware flowerpots are used for the mass culture cultivation. An appropriate host can be used. A sterile soil–sand (3:1) mixture can be used as substratum. The soil was autoclaved thrice on alternate days and air-dried. The river bed sand is soaked in 10 % HCl overnight and then washed under running tap water

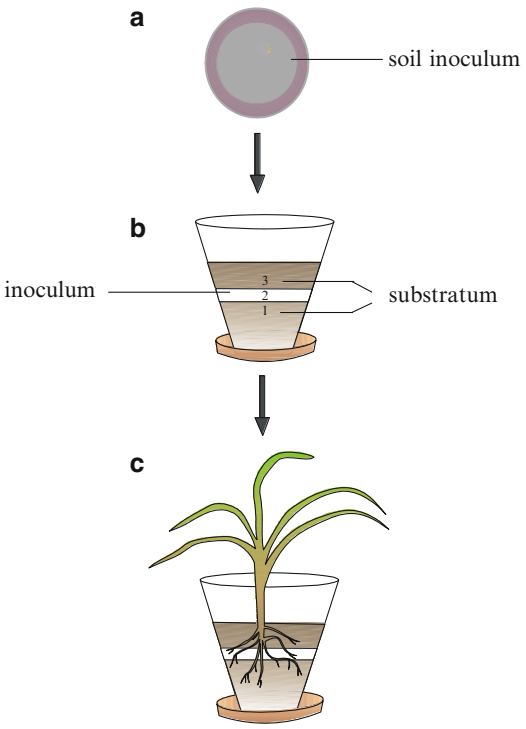

Fig. 22.1 Polyethylene pots of ½ kg capacity contained autoclaved sand and soil mixture (1:3) of pH 7.0. (**a**) Soil inoculum consisting of spores, hyphae and colonized root propagules, (**b**) 1 cm layer of inoculum was sandwiched, (**c**) micropropagated plantlets were plated upto second layer towards the upward direction. Gently little sterile tap water was sprinkled to moisten the upper soil layer

till the pH attains to neutrality. Air-dried mixture of soil and sand in the ratio of 3:1 (Feldmann and Idczak 1994) is added in the flowerpots. The ½ kg flowerpots are also sterilized by 70 % ethanol and are half filled with this mixture and the inoculum is layered over it. The pots are then filled with soil–sand mixture of pH 7.0. Five holes are made in each pot and in each hole approximately 1 g of endomycorrhizal inoculum (80 spores/10 g soil/fungal propagules) is added. Then, five germinated seeds of 10-mm length are placed 1–2 cm above the inoculum layer in the marked holes in each pot (Fig. 22.1a). The pots are maintained in green house at 30 ± 2 °C with 16 h light/8 h dark and relative humidity 60–70 %, with light intensity of 1,000 lux. The roots are checked for colonization after 15–20 days. Root pieces with spores and hyphal fragments can be used as live propagule (inoculum) for experiments or to introduce fungi into soils. The soil cultures along with the root propagules obtained after 4 months can be stored in cold room for further use.

For the comparative photomycobiont growth of Sebacinales (*P. indica* and *S. vermifera* sensu), a disc (4-mm diameter) of inoculum infested with hyphae and spores can be taken and placed at the marked hole at the depth of 2 cm per plant (Fig. 22.1b). After the placement of the seedling, the soil substratum is used to cover the hole.

22.2.4 Root Organ Culture

Glomales are symbiotic microorganisms that obligatorily live in association with plants. As a result inocula of AMF are grown in pot-culture maintained under greenhouses or growth chamber conditions, with the constant risk of inocula contamination. Consequently, several species are now routinely cultivated in vitro propagation techniques, allowing the development of contaminant-free culture. Glomales cultures can be maintained with excised roots in pure and viable for long-term periods. But only half-dozen strains are currently available in monoaxenic culture. Thus a very promising avenue for the development of studies on biodiversity, taxonomy, ontogeny of spores, and verification of the mycorrhizal potential of the isolated AMF strains have been opened. The positive point is that the same fungus can be grown on a large number of plant species.

Growth media commonly used are the modified Strullu-Romand (MSR) medium (Declerck et al. 1998) which is a modification of the Strullu and Romand (1986) medium and the minimal medium of Bécard and Fortin (1988).

Mugnier and Mosse (1987) developed Ri-plasmid-transformed root cultures. Later the culture method has been developed by Yve Piché and colleagues in Canada (Becard and Piche 1989, 1990; Chabot et al. 1992) and by Wu in Taiwan and Declerck and colleagues in Belgium. The two most widely used species in this system are *Glomus intraradices* and *Gigaspora margarita* and others are like *G. proliferum* (Declerck et al. 2000), *G. cerebriforme*, *G. lamellosum*, and *G. rosea* in the axenic cultures. The multidisciplinary approach has been recently used for the description of the new species *G. proliferum* (Declerck et al. 2000). Although the use of mycorrhizal root organ cultures has allowed the clarification of many aspects of the AM symbiosis, the in vitro system has obvious limitations. Possibly the most important fact is that a root organ replaces the plant host. As a result, the absence of photosynthetic tissues, a normal hormonal balance, and physiological source–sink relationships affect the symbiotic benefit to the plant. To compensate for the absence of photosynthates, sucrose is added to the culture. Therefore, the root–fungus interface is bathed in a sugar solution. In this case, carbohydrates reach the cortex and the presence of sugars at this interface modifies the biochemistry of the plant–fungal interaction. This might explain why arbuscules and vesicles are often scarce in Ri T-DNA transformed carrot roots, despite abundant intracortical mycelium. However, this hypothesis is not supported by recent work performed with *M. truncatula* hairy roots inoculated with *G. intraradices*, which exhibit colonization levels of up to 40 %. This being mostly arbuscular fungi (G. Bécard, unpublished data). Despite the artificial nature of this in vitro system, there are several legitimate reasons for its continued use in the study of AMF. The fungus forms typical colonization structures (i.e., appressoria, arbuscules, and vesicles) and produces profuse extraradical mycelium and spores. The production of spores, morphologically and structurally similar to those produced in pot cultures, and of intraradical structures capable of initiating new mycorrhizal symbiosis following subculturing indicates that the fungus is able to complete its life cycle. Therefore, it can be assumed that the mechanisms controlling the early colonization steps reflect those occurring in vivo.

The use of the AM root organ culture technique has important implications for the production of AM inocula for research and commercial purposes. Although the results from most industry-based research are not generally publicly available. Moutoglis and Béland (2001) provided a brief insight into some of the potential techniques, and Jolicoeur et al. (1999) and Jolicoeur and Perrier (2001) proposed a bioreactor-based production technique using root organ cultures. The mycorrhizal root organ culture has proven useful for taxonomists and physiologists and potentially useful for geneticists (Fortin et al. 2002).

Piriformospora indica, due to its characteristics spore morphology, improves the growth and overall biomass production of different plants, herbs, trees, etc., and can easily be cultivated on a number of complex and synthetic media (Singh et al. 2003a, b; Varma et al. 1999, 2001). Significant morphological and quantitative changes have been detected when the fungus was grown on different nutrient composition with no obvious negative effect on plants. Various media can be used to study the morphological, physiological, biochemical, molecular, immunological-signal transduction, and biotechnological applications.

22.3 Cultivation of Sebacinales

Fungi are heterotropic for carbon compounds and these serve two essential functions in fungal metabolism. The first function is to supply the carbon needed for the synthesis of compounds which comprise living cells, proteins, nucleic acids reserve food materials, etc. Second, the oxidation of carbon compounds produces considerable amounts of energy. Fungi can utilize a wide range of carbon sources such as monosaccharides, disaccharides, oligosaccharides, polysaccharides, organic acids, and lipids. Carbondioxide can be fixed by some fungi but cannot be used as an exclusive source of carbon for metabolism. Fungus can be successfully cultivated on a wide range of synthetic solidified and broth media, e.g., MMN1/10, modified aspergillus, M_4N, MMNC, MS, WPM, MMN, Malt–Yeast Extract, PDA, and aspergillus. Among the tested media, most optimum was aspergillus (Kaefer 1977). However, other media were helpful in carrying out several physiological and molecular experiments (Pham et al. 2004). A typical growth on solidified aspergillus medium after 7 days show a rhythmic growth. Mycelium at the terminal end produced a large number of chlamydospores and after 24–48 h, they germinate. Then growth was stopped and spores were produced. Physiological reason for this unique phenomenon is not yet known, although this tendency has been recorded for several other members of Basidiomycetes. Fungus grows profusely on shaking broth aspergillus medium. The temperature range of the fungal growth is 30 °C (25–35 °C); the optimum temperature is 30 °C and pH 5.8 (4.8–6.8), respectively. On Modified Melin-Norkran medium sparsely running hyaline hyphae on the agar surface was seen, while on potato dextrose agar deep furrows with a strong adhesion to the agar surface was visible. This sharp mode of growth was not observed when fortified with malt extract and normal aspergillus

(Kaefer) medium. In contrast to aspergillus medium, shaking conditions on MMN broth medium invariably inhibited the growth. The explanation for this observation is not known. Fungal growth acidifies the medium within 5 days to pH 4.4. Buffered medium prevented the reduction of pH. MES in the range of 25–100 mM was used.

P. indica colonizes the root of a wide range of hosts but does not invade the root of myc$^-$ plants like pea (*Pisum sativum*) and soyabean (*Glycin max*). When the fungus was confronted with mutants, the plants were suppressed and the fungal morphology was severely affected. Homogenous sporulation was observed on scanning electron microscope, while they were heterogeneous in myc$^-$ plants. Mycelia turned brown and also produced a copious amount of mucilage.

22.4 Carbon and Energy Sources

Individual sugars were homogeneously added into the minimal broth at a rate of 1.0 % (w/v) in all the treatments. They were included into the medium separately before sterilization. In all the sugar-added media, the growth was recorded better than the control. There were not many changes in the growth except in raffinose. In case of raffinose, the growth was less than the other mono- and disaccharides. There were no changes in the color of mycelium. Good growth was recorded in media containing maltose followed by xylose, sucrose, rhamnose, arabinose, glucose, lactose, and mannose, respectively. The end pH did not alter significantly, however, was lower than the control.

Fungus grew better when glucose was used as carbon and energy nutrients as compared to sucrose, and followed by fructose (Fig. 22.2). On supplementation of glucose (1.5 % w/v) and sucrose (0.5 % glucose, sucrose, respectively) together, the former was consumed completely and then the later was metabolized by active production of invertase. In summary, it may be stated that (1) *P. indica* metabolizes sucrose, glucose, and fructose; (2) apparently good growth was observed on glucose, followed by sucrose and fructose, respectively, and (3) glucose (0.5 %) and fructose (0.5 %) when used in combination, glucose was preferentially taken up, fructose largely remained unutilized. Morphology of the colonies was quite different on respective sugar supplementation. Sporulation was severely affected.

22.5 Biomass on Individual Amino Acids

Addition of glycine promoted the fungal growth. Although initial pH of the broth was set to 4.82, but after the termination of the experiment, the end pH was 5.01. In methionine-added medium, the growth was comparatively better than other amino acid and end pH 4.71, but was lower than glycine. In serine, the fungal biomass was higher than alanine but reported less than methionine, end pH in case of serine was recorded as 5.82. Phenylalanine did not support growth and the end

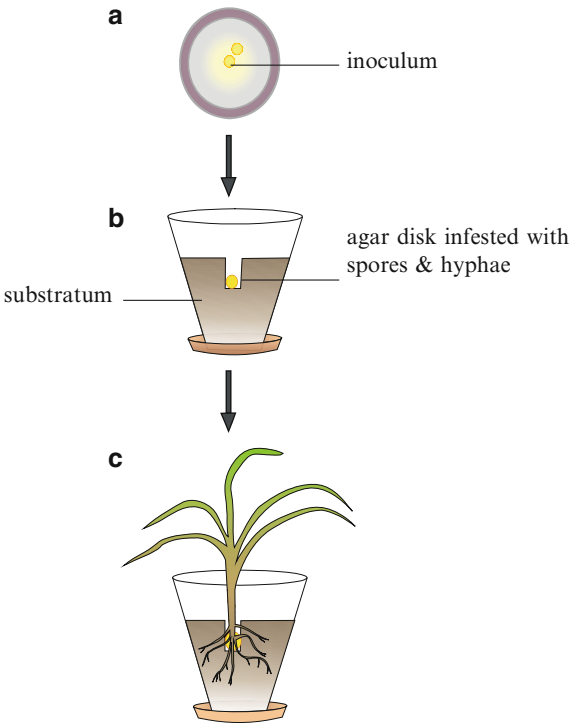

Fig. 22.2 Polyethylene pots of ½ kg capacity contained autoclaved sand and soil mixture (1:3) of pH 7.0. (**a**) Culture inoculum pertidish consisting of spores and hyphae, (**b**) a hole was made in the centre of the pot upto 2 cm deep with a help of surface sterilized especially designed plastic rod. Agar disc (4 mm diameter) infested with spores and hyphae was placed in the hole, (**c**) micropropagated plantlets were inserted into the hole towards the upward direction and top was covered with the same substratum. Gently little sterile tap water was sprinkled to moisten the upper soil layer

pH was extremely acidic. Not much difference in mycelial growth was observed in media containing glutamine, asparagine, and histidine although substantial difference in the end pH of these amino acids fortified culture broth was recorded. Compared to control, growth was not good in the incubation media containing arginine, pH changed to alkaline (8.93) than the expected value of 4.82 pH and was low in aspartic acid. Growth in cysteine was poor and the end pH was extreme acidic 1.90.

22.6 Growth on Complex Media

Fungus grown in minimal broth was transferred onto one set of fresh minimal media containing agar and one set of fresh minimal media containing agarose. In the minimal broth, the complex substances like soil-extract, malt-extract, peptone, beef-extract, yeast-extract, and casamino hydrolysate were added individually at a rate of 1 % (w/v). pH of the media was adjusted to 4.8 and autoclaved. Compared to all other media used, excellent growth of mycelium was recorded in the incubation broth fortified with casamino hydrolysate–HCl. Growth in beef, yeast, malt

extracts, and peptone showed moderate growth. Soil extracts did not support the fungus growth.

22.7 Phosphatic Nutrients

Phosphorus is an essential mineral for the growth of the Sebacinales. Optimum growth was obtained on supplementing the modified aspergillus medium with monohydrogen potassium/sodium phosphate in equimolar concentration. Interestingly, the fungus utilized tri-poly phosphate and solubilized insoluble calciumhydrogen phosphate. Acid phosphatases were observed to be active in *P. indica* mycelium (Varma et al. 2001). The fungus could utilize a variety of inorganic and organic phosphate sources which is in accordance with the broad range of the substrates utilized by the acid phosphatases of many fungi. Besides this, phosphate starvation of *P. indica* led to an overall (27 %) increase in the intracellular acid phosphatase activity. This increase was probably due to the appearance of a P-repressible isoform of acid phosphatase in addition to the constitutive one observed in the enzyme staining of the native polyacrylamide gels. The significance of these enzymes in the phosphate transport needs to be further substantiated by the studies on the plant roots colonized with *P. indica*.

22.8 Composition of Media

(a) MMN 1/10 (Herrmann et al. 1998)

Composition	*g/l*
$CaCl_2 \cdot 2H_2O$	0.07
$MgSO_4 \cdot 7H_2O$	0.15
NaCl	0.03
$(NH_4)_2HPO_4$	0.03
KH_2PO_4	0.05
Trace elements	*mg/l*
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.09
H_3BO_4	1.55
$CuSO_4 \cdot 5H_2O$	0.13
KCl	3.73
$MnSO_4 \cdot H_2O$	0.84
$ZnSO_4 \cdot 7H_2O$	0.58
Fe-EDTA	*mg/l*
$FeSO_4$	8.50
EDTA	1.50
Agar	20.0 g

(b) Modified aspergillus (Varma et al. 2001)
The media composition was same, except yeast extract, peptone, and casamino acid were reduced to 1/10 in quantity.

(c) M_4N (Mukerji et al. 1998)

Composition	*g/l*
D-Glucose	10.0
$(NH_4)_2HPO_4$	0.25
KH_2PO_4	0.50
$MgSO_4{\cdot}7H_2O$	0.15
$CaCl_2{\cdot}2H_2O$	0.05
Ferric citrate (2 % ferric citrate, 2 % citric acid v/v)	7.0 ml
NaCl	0.025
Thaimine HCl	100.0 µg
MES	2.5
Malt extract	1.5
Yeast extract	1.5
Agar	15.0
pH	5.6

(d) MMNC (Kottke et al. 1987; Marx 1969)

Composition	*g/l*
Glucose	10.0
$CaCl_2{\cdot}2H_2O$	0.07
$MgSO_4{\cdot}7H_2O$	0.15
NaCl	0.03
$(NH_4)_2HPO_4$	0.25
KH_2PO_4	0.5
Casein-hydrolysate	1.0
Malt extract	5.0
Trace elements	*mg/l*
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	0.02
H_3BO_4	1.55
$CuSO_4{\cdot}5H_2O$	0.13
KCl	3.73
$MnSO_4{\cdot}H_2O$	0.85
$ZnSO_4{\cdot}7H_2O$	0.58
Fe-EDTA	*mg/l*
$FeSO_4$	8.5
EDTA	1.5
Vitamins	*mg/l*
Thiamine	0.1
Riboflavin	0.1
pH	5.6
Agar	20.0 g

(e) MS (Murashige and Skoog 1962)

Chemicals	*mg/l*
Macronutrients	
NH_4NO_3	0.5
KNO_3	1650.0
$CaCl_2{\cdot}2H_2O$	900.0
$MgSO_4{\cdot}7H_2O$	440.0
KH_2PO_4	370.0
Micronutrients	
KI	170.0
H_3BO_3	0.83
$MnSO_4{\cdot}H_2O$	6.20
$ZnSO_4{\cdot}7H_2O$	15.60
$NaMoO_4{\cdot}2H_2O$	8.60
$CuSO_4{\cdot}5H_2O$	0.25
$CoCl_2{\cdot}H_2O$	0.025
Iron source	
$Na_2{\cdot}EDTA$	0.025
$FeSO_4{\cdot}7H_2O$	37.30
Vitamins	
Nicotinic acid	27.8
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Myo-inositol	100.0
Agar	0.7 % (w/v)
Sucrose	3.0 % (w/v)
PH	5.6–5.7

Each chemical was dissolved in bidistilled water. pH of the medium was adjusted using 1N NaOH/HCl before autoclaving at 121 °C, 15 lbs for 20 min. Stock solutions were stored at 4 °C except organic supplements, which were stored at −20 °C.

(f) WPM ("Woody Plant Medium" for *Populus*) Ahuja (1986)

Composition	*g/l*
Sucrose	20.0
K_2SO_4	1.00
$Ca\ (NO_3)_2{\cdot}4H_2O$	0.73
NH_4NO_3	0.40
$MgSO_4{\cdot}7H_2O$	0.37
Myo-inositol	0.10
Agar	7.00

Add 700 ml H_2O, adjust pH to 5.8 using 3.7 % HCl (ca. 9.5 µl)
Add after autoclaving sterile phosphate solution (0.17 g KH_2PO_4 dissolved in 270 ml H_2O) + 15 µl NaOH (saturated)
10 ml of trace element stock solution (see below)
10 ml Fe-EDTA (see below)

10 ml Glycine stock solution (100×: solve 20 mg in 100 ml)
1 ml Thiamine stock solution (1,000×: solve 10 mg in 100 ml)
1 ml Nicotinic acid stock solution (1,000×: solve 50 mg in 100 ml)
1 ml $CaCl_2$ stock solution (1,000×: solve 3.6 g in 50 ml)
250 µl Pyridoxine stock solution (4,000×: solve 40 mg in 100 ml)
100 µl $CuSO_4$ stock solution (10,000×: solve 25 mg in 100 ml)
Sterilize by filtration before adding
100× trace element stock solution (g/l, autoclave, store at 4 °C):

$MnSO_4 \cdot H_2O$	2.23
$ZnSO_4 \cdot 7H_2O$	0.86
H_3BO_4	0.62
Ammonium molybdate	0.10
KI	0.09

100× Fe-EDTA stock solution
Dissolve 0.128 g $FeSO_4$ and 0.172 g EDTA at 60 °C in 100 ml H_2O store at 4 °C, 20 g

$CaCl_2 \cdot 2H_2O$	0.07
$MgSO_4 \cdot 7H_2O$	0.15
NaCl	0.03
$(NH_4)_2HPO_4$	0.03
KH_2PO_4	0.05
Trace elements	*mg/l*
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.018
H_3BO_4	

(g) MMN (Modified Melin-Norkrans) (Johnson et al. 1957)

Composition	*g/l*
NaCl	0.025
KH_2PO_4	0.5
$(NH_4)_2HPO_4$	0.25
$CaCl_2$	0.05
$MgSO_4$	0.15
$FeCl_3$	0.001
Thiamine hydrochloride	83.0 µl
Trypticase peptone	0.1 % (w/v)
Glucose monohydrate	1.0 % (w/v)
Malt extract	5.0 % (w/v)
Trace elements from stock	10.0 ml/l
Trace elements (*stock*)	*g/l*
KCl	3.73
H_3BO_3	1.55
$MnSO_4 \cdot H_2O$	0.85
$ZnSO_4$	0.56
$CuSO_4$	0.13

pH was adjusted to 5.8 with 1N HCl/NaOH.
All the stocks were stored at 4 °C except Thiamine hydrochloride which was stored at −20 °C.

(h) Malt Extract (Gallowey and Burgess 1962)

Composition	*g/l*
Malt extract	30.0
Mycological peptone	5.0
Agar	15.0
pH	5.4

(i) Potato Dextrose Agar (PDA) (Martin 1950)

Composition	*g/l*
Potato peel	200.0
Dextrose	20.0
Agar	15.0
Distilled water	1.0

Skin of potatoes was peeled-off, cut into small pieces, and boiled (200 g) in 500 ml of water, till they were easily penetrated by a glass rod. Filtered through cheese cloth and dextrose was added to the filtrate. Agar was dissolved and the required volume (1 l) was made up by the addition of water. The medium was autoclaved at 15-lb pressure for 20 ml

(j) Aspergillus (Kaefer 1977)

Composition	*g/l*
Glucose	20.0
Peptone	2.0
Yeast extract	1.0
Casamino acid	1.0
Vitamin stock solution	1.0 ml
Macroelements from stock	50.0 ml
Microelements from stock	2.5 ml
Agar	10.0
$CaCl_2$ 0.1 M	1.0 ml
$FeCl_3$ 0.1 M	1.0 ml
pH	6.5
Macroelements (major elements)	*Stock (g/l)*
$NaNO_3$	120.0
KCl	10.4
$MgSO_4 \cdot 7H_2O$	10.4
KH_2PO_4	30.4
Microelements (trace elements)	*Stock (g/l)*
$ZnSO_4 \cdot 7H_2O$	22.0
H_3BO_3	11.0
$MnCl_2 \cdot 4H_2O$	5.0
$FeSO_4 \cdot 7H_2O$	5.0

(continued)

$CoCl_2 \cdot 6H_2O$	1.6
$CuSO_4 \cdot 5H_2O$	1.6
$(NH_4)_6Mo_7O_{27} \cdot 4H_2O$	1.1
Na_2EDTA	50.0
Vitamins	*Percent*
Biotin	0.05
Nicotinamide	0.5
Pyridoxal phosphate	0.1
Amino benzoic acid	0.1
Riboflavin	0.25

pH was adjusted to 6.5 with 1N HCl. All the stocks were stored at 4 °C except vitamin which was stored at −20 °C.

The fungus grew on a wide range of synthetic and complex media, e.g., on minimal medium (MMN) normally used for in vitro germination of AMF with 10 % sucrose or glucose as a carbon source, on two different media for *Aspergillus* sp. (CM and MM2) and on Moser B medium.

Significant quantitative and morphological changes were detected when the fungus was challenged to grow on different media. Shaking during incubation retarded growth in MMN broth cultures (7–12 g fresh wt/l, after 2 week at 30 °C), whereas no such negative effect was ever observed during cultivation on any other substrates. There was practically no growth when mycelia were incubated under shaking conditions, whereas in stationary conditions, normal growth was obtained. Hyphae did not adjust to even slow rate of shakings. In fact, the fungal biomass was considerably enhanced on shaking cultures with aspergillus medium (CM), sometimes upto 50 g fresh wt/l after 2 week at 30 °C. On aspergillus and Moser b media (photograph not given here), the colonies appeared compact, wrinkled with furrows, and constricted. The mycelium produced fine zonation and a great amount of white aerial hyphae. Hyphae were highly interwoven, often adhered together, and gave the appearance of simple cords. New branches emerged irregularly and the hyphal walls showed some external deposits at regular intervals, perhaps polysaccharides and/or some hydrophobic proteins, which stained deeply with toluidine blue. Since septation was irregular, the single compartment could contain more than one nucleus. The chlamydospores appeared singly or in clusters and were distinctive due to their pear-shaped structure. The fungus produced chlamydospores at the apex of undifferentiated hyphae.

22.9 Conclusions

Mycorrhizae do not always promote the growth of agricultural crops. In phosphorus-rich soils, they can parasitize citrus plants, wheat, and maize by tapping sugars from these plants without giving anything back. Researchers ignore this darker side of the mycorrhiza, claims Dr. James Graham from the University of Florida. Theoretically, mycorrhiza can also harm biodiversity. In the long run,

specific mycorrhiza can promote the growth of one plant at the expense of another. "What exactly happens probably depends on the system itself," states Van der Heijden. In any case, the interactions between plants and mycorrhiza clearly have at least as great an effect on the ecosystem's species composition as the interactions between plants themselves.

P. indica acts as biofertilizer, bioregulator, and bioprotector, can be easily massmultiplied on defined synthetic media. Since *P. indica* is the first symbiotic fungus, known in the literature which can be grown on root of a living plant and under axenic culture. Scientists can take advantage to carry out certain basic research to understand the molecular basis of plant–microbes interaction. Plant industry may produce this fungus under aseptic conditions for commercial propositions and biological hardening of tissue-culture-raised plants.

Acknowledgment Authors are thankful to DBT, DST, CSIR, ICAR, UGC, Government of India for partial financial assistance. Further authors are thankful to Dr. Michael Weiss, Germany for providing 28s rDNA analysis of *P. indica*.

References

Ahuja MR (1986) Aspen. In: Evans DA, Sharp WR, Ammirato PJ (eds) Handbook of plant cell culture 4, techniques and applications. Macmillan, New York, pp 626–651

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Becard G, Piche Y (1989) New aspects of the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, Gigaspora margarita. New Phytol 112:77–83

Becard G, Piche Y (1990) Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and non-host Ri T-DNA transformed roots. Can J Bot 68:1260–1264

Chabot S, Becard G, Piche Y (1992) Life cycle of Glomus intraradix in root organ culture. Mycologia 84:315–321

Declerck S, Strullu DG, Plenchette C (1998) Monoaxenic culture of intraradical forms of *Glomus* sp isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) Glomus proliferum sp. nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187

Feldmann F, Idczak E (1994) Inoculum production of vescicular-arbuscular mycorrhizal fungi for use in tropical nurseries. In: Norris JR, Read DJ, Varma A (eds) Methods in microbiology, vol 24. Academic, London, pp 339–358

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughan AP, Piché Y (2002) Arbuscular mycoorhiza on root-organ cultures. Can J Bot 80:1–20

Gallowey LD, Burgess R (1962) Applied mycology and bacteriology, 3rd edn. Leonard Hill, London, pp 54–57

Herrmann S, Munch JC, Buscot F (1998) A gnotobiotic culture system with oak microcuttings to study specific effects of mycobionts on plant morphology before, and in the early phase of, ectomycorrhiza formation by *Paxillus involutus* and *Piloderma croceum*. New Phytol 138:203–212

Johnson CN, Stout PR, Broyer RC, Carlton AB (1957) Comparative chlorine requirements of different plant species. Plant Soil 8:337–353

Jolicoeur M, Perrier M (2001) Study of nutrient and water availability of mycorrhizal Daucus carota hairy roots on Glomus intraradices AM fungus development. In: Proceedings of the ICOM-3 conference, Adelaide, South Austria, 8–13 July 2001, Section PI, p 122

Jolicoeur M, Williams RD, Chavarie C, Fortin JA, Archambault J (1999) Production of Glomus intraradices propagules, an arbuscular mycorrhizal fungus, in an airlift bioreactor. Biotechnol Bioeng 63:0224–0232

Kaefer E (1977) Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv Genet 19:33–131

Kottke I, Guttenberger M, Hampp R, Oberwinkler F (1987) An in vitro method for establishing mycorrhizae on coniferous tree seedlings. Trees 1:191–194

Martin JP (1950) Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. Soil Sci 69:215–232

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Moutoglis P, Béland M (2001) PTB's research report. In Proceedings of the ICOM-3 conference, Adelaide, South Austria, 8–13 July, Section PI, p 26

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Mukerji KG, Mandeep, Varma A (1998) Mycorrhizosphere microorganisms: screening and evaluation. In: Varma A (ed) Mycorrhiza manual. Springer, Berlin, pp 85–98

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:431–487

Pham GH, An S, Malla R, Kumari R, Saxena AK, Rexer KH, Kost G, Louis P, Kaldorf M, Buscot M, Herrmann S, Pescan T, Oelmueller R, Mittag M, Declerck S, Hell S, Varma A (2004) Interaction of *Piriformospora indica* with diverse microorganisms and plants. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Germany, pp 237–265

Singh A, Singh A, Kumari M, Rai MK, Varma A (2003a) Biotechnological importance of *Piriformospora indica* Verma et al. - A novel symbiotic mycorrhiza - like fungus: an overview. Ind J Biotechnol 2:65–75

Singh A, Singh A, Kumari M, Kumar S, Rai MK, Sharma AP, Varma A (2003b) Unmasking the accessible treasures of the hidden unexplored microbial world. In: Prasad BN (ed) Biotechnology in sustainable biodiversity and food security. Science Publishers, Inc., Enfield, NH, pp 101–124

Strullu DG, Romand C (1986) Méthode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques. C.R. Acad. Sc. Paris, t. 303, Série III, vol 6, pp 245–250

Varma A, Verma S, Sudha, Sahay NS, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sudha, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharati K, Franken P, Hurek T, Blechert O, Rexer KH, Kost HA, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) Piriformospora indica: a cultivable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) Mycota IX series. Springer, Berlin, pp 123–150

Index

A. Varma et al. (eds.), *Piriformospora indica*, Soil Biology 33,
DOI 10.1007/978-3-642-33802-1,