A functional study on the multilateral symbiosis of the fungal order *Sebacinales* with plant hosts and bacteria

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1 Introduction

1.1 Rhizosphere

The region of soil surrounding a plant root is known as the ‘rhizosphere’. This is the most complex area within the soil environment and also represents the site with the highest microbial biomass and activity. It is here that interactions between plants and microorganisms are most intense and variable (Kiely et al., 2006). The plant exerts a major influence on microbial communities through the active release of a range of organic compounds, as root exudates, or eventually through nutrients released during roots decomposition. The release of root exudates and decaying plant material provide sources of carbon compounds for the heterotrophic soil biota either as growth substrates, structural material or signals for the root associated microbiota (Barea et al., 2005). Plants benefit from releasing root exudates into the rhizosphere by the dual effects of improving microbial turnover and together with other soil organic and inorganic matter enhancing the soil structure. In addition, microbial activity in the rhizosphere affects rooting patterns and the supply of available nutrients to plants, thereby modifying the quality and quantity of root exudates (Bowen and Rovira, 1999; Barea et al., 2005). In some cases, correlations have been reported between particular plants (e.g., *Ammophila arenaria*, Kowalchuk et al., 2002), or plant communities, and the species composition of microbial communities colonizing the rhizosphere (Wardle, 2005), but these links are less clear in complex natural ecosystems (McCaig et al., 1999). Root-microbe communications are of continuous occurrence in this biologically active soil zone (rhizosphere).

1.2 Symbiosis

The term symbiosis (from the Greek: sym, "with"; and biosis, "living") commonly describes close and often long-term interactions between different biological species. The term was first used in 1879 by the German mycologist, Heinrich Anton de Bary, who defined it as: "the living together of unlike organisms". The definition of symbiosis is in flux and the term has been applied to a wide range of biological interactions. In symbiosis, at least one member of the pair benefits from the relationship. Some people restrict the term symbiosis to only the mutually beneficial interactions but in broadest

sense, symbiosis refers to organisms living together, whether the interaction is mutualistic, commensal or parasitic (Parniske, 2004). Nitrogen fixing root-nodulating bacteria and mycorrhizal associations are some of the best studied examples of mutualistic symbiosis, and will be described in more details in the following chapters. The broadest definition of symbiosis (e.g. living together of two or more organisms) applies universally to mycorrhizal associations (Lewis, 1985; Smith and Read, 1997).

1.2.1 Rhizobium-Legume symbiosis

Soil bacteria belonging to α-proteobacteria and the order *Rhizobiales*, collectively called rhizobia, invade the roots of leguminous plants in nitrogen-limiting environments and forms a highly specialized organ-the nitrogen-fixing root nodule (Spaink, 2000). About 90% of legumes can become nodulated. Nodule formation is as complex on the plant side as for the bacterial partner (Schultze and Kondorosi, 1998) and requires a continuous and adequate signal exchange between plant and bacteria. Rhizobia are attracted by root exudates and colonize plant root surfaces. Root exudates contain Flavonoids, e.g. luteolin, which activates the expression of rhizobial *nod* genes. Induction of these genes leads to the production and secretion of return signals, the nodulation factors (Nod signals or Nod-factors (NF)), which are lipochito-oligosaccharides of variable structure (Lerouge et al., 1990). These NF are recognized by the plant which trigger root hair curling (Schultze et al., 1994) followed by cell wall invagination and the formation of an infection thread that grows within the root hair. The infection thread grows towards the root cortex and reaches the nodule primordium, which is initiated by the reactivation of differentiated cells of the root cortex for division. Within the infection thread the rhizobia multiply but remain confined by the plant cell wall (Schultze and Kondorosi, 1998). As the primordium develops to a nodule, bacteria are released from the tip of the infection thread by endocytosis and differentiate into bacteroids surrounded by the peribacteroid membrane. These bacteroids can fix gas phase nitrogen into ammonia (Kaminski et al., 1998), which is used by the plant. In turn, the bacteria are supplied with various nutrients in a protected environment (Soto et al., 2006).

1.2.2 Mycorrhiza

Mycorrhiza refers to associations or symbioses between plants and fungi that colonize the cortical tissue of roots during periods of active plant growth. Generally, these symbioses are often characterized by bi-directional exchange of plant-produced carbon to the fungus and fungal-acquired nutrients to the plant thereby providing a critical linkage between the plant root and soil. All mycorrhizal associations are symbiotic, but some are not mutualistic (Brundrett, 2004). To avoid the problems resulting from inconsistent use of the terms symbiosis and mutualism, the terms 'balanced mycorrhizae' ' and 'exploitative mycorrhizal associations' were proposed (Brundrett, 2004) for mutualistic and non-mutualistic mycorrhizal associations. The term 'balanced mycorrhizae' has been proposed to situations where bidirectional flow of nutrients occurs and both organisms receive beneficial effects. The term "exploitive mycorrhizal associations" was suggested for situations in which unidirectional nutrition flow occurs and plant gains the main beneficiary effect (Peterson and Massicotte, 2004).

The term mycorrhiza, which literally means 'fungus-root', was first applied to fungus-tree associations described in 1885 by the German forest pathologist A.B. Frank (Trappe, 2005). Since then a vast majority of land plants have been reported to form symbiotic associations with fungi. 80% of land plant species and 92% land plant families, surveyed by Wang and Qiu (2006) were shown to have mycorrhizal associations. The benefits afforded to the plants from mycorrhizal symbioses can be characterized agronomically by increased growth and yield and ecologically by improved fitness (i.e., reproductive ability). Mycorrhizal plants are often more competitive and exhibit enhanced tolerance against biotic and abiotic stresses compared to non-mycorrhizal plants (Marler et al., 1999; Peterson and Massicotte, 2004).

Early morphological classifications separated mycorrhizas into endomycorrhizal, ectomycorrhizal and ectendomycorrhizal associations based on the relative location of fungi in roots (Peyronel et al., 1969). These three types were not enough to describe the diversity of mycorrhizal associations. Harley and Smith (1983) recognized seven types that, for the most part, still comprise the generally accepted classification. These include Ectomycorrhizae, Endomycorrhizae, Ectendomycorrhizae, Arbutoid mycorrhizae, Monotropoid mycorrhizae and Orchid mycorrhizae. However, differnt people use

different criteria and hence describe different types and categories of mycorrhizal associations. The following terms are most commonly used in the mycorrhizal studies:

1) **Ectomycorrhizae (ECM)**: The diagnostic feature of ectomycorrhizae ("outside" mycorrhizas) is the presence of hyphae between root cortical cells producing a netlike structure called the Hartig net (Scheidegger and Brunner, 1993)**.** Hyphae of the Hartig net completely envelope the host cells to provide maximum contact between host and fungus. The Hartig net exhibits a complex labyrinthine growth mode with finger-like structures termed palmettes and with rare hyphal septations (Blasius et al., 1986).
2) **Endomycorrhizae**: Endomycorrhizae ("inside" mycorrhizas) grow within cortical cells and do not form a mantle around the root, but instead the fungal hyphae establish between the cortex cells, and often enter them.
3) **Arbuscular Mycorrhizae (AM)**: It is a member of endomycorrhizae. The diagnostic feature of arbuscular mycorrhizae (AM) is the development of a highly branched arbuscule within root cortical cells. The fungus initially grows between cortical cells, but soon penetrates the host cell wall and grows within the cell. As the fungus grows, the host cell membrane invaginates and envelops the fungus, creating a new compartment where material of high molecular complexity is deposited. This apoplastic space/compartment prevents direct contact between the plant and fungus cytoplasm and allows for efficient transfer of nutrients between the symbionts. The arbuscules are relatively short lived, less than 15 days. The fungi that form AM were all classified as members of the order *Glomales* (Morton, 1988), which was further subdivided into suborders based on the presence or absence of vesicles. Scheussler et al (2001) described a new phylum *Glomeromycota* which includes AMF. AM can be divided into two main types, the *Arum*-type and the *Paris*-type (Smith and Smith, 1997). In the *Arum*-type, usually one arbuscule develops through repeated branching of a hypha that penetrates through the cortical cell wall (Bonfante and Perotto 1995) whereas in *Paris*-type, penetration of the cortical cell wall by a single hypha is followed by extensive coiling of this hypha from which lateral branches are initiated to form arbusculate coils (Cavagnaro et al., 2001). Originally, the term ‘vesicular-arbuscular mycorrhiza’ (VAM) was applied to symbiotic associations formed by all

Glomeromycota mycorrhizal fungi. However, since a major proportion of fungi lacks the ability to form the vesicles in roots, AM is now the preferred acronym.

4) **Ectendomycorrhizae**: The ectendomycorrhizae form typical ECM structures, except that the mantle is thin or lacking and hyphae in the Hartig net may penetrate root cortical cells. The ectendomycorrhiza is replaced by ECM as the seedling matures.
5) **Ericaceous Mycorrhizae**: The term ericaceous is applied to mycorrhizal associations found in plants of the order *Ericales*. The hyphae in the root can penetrate cortical cells (endomycorrhizal habit); however, no arbuscules are formed. Three major forms of ericaceous mycorrhiza have been described:
   a) Ericoid mycorrhizae (ERM): Cells of the inner cortex become packed with fungal hyphae. A loose welt of hyphae grows over the root surface, but a true mantle is not formed. The ericoid mycorrhizae are found on plants such as *Calluna* sp. (heather*), Rhododendron* sp. (*Azaleas* and rhododendrons) and *Vaccinium* sp. (blueberries) that have very fine root systems and typically grow in acid, peaty soils. The fungi involved are ascomycetes of the genus *Hymenoscyphus*.
   b) Arbutoid mycorrhizae: In this type of association, characteristics of both ECM and endomycorrhizae are found. Intracellular penetration can occur, a mantle forms, and a Hartig net is present. These associations are found on *Arbutus* sp. (e.g., Pacific madrone), *Arctostaphylos* sp. (e.g., bearberry), and several species of the *Pyrolaceae*. The fungi involved in the association are basidiomycetes.
   c) Monotropoid mycorrhizae: In this association, mycorrhizal fungi colonize achlorophyllous plants of *Monotropaceae* (e.g. Indian pipe), producing the Hartig net and mantle. The same fungi also form ECM associations with trees thereby forming a link through which carbon and other nutrients can flow from the autotrophic host plant to the heterotrophic, parasitic plant.
6) **Orchidaceous Mycorrhizae**: The association between orchids and mycorrhizal fungi is included in this category. These fungi enter plant cells by invaginating the cell membrane and forming hyphal coils within cells of the protocorm and developing root. These coils are active for only a few days, after which they loose turgor and degenerate while nutrient contents are absorbed by the developing orchid. The fungi participating in this type of symbiosis are basidiomycetes similar to those involved in

decaying wood (e.g., *Coriolus* sp., *Fomes* sp., *Marasmius* sp.) and pathogenesis (e.g., *Armillaria* sp. and *Rhizoctonia* sp.). In mature orchids, mycorrhizae also have roles in nutrient uptake and translocation. Orchid mycorrhizas support orchid development and initial root development by delivering nutrients for germination, protocorm and initial root development (Peterson and Massicotte, 2004).

More recently, Brundrett (2004) recommended that mycorrhizal associations are defined and classified primarily by anatomical criteria regulated by the host plant. A revised classification scheme for types and categories of mycorrhizal associations defined main categories of vesicular-arbuscular mycorrhizal associations (VAM) as 'linear' or 'coiling', and of ectomycorrhizal associations (ECM) as 'epidermal' or 'cortical'. Subcategories of coiling VAM and epidermal ECM occur in certain host plants. Fungus-controlled features result in 'morphotypes' within categories of VAM and ECM. Following this classification, arbutoid and monotropoid associations should be considered subcategories of epidermal ECM and ectendomycorrhizas should be relegated to an ECM morphotype.

1.3 Bacteria-fungi interactions

The various microorganisms found routinely in the rhizosphere and known to contribute to soil fertility and crop yield include mycorrhizal fungi, free nitrogen-fixing bacteria and other plant growth promoting rhizobacteria (PGPR), such as rhizobia and pseudomonads. The beneficial traits of root-colonizing bacteria and fungi have been almost separately studied. However, the synergistic effects of bacteria and mycorrhizal fungi have recently been started to study with respect to their combined beneficial impacts on plants. Linkages between plant roots and their microbial communities exist in a complex web of interactions that act at individual and at community levels (Singh et al., 2008). A better understanding of interactions of soil microorganisms with each other and with plants is crucial for the development of sustainable strategies for soil fertility and crop production. To date, many bacterial strains have been reported to be able to promote either AM or ECM symbioses.

1.3.1 Interaction between ectomycorrhizal fungi and bacteria

The symbiotic establishment of mycorrhizal fungi on plant roots is affected in various ways by other microorganisms of the rhizosphere, including especially bacteria. There are several reports showing stimulatory effects of bacteria on mycorrhizal development. Garbaye (1994) proposed the term 'mycorrhization helper bacteria' (MHB) to define bacteria associated with mycorrhizal roots and mycorrhizal fungi, which selectively promote the establishment of mycorrhizal symbioses. Since then, associations of MHB with many ECM fungi have been discovered. The MHB concept is generic. It depends neither on the type of the mycorrhizal symbiosis nor on the taxonomy of the MHB strains (Frey-Klett et al., 2007). The MHB strains associated with ECM that have been identified so far belong to many bacterial groups and genera such as gram-negative Proteobacteria (*Pseudomonas* and *Burkholderia*), gram-positive Firmicutes (*Bacillus* and *Paenibacillus*) and gram-positive Actinomycetes (*Streptomyces*, *Rhodococcus* and *Arthrobacter*). Positive interactions of MHB with ECM fungi belonging either to Basidiomycetes or Ascomycetes have been reported.

MHB promote the establishment of plant-fungal symbioses by interacting with both, plant organs and fungal structures, through the enhancement of spore germination, mycelial extension and root development. In addition, recognition process between roots and fungi are supported eventually leading to increased root-fungus contacts and colonization in combination with a reduction of the impact of adverse environmental conditions on fungal mycelium.

Aspray et al. (2006) reported the non-specificity of MHB on mycorrhiza formation but specific effects on mycorrhiza architecture (e.g., dichotomous short root branching) and further suggested that different mechanisms operate for different MHB. MHB strains have also been shown to induce changes in the transcriptome of mycorrhizal fungi. Schrey et al. (2005) showed that the MHB *Streptomyces* AcH 505 affects gene expression of the ECM *Amanita muscaria* including fungal transcripts whose gene products are involved in signaling pathways, metabolism (carbon, sulphur and nitrogen metabolisms), cell structure, and the cell growth response. Deveau et al. (2007) reported that MHB *Pseudomonas fluorescens* BBc6R8 strain has a specific priming effect on growth, morphology and gene expression of its fungal associate *Laccaria bicolor* S238N.

At present, there is no clear evidence to show whether the developmental changes in mycorrhizal fungi do benefit bacteria. However, it seems likely that enhanced mycorrhization, and associated increases in nutrient availability for plants and fungi may benefit plants, bacteria and fungi in a tri-trophic interaction.

1.3.2 Interaction between arbuscular mycorrhizal fungi and bacteria

Most of arbuscular mycorrhizal fungi (AMF) have been recently classified in a new taxon, the *Glomeromycota* (Scheussler and Kluge, 2001). Many examples of association between AMF and MHB have been described since their first discovery in the genus *Glomus* by Mosse (1962). The MHB strains associated with AMF have been identified to date belong to many bacterial groups and genera such as gram-negative Proteobacteria (*Agrobacterium*, *Azospirillum*, *Azotobacter*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas*, *Klebsiella* and *Rhizobium*), gram-positive Firmicutes (*Bacillus*, *Brevibacillus*, and *Paenibacillus*) and gram-positive Actinomycetes (*Streptomyces*). The interactions between bacteria and AMF have potentially beneficial functions, including the majority of those where PGPR including N_2-fixing bacteria are involved. Mycorrhizosphere bacteria may affect AMF and their plant hosts through a variety of mechanisms (Johansson et al., 2004). Some of these include (1) effects on the germination of fungal propagules, (2) effects on root cell permeability and root receptivity, (3) effects on root-fungus recognition, (4) effects on fungal growth, (5) modification of the chemistry of rhizosphere soil, (6) nutrient acquisition and (7) inhibition of plant pathogenic fungi in the rhizosphere. The AMF themselves have also been shown to have an impact on the composition of bacterial communities (Artursson et al., 2006). This impact may be relayed through the plant root because mycorrhizal establishment has been shown to change the chemical composition of root exudates and these are often a source of nutrients to associated bacteria in the mycorrhizosphere. A few studies have shown that some bacterial species respond to the presence of certain AMF (Andrade et al., 1997; Artursson et al., 2005) suggesting a high degree of specificity between bacteria associated with AMF. Different bacteria vary in their attachment properties to AMF. Bianciotto and colleagues (1996a) reported that some *Rhizobium* sp. and *Pseudomonas* sp. species attached to germinated AMF spores and hyphae under

sterile conditions, and that the degree of attachment varied with the bacterial strain. However, no specificity for either fungal or inorganic surfaces could be detected among the bacteria tested. However, Artursson and Jansson (2003) demonstrated that a *Bacillus cereus* strain, isolated from a Swedish soil containing abundant AMF, attached to hyphae of the AMF *Glomus dussii* at significantly higher levels than a number of bacterial control strains. Toljander et al. (2006) compared the attachment of five different Green fluorescent protein (GFP)-tagged bacterial strains to vital and non-vital hyphae of the AMF *Glomus claroideum* and indicated major differences between the bacterial strains in their ability to attach to different physiological states of hyphae.

1.3.3 Fungal endosymbiotic bacteria

Endocellular bacteria are reported for a few fungi including some *Glomeromycota* species (AMF and *Geosiphon pyriforme*), ECM Basidiomycetes like *Laccaria bicolor* and *Tuber borchii,* the phytopathogenic Zygomycete *Rhizopus microsporus* and the edible white-rot fungus *Pleurotus ostreatus*. Those AMF, which are themselves obligate plant symbionts, represent a specialized niche for rod-shaped bacteria, consistently found in many of the Gigasporaceae through all the steps of fungal life cycles. AMF host bacteria in their cytoplasm. Intracellular structures very similar to bacteria and bacteria-like organisms (BLOs) were first described in the 1970s (Scannerini and Bonfante, 1991). Using a combination of electron microscopy, confocal microscopy and molecular analyses of bacterial 16S-rRNA gene sequence, these BLOs were identified as true bacteria (Bianciotto et al., 1996b). Earlier, on the basis of 16S-rRNA gene sequences, the bacterial endosymbionts living in *Gigaspora margarita* (BEG 34) were believed to be members of the genus *Burkholderia*, but were subsequently classified as a new bacterial taxon, *Candidatus* Glomeribacter gigasporarum (Bianciotto et al., 2003).

Some endocellular biotrophs are vertically transmitted, while facultative endocellular biotrophs possess mechanisms for invading and subverting fungal cells. The symbiosis between *Geosiphon pyriformis* and *Nostoc* is in some respects a relatively primitive and unstable symbiosis, which involves horizontal acquisition of a free-living bacterium. By contrast, the vertically transmitted obligate endocellular bacterium *Ca.* G. gigasporarum colonizes the spores of *Gigaspora margarita* at densities ranging from 3700 to 26000

bacteria per spore (Jargeat et al., 2004). These endobacteria are always inside a vacuole-like compartment surrounded by a membrane (Bianciotto et al., 1996b). *Ca*. G. gigasporarum has an estimated genome size of 1.35 Mbp (Bianciotto et al., 2003; Jargeat et al., 2004). The small genome size suggests that this bacterium, like other obligate pathogens and symbionts, is entirely dependent on fungal cells for many metabolic functions. However, this dependence is not only restricted to the bacterium. Lumini and collaborators (2007) cured a strain of *G. margarita* of its endogenous endocellular bacteria, and found that although the fungus could still colonize plants and complete its lifecycle under laboratory conditions, the cured strain showed altered spore morphology, reduced presymbiotic hyphal growth and reduced branching, which is associated with reduced competitive fitness. In analogy, seedling blight fungus *R. microsporus* harbours an endosymbiotic bacteria (Partida-Martinez and Hertweck, 2005) belonging to the genus *Burkholderia* (*B. rhizoxinica* sp. nov. and/or *B. endofungorum* sp. nov.). The endosymbiotic bacterium can be cultured *in vitro* and produces the toxins rhizoxin and rhizonin, causal agents of seedling blight, which were earlier thought to be produced by the fungus. In the absence of endosymbionts, the host is not capable of vegetative reproduction. Formation of sporangia and spores is restored only upon reintroduction of endobacteria (Partida-Martinez et al., 2007b).

Several studies propose that the ECM fungi could also harbour intracellular bacteria (Bertaux et al., 2005). For the ECM of *Pinus strobus-Endogone flamicorona* (Bonfante-Fasolo and Scannerini, 1977), *Picea abies*-'Type F' (Buscot, 1994) and *Pinus sylvestris-Suillus bovines* (Nurmiaho-Lassila et al., 1997), the endobacteria were observed in living cells. However, the 'Type F' ECM were senescing, and in the case of *Fagus sylvatica-Lactarius rubrocinctus* (Mogge et al., 2000), endobacteria were detected only inside damaged cells. Intracellular bacteria were also observed in axenic cultures of *Tuber borchii* (Barbieri et al., 2000) and *Laccaria bicolor* S238N (Bertaux et al., 2003).

1.4 *Sebacinales*

The order *Sebacinales* houses a great variety of ERM, orchid, jungermannioid and ECM. Recent works on mycorrhizal communities have stimulated considerable interest in a neglected group of fungi related to the genus *Sebacina*, recently raised to the order

Sebacinales (Weiss et al., 2004). 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. Most of our knowledge on *Sebacinales* and their diverse host species comes from molecular ecology studies during the last four years by comparing the sequence of fungal ribosomal DNA (rDNA) of environmental samples. Phylogenetically, *Sebacinales* are divided into two clades A and B. In the study of Selosse et al. (2007), all sequences obtained from ERM clustered within *Sebacinales* clade B, the clade that contains the sequences of *Sebacina vermifera* isolates (cultivable isolates obtained mostly from Australian green, autotrophic orchids) (Warcup, 1988) and of *Piriformospora indica* as well as also those sequences obtained from samples of cavendishioid mycorrhizas and liverwort thalli.

1.4.1 *Piriformospora indica*

The root-colonizing fungal mutualist *Piriformospora indica* was discovered in the rhizosphere of the woody shrubs *Prosopsis juliflora* and *Zizyphus nummularia* in the Indian Thar desert in 1997 by Ajit Varma and his collaborators. Depending on the ultra structure of hyphae (presence of dolipore septa) and 18s-rRNA gene sequence, *P. indica* was grouped in the class Hymenomycetes (Basidiomycota) (Varma et al., 1998). Weiss et al. (2004) classified the fungus as a member of the order *Sebacinales* depending on nuclear rDNA sequence for the 5´ terminal domain of the ribosomal large subunit (nucLSU). *P. indica* infests roots of a broad range of mono- and dicotyledonous plants (Varma et al., 1998; Pham et al., 2004). Endophytic root colonization by this fungus confers enhanced growth to the host plant (Varma et al., 1999; Peskan-Bergheofer et al., 2004) and provides protection against biotic and abiotic stresses. *P. indica* enhances salt stress tolerance, confers disease resistance and increases grain yield in barley (Waller et al., 2005). Disease resistance is provided not only to the roots but also to the shoot. As endophytic growth of *P. indica* is restricted to the root, the fungus is able to provide systemic protection due to a yet unknown mechanism of induced resistance. As *P. indica* can easily be cultured without a host plant (Varma et al., 1999), it is suitable as a model system to study compatible plant–microbe interactions. Like other mutualistic

endophytes, *P. indica* colonizes roots in an asymptomatic manner. PCR-based quantification of *P. indica* revealed that root colonization gradually increases with tissue maturation. The root tip meristem shows no colonization and the elongation zone shows mainly intercellular colonization. The differentiation zone is usually heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. Fungal growth within plant roots is characterized by extensive cellular and extracellular fungal growth in epidermal and cortical tissue that became completely filled with chlamydospores at later interaction stages, although fungal mycelium never reaches the stelar tissue. Interestingly, cellular mycelial proliferation of *P. indica* in barley was shown to coincide with the repressed expression of (*HvBI-1*) gene, an inhibitor of plant cell death in barley. In analogy, fungal proliferation was strongly inhibited in transgenic barley overexpressing GFP-tagged barley *BAX Inhibitor-1* (*HvBI-1*), which shows that *P. indica* requires host cell death for barley root colonization (Deshmukh et al., 2006).

1.5 Objectives

The basidiomycetous fungus *P. indica* is a model organism for species of the order *Sebacinales*, fungi that are involved in a uniquely wide spectrum of mutualistic symbioses (mycorrhizae) with plants in nature. *P. indica* confers considerable beneficial effects ranging from growth augmentation to tolerance against various abiotic stresses (e.g., salt stress) and biotic stresses (e.g., root and shoot diseases). However, the interaction of other *Sebacinales* with various crop plants and their effect on plant health and vigor has not been studied yet. As first part of this study, morphological and phylogenetic analyses of different isolates of *Sebacina vermifera* species complex were carried out. Furthermore, the interaction between various strains/isolates of *S. vermifera* with barley were investigated analyzing whether these strains exhibit comparable biological activities as *P. indica*.

Many recent reports on mycorrhizal interactions have suggested that biological activities brought about by the symbiosis ought to be considered under the premise of a more complex tripartite interplay of the host plant with the mycorrhiza fungus and fungus-associated bacteria. These bacteria can associate extracellular as well as intracellular with fungal structures. The effects of bacteria on fungal growth and mycorrhization frequency

have been studied, but the contribution of the symbiotic partners on plant's physiology and - on another scale - on natural ecosystems is almost unknown. While culturing *P. indica*, it had been observed on several occasions that mechanical rupturing of fungal hyphae release some bacteria. Hence, the second major focus of this study was aimed at the study of the functional roles of the bacteria associated with *P. indica*. The hypothesis that members of *Sebacinales* associate with bacteria was tested. Isolation and identification of associated bacteria were performed using various cultural, molecular and cytological methods. Furthermore, the biological activity of fungus-associated bacteria was determined in barley and *Arabidopsis thaliana* and mechanisms of systemic resistance induced by bacteria was elucidated.

2 Materials and Methods

2.1 Fungal material

Piriformospora indica isolates were obtained from the following sources: *P. indica*-DSM11827 from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; *P. indica*-JE1 from Dr. Ralph Ölmüller, Jena, Germany; *P. indica*-HA from Dr. Holger Deising, Halle, Germany; *P. indica*-ND from Dr. Ajit Varma, New Delhi. All isolates stem from one original sample collected in the Thar desert, India in 1997 (Verma et al., 1998). They were propagated in liquid modified *Aspergillus* minimal medium at room temperature (Pham et al., 2004). Six *Sebacina vermifera* strains (Table 2.1) were obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan). One more strain (DAR29830) of *S. vermifera* complex (deposited as Multinucleate Rhizoctonia in culture collection) was kindly provided by Karl-Heinz Rexer (University of Marburg, Marburg, Germany). All strains of *S. vermifera* complex were propagated in Malt-Yeast-Extract-Pepton medium (aqueous solution of 7 g l^{-1} malt extract, 1 g l^{-1} peptone, 0.5 g l^{-1} yeast extract, with or without 15 g l^{-1} agar-agar).

Table 2.1 *Sebacinales* isolates

Isolate	Host
Piriformospora indica DSM11827[a]	*Prosopis juliflora* and *Zizyphus nummularia*[d] (Woody shrubs)
S. vermifera [b]MAFF305830	*Crytostylis reniformis* (Orchid)
S. vermifera [b]MAFF305842	*Microtis uniflora* (Orchid)
Multinucleate *Rhizoctonia* [c]DAR29830	
S. vermifera [b]MAFF305828	*Eriochilus cucullatus* (Orchid)
S. vermifera [b]MAFF305837	*Caladenia dilatata* (Orchid)
S. vermifera [b]MAFF305835	*Caladenia catenata* (Orchid)
S. vermifera [b] MAFF305838	*Caladenia tesselata* (Orchid)

[a] Type species *Piriformospora indica* was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; [b] Culture collection numbers: Isolates of *Sebacina vermifera* (S. v.) were obtained from the National Institute of Agrobiological Sciences, Tsukuba, Japan; [c] The isolate DAR29830 was kindly provided by Karl-Heinz Rexer, University of Marburg, Germany; [d] Fungus was originally isolated from rhizosphere of these woody shrubs.

CM medium (modified *Aspergillus* minimal medium) for *P. indica*

20x salt solution	50 ml
Glucose	20 g
Peptone	2 g
Yeast extract	1 g
Casamino-acid	1 g
Microelements	1 ml
Agar-agar	15 g
A. dist.	950 ml

20X salt solution

$NaNO_3$	120 g
KCl	10.4 g
$MgSO_4 \times 7H_2O$	10.4 g
KH_2PO	430.4 g
A. dist.	1000 ml

Microelements

$MnCl_2 \times 4H_2O$	6.00 g
H_3BO_3	1.50 g
$ZnSO_4 \times 7H_2O$	2.65 g
KI	0.75 g
$Na_2MoO_4 \times 2H_2O$	2.40 mg
$CuSO_4 \times 5H_2O$	130 mg
A. dist.	1000 ml

MYP Medium

Malt-extract	7.0 g
Peptone (Soya)	1.0 g
Yeast-extract	0.5 g
A. dist.	1000 ml

Agar-Agar 15.0 g

2.2 DNA isolation

DNA was isolated using three different approaches during the present study:

1. Fungal mycelia were harvested either from agar-medium or from liquid cultures. Frozen mycelium was grinded in liquid nitrogen and approximately 20 mg of the powder was used for DNA isolation using Plant DNeasy kit (QIAGEN GmbH, Hilden, Germany), according to manufactures' instructions.
2. Genomic DNA was isolated from two-week-old axenic cultures of *P. indica* and *S. vermifera* isolates and 24-hour-old bacterial cultures using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany). The fungal samples were first crushed with micro-pestle in Eppendorf tube containing buffer T1. Subsequent steps in total genomic DNA preparation procedure were performed by using the instructions supplied with the NucleoSpin® Tissue Kit (Macherey-Nagel).
3. DNA extraction was performed using the FastDNA® Spin Kit for soil (MP Biomedicals, LLC., Illkirch, France) according to the manufacturer's protocol.

2.3 PCR and Sequence analysis

Internal-transcribed-spacer-region (ITS) 1 and 2, including the 5.8S-rRNA gene (Fig 2.1), were amplified using the primers (ITS1 and ITS4; Table 2.2) for all isolates of the *Sebacinales*. Cloning of the PCR products were carried out in pGEM-T vector (Promega) using standard procedure and were sequenced. Phylogenetic analysis was carried out using PAUP software.

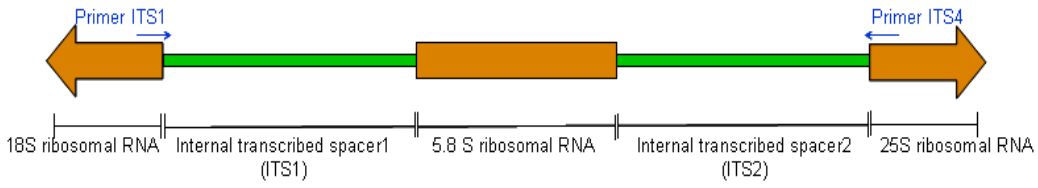

Fig 2.1. Schematic representation of internal transcribed spacer (ITS1-5.8S-ITS2) region.

Table 2.2 Primer sequences

Primer Name	**Primer Sequence (5' ⟶ 3')**
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	CTCCGCTTATTGATATGCT
27f	GAGAGTTTGATCCTGGCTCAG
1495r	CTACGGCTACCTTGTTACGA
R518	ATTACCGCGGCTGCTGG
F357	TACGGGAGGCAGCAG
9f	GAGTTTGATCMTGGCTCAG
1492r	ACGGYTACCTTGTTACGACTT
616F	AGAGTT TGATYMTGGCTCAG
630R	CAKAAAGGAGGTGATCC
ITSFeub	GTCGTAACAAGGTAGCCGTA
ITSReub	GCCAAGGCATCCACC
ITS_Rhf	TCAGCACATAACCACACCAATCGCG
ITS_Rhr	TGCTTTGTACGCTCGGTAAGAAGGG
ITS_PiBi F	GCACATAACCACACCAATCG
ITS_PiBi R	GGTTATGCTGTCGGGTGTTT
ros1	CGCGGGCTACAAGTTGAATC
ros3	GACCGAGACCCATTTCCTTG
ChvA_F1	ATGCGCATGAGGCTCGTCTTCTTCGAG
ChvA_R1	GACGCAACGCATCCTCGATCAGCT
ChvA_F2	TTGACCTTGTTTCAGGTTTACACA
ChvA_R2	GACGAGGATAATCATCATCGAAAC
R16-1	CTTGTACACACCGCCCGTCA
R23-3R	GGTACTTAGATGTTTCAGTTC
$VirD_2A$	ATGCCCGATCGAGCTCAAGT
$VirD_2C$	TCGTCTGGCTGACTTTCGTCATAA
$VirD_2E$	CCTGACCCA ACATCTCGGCTGCCCA
ipt F	GATCG(G/C)GTCCAATG(C/T)TGT
ipt R	GATATCCATCGATC(T/C)CTT
NifH1	AAGTGCGTGGAGTCCGGTGG
NifH2	GTTCGGCAAGCATCTGCTCG
NifH3	GCCAACAACATCGCCAGGGGTAT
NifH4	GCAGCCAGCGCTTCGGCGAG
NifH5	GCGGC(AC)AGTGCCTCGGCGAG
fliG F	CCGGCAAGCTGCTGAAAT
fliG R	CGTCTGAGCCGAGGAAATGA
rol F	GGCGATAAAACCTTCCAGATCA
rol R	GTCCGTGCTCACAACATTGC

Metagenomic fungal DNA was used to amplify bacterial 16S-rRNA gene using the bacterial universal primer pair 27f and 1495r. A conventional PCR amplification was

performed in a Gene Amp® PCR System 9700 PE Applied Biosystem thermo cycler in a total volume of 25 µl containing 2 x PCR Master Mix (Fermantas, Life Sciences, St. Leon-Rot, Germany), 75-100 ng DNA and 1 µM of each primer. After an initial denaturation step at 95°C for 5 min, 34 cycles with denaturation at 95°C for 1 min, primer annealing at 59°C for 1 min, elongation at 72°C for 1.45 min, and a final extension at 72°C for 10 min were performed. The obtained PCR products were purified using a Gel Extraction Kit (Promega, Mannheim, Germany) and cloned into the pGEM-T vector following the manufacturer's instructions. DNA from 25 plasmids was extracted with Wizard® Plus SV Minipreps (Promega) and submitted for sequencing to AGOWA GmBH, Berlin, Germany. Direct sequencing of PCR products was also performed with the primer pair 27f and 1495r. Sequences were assembled with the Sequencer 3.1.1 software (Gene Codes Corporation) and analyzed with the ARB software package (www.arb-home.de) (Ludwig et al., 2004).

2.3.1 Phylogenetic analysis

The 16S-rRNA coding gene sequences obtained from the sequenced plasmids and from direct sequencing were added to an existing database of well aligned small-subunit rRNA gene sequences by using the fast alignment tool implemented in the ARB software package (http://www.arb-home.de) (Ludwig et al., 2004). Sequences were proof read according to the chromatograms and wrong positions in the alignments were manually corrected if needed. Phylogenetic analyses were performed by applying maximum likelihood, maximum parsimony, and neighbor joining methods by use of respective tools in the ARB software package.

2.4 Isolation of bacteria

Mycelia of 14-day-old *P. indica* DSM11827 and *P. indica*-JE1 cultures were crushed in Gamborg B5 medium (Duchefa Biochemie, Netherland) supplemented with 0.45 M mannitol using a fine blender. Homogenate was filtered through a miracloth (22-25 µm) filter and centrifuged at 100 g for seven min. The supernatant was collected and subsequently centrifuged at 3,200 g for ten min. The bacterial cell pellet was resuspended in LB medium containing 0.8% sucrose and inoculated in the same medium at 22°C for

two days under gentle shaking. The bacterial culture was streaked on LB medium plate and incubated for two days at 25°C. Thirty bacterial clones were randomly picked from the plates and identified by sequencing using a universal primer pair for the 16S-rRNA gene as described above.

Luria-Bertani (LB) medium

Tryptophan	10 g
Yeast-extract	5 g
NaCl	10 g
A. dist.	1000 ml
Adjust pH to 9.0	
Agar-agar	10 g

2.5 Denaturing gradient gel electrophoresis (DGGE)

DNA extraction for DGGE analysis was performed using the FastDNA® Spin Kit for soil (MP Biomedicals, LLC., Illkirch, France) according to the manufacturer's protocol. A semi-nested PCR was performed to amplify a 500 bp region of the bacterial 16S-rRNA coding gene. First, almost the entire 16S-rRNA gene was amplified using the above mentioned bacterial primer pair. The cycle conditions differ in 25 cycles of amplification. Subsequently, next PCR was performed using 27f (with a 42 bp GC clamp on the 5' end), and reverse R518 universal primers (Table 2.2) (Vanhoutte et al., 2005). The PCR mix contained (final concentrations) 1 x Thermophilic DNA Polymerase Buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 0.025 mM of each dNTP (Fermantas, Life Sciences, St. Leon-Rot, Germany), 0.25 μM of each primer, and 0.05 U/μl of Taq DNA polymerase (Promega). 1 µl of first PCR product was used as template in a total volume of 50 µl reaction. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 25 amplification cycles with heat denaturation at 94°C for 1 min, primer annealing at 59°C for 45 sec, and extension at 72°C for 40 sec. A final elongation step at 72°C for 10 min completed the reaction. The PCR products were analyzed with standard horizontal agarose gel electrophoresis on a 1% agarose gel.

Additionally a single step PCR amplification of a 500 bp region of the 16S-rRNA gene was performed using the forward primer 27f or F357 (Table 2.2) (Vanhoutte et al., 2005),

with a 42 bp GC-clamp on the 5' end, in combination with the reverse primer R518. PCR was performed under the above mentioned conditions. The cycle conditions differ only in 32 cycles of amplification.

DGGE analyses were performed using an 8% (wt/vol) acrylamide-bisacrylamide gel (Liqui-Gel™ 37.5:1; MP Biomedicals) with a 35 to 75% linear urea-formamide (Fluka, Seelze, Germany) denaturing gradient (100% denaturant corresponds to 40% formamide plus 7 M urea). After adding the loading buffer (0.05% bromophenol blue and 0.05% xylene cyanol in 70% glycerol), 20 µl of each sample were loaded on the DGGE gel and submitted to electrophoresis in 1 x TAE buffer at 60°C with a constant voltage of 50 V for 20 h using a Bio-Rad DCode™ Universal Mutation Detection System. The gels were stained in the dark for 20 min in ethidium bromide and subsequently washed with 1 x TAE buffer. The following bacterial strains were used as reference: *Escherichia coli*, *Herbaspirillum frisingense* strain Mb11 and *Acinetobacter* sp. The obtained DGGE bands for *P. indica* DSM11827, *P. indica*-JE1, PABac-DSM and PABac-JE isolate were excised, the DNA fragments were purified using Wizard® Plus SV Minipreps (Promega) and submitted for sequencing to AGOWA GmbH, Berlin, Germany.

Solutions for DGGE

8 % acrylamide:bis-acrylamide with 0% denaturants

40% acrylamide:bis-acrylamide	20 ml
50X TAE Buffer	2 ml
A. dist. to bring to 100ml	78 ml

8 % acrylamide:bis-acrylamide with 100% Denaturants

40% acrylamide:bis-acrylamide	20 ml
50X TAE Buffer	2 ml
100 % Formamide	40 ml
Urea	42 g
Little water to bring to 100 ml.	

To make gradient

13 ml 35 % (Low) mixture	**13 ml 75 % (High) mixture**
4.6 ml 100 % solution	9.8 ml 100 % solution
8.6 ml 0 % solution	3.2 ml 0 % solution

13 ml 50 % (Low) mixture	**13 ml 70 % (High) mixture**
6.5 ml 100 % solution	9.1 ml 100 % solution
6.5 ml 0 % solution	3.9 ml 0 % solution

13 ml 30 % (Low) mixture	**13 ml 60 % (High) mixture**
3.9 ml 100 % solution	7.8 ml 100 % solution
9.1 ml 0 % solution	5.2 ml 0 % solution

59 µl 10 % APS (Ammonium persulfate) and 12.5 µl TEMED was added just before making gel.

Casting gel

After making gradient part of gel, casting gel was put as top solution (1-2 hrs).

0 % solution	5 ml
10 % APS (Ammonium persulfate)	23 µl
TEMED	5 µl

Pockets were installed and let it stood for night. (The glasses were washed with water, soap and ethanol and dried nicely). After removing the comb, the gel was put in buffer tank (with 1X TAE buffer). The wells were washed with buffer by putting some buffer on it two or three times. The system was started and samples were loaded when temperature reached at 55-60^{0}C.

For 2X gel loading buffer for 10 ml

1% Bromphenol Blue	0.5 ml
1% Xylene cyanol	0.5 ml

86% Glycerol (or 7ml glycerol + 2.5 ml water) 8.2 ml

2.6 Real-Time PCR quantification

Various other primer pairs were tested for amplifying bacterial genes from genomic DNA of PABac (*R. radiobacter*) and *P. indica* (Table 2.2). To design specific primers for PABac (*R. radiobacter*), genomic DNA of PABac-DSM was used as template to amplify the 16S-23S rRNA intergenic transcribed spacer region, using primer ITSFeub and ITSReub (Table 2.2) (Cardinale et al., 2004). A PCR product of the expected size (1.4 kb) was cloned and sequenced as described earlier. The sequences obtained were used to design the *Rhizobium / Agrobacterium* specific primer pair ITS_Rh (Table 2.2). Primer pair ITS_Rh and virD2 were used in real-time PCR to quantify the amount of bacterium in *P. indica* cultures. Amplifications were performed in 25 µl SYBR® Advantage® qPCR Premix (Clontech Laboratories, Inc., CA, USA) according to manufacturer's instructions with 200 nM oligonucleotides, 100-300 ng fungal genomic DNA, and carried out with a Stratagene-Mx3000P® QPCR SystemMx3000P (Stratagene Research, La Jolla, CA, USA). After an initial activation step at 95°C for 1 min, 45 cycles (95°C for 5 s and 65°C for 25 s) were performed and a single fluorescent reading was obtained at 65°C of each cycle step. A melting curve was determined at the end of cycling to ensure the amplification of a single PCR product. Cycle threshold (Ct) values were determined with the Mx3000P V2 software supplied with the instrument. A standard curve using different dilutions of bacterial DNA was prepared and was used to calculate the amount of bacterial DNA in fungal samples.

2.7 Treatment of *P. indica* with antibiotics

In order to cure *P. indica* form bacteria, fungal hyphae were cultured for five generations in the presence of either spectinomycin (300 µg ml^{-1}) or ciprofloxacin (200 and 500 µg ml^{-1}), and in combination. Both antibiotics were effective against PABac-DSM *in vitro* (Table 3.6). Additionally, single spore culturing was performed in the presence of these antibiotics. Chlamydospores were harvested from four-week-old plates using a 0.05% Tween-20 solution, and purified three times by centrifugation at 100 g for seven min. The pellet was resuspended before each centrifugation step in 0.05% Tween-20 containing

300 µg ml^{-1} spectinomycin. Spores were finally treated with spectinomycin (300 µg ml^{-1}) for three hrs and subsequently plated on *Aspergillus* minimal medium plates containing spectinomycin (300 µg ml^{-1}). A single germinating spore was picked using a stereomicroscope (MZ16F, Leica, Germany) and used as inoculum on antibiotic containing agar plates. This was termed generation 1 (G1) of the single spore culture. Spores were harvested after three weeks from these plates and plated to produce further generations of single spore cultures (G2-G5) in the same way. After every generation, fungal samples were taken and bacterial detection was performed by conventional PCR using universal eubacterial primers and with real-time PCR using specific primers as described above.

Additionally, young growing mycelium from *P. indica* was picked with the help of a stereomicroscope and transferred to new plates containing *Aspergillus* minimal medium with antibiotics (spectinomycin 300µg ml^{-1} and ciprofloxacin 300µg ml^{-1}) and incubated at 24°C. Every fourth day the growing mycelium was transferred alternatively to fresh plates or to liquid *Aspergillus* minimal medium containing antibiotics for a total of five times. Finally young mycelium was transferred on *Aspergillus* minimal medium plates and liquid medium without antibiotics, grown for three weeks and used for DNA isolation. Seven independent treated colonies were checked for bacterial presence as described above.

2.7.1 *P. indica* protoplast isolation and treatment with antibiotics

Fresh mycelia from *P. indica*-DSM11827 were crushed and filtered through miracloth. The filtrate was collected by centrifugation and resuspended in liquid *Aspergillus* minimal medium. After three days the young mycelium was collected using a miracloth filter, washed twice with 0.9% NaCl and resuspended in SMC buffer (1.33 M sorbitol, 50 mM $CaCl_2$, 20 mM MES buffer pH 5.8) containing 2.5% lysing enzymes from *Trichoderma harzianum* (L1412 Sigma). The suspension was incubated for 1 h at 37°C. The activity of the lysing enzymes was stopped by adding STC buffer (1.33 M sorbitol, 50 mM $CaCl_2$, 10 mM TrisHCl; pH 7.5). Protoplasts were filtered through miracloth and collected by centrifugation. The pellet was washed thrice in STC buffer containing spectinomycin (300 µg ml^{-1}) and ciprofloxacin (300 µg ml^{-1}) and diluted to a final

concentration of $1x10^8$ cells ml^{-1}. The preparation was checked under a phase contrast microscope to ensure the absence of any mycelial fragments or spores. Liquid *Aspergillus* minimal medium containing 0.3 M sucrose, spectinomycin (300 µg ml^{-1}) and ciprofloxacin (300 µg ml^{-1}) was used for regeneration of protoplasts. Regeneration was observed after 72 h incubation at 30°C. Young growing mycelium from seven colonies were picked with the help of a stereomicroscope and transferred to new plates containing *Aspergillus* minimal medium with antibiotics. Every second day for eight consecutive days the growing mycelium was transferred to fresh *Aspergillus* minimal medium plates containing antibiotics. Finally young mycelium was transferred on medium plates without antibiotics, grown for three weeks and used for DNA isolation and checked for bacterial presence as described above.

2.8 Fluorescence *in situ* hybridization (FISH)

Based on a protocol described by Manz et al. (1996), a procedure for FISH was adopted, with some modification, as described below. All steps of FISH with fungal material were carried out in Eppendorf tubes (humid chamber). Two µl of the fixed bacterial suspension was immobilized on hydrophobic Teflon-coated slides in 8 mm hybridization wells (Roth GmbH, Karlsruhe) and hybridization was carried out as described above.

Procedure for Fluorescence *in situ* hybridization (FISH)

1. **Fixation.** Two to four-week-old fungal cultures or overnight grown bacterial cultures were fixed by adding 50% ethanol and incubation at 4°C for 3-4 h. Thereafter, cultures were washed three times in 1 x PBS and finally resuspended in a 1:1 mixture of 1 x PBS and $Ethanol_{absolute}$. Samples were stored at -20°C.
2. **Dehydration.** Fixed fungal material was dehydrated in an increasing ethanol series (50%, 80%, and 96% ethanol, three min each).
3. **Probe hybridization.** 8 µl of hybridization buffer (Table 2.3) and 1 µl of each probe were applied to the samples. The samples were incubated for 90 min at 46°C in dark.
4. **Washing.** The washing buffer (Table 2.4) was prepared and preheated at 48°C (in water bath). The hybridization buffer from the samples was rinsed with the pre-warmed washing buffer and the samples were incubated in the washing buffer for

10-20 min. in a preheated water bath (48°C). Afterwards, the washing buffer was removed with cold distilled water. After the washing step, the fungal material was spread onto glass slides and dried. The bacterial slides were dried quickly. The slides were then mounted in AF1 antifading reagent (Citifluor Ltd., London, United Kingdom) and observed in confocal laser scanning microscope.

Hybridization buffer

5M NaCl	360 µl
1M Tris-HCl (pH 8.0)	40 µl
Formamide	According to applied stringency (Table 2.3)
A. dest.	According to applied stringency (Table 2.3)
10 % (w/v) SDS	2 µl

(The buffer should always be freshly prepared)

Table 2.3 Hybridization buffer for FISH

% Formamide (v/v)	Formamide (µl)	A. dist. (µl)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
60	1100	500
65	1200	400
70	1300	300

Washing buffer

1M Tris-HCl (pH 8.0)	1000 µl
5M NaCl	According to applied stringency (Table 2.4)

0.5 M EDTA (pH 8.0)	500 µl
10 % (w/v) SDS	50 µl
A. dest.	Add to make final volume 50 ml.

(Preheat the buffer at 48°C prior to use)

Table 2.4 Washing Buffer for FISH

% Formamide in hybridization buffer	NaCl in mol/l	NaCl (µl)
0	0.900	9000
5	0.636	6300
10	0.450	4500
15	0.318	3180
20	0.225	2150
25	0.159	1490
30	0.112	1020
35	0.080	700
40	0.056	460
45	0.040	300
50	0.028	180
60	0.020	100
65	0.008	40
70	0.000	0

10 X PBS

NaH_2PO_4	12 g
Na_2HPO_4	14.2 g
NaCl	75.70 g
A. dist.	1000 ml
pH	7.2 - 7.4

5M NaCl

NaCl	292.0 g
A. dist.	1000 ml

1M Tris-HCl

Tris	121.14 g

($NH_2C(CH_2OH)_3$)

A. dist. 900 ml

Adjust pH 8.0 with HCl

Fill with A. dist. to 1000 ml

0.5M EDTA

EDTA 146.10 g

($C_{10}H_{16}N_2O_8$)

A. dist. 900 ml

Adjust pH 8.0 with NaCl

Fill with A. dist. to 1000 ml

10 % SDS (sodium dodecyl sulphate)

SDS 10 g

A. dist. 100 ml

Filter sterilization

The fluorescent tagged oligonucleotide probes used in this study were purchased from Thermo Electron Corporation GmbH, Ulm, Germany. These were EUB-338-mix (an equimolar mixture of EUB-338, [Amann et al., 1990], EUB-338-II, and EUB-338-III [Daims et al., 1999]), LGC-354-mix (an equimolar mixture of LGC-354-a, LGC-354-b, and LGC-354-c [Meier et al., 1999]), Rhi-1247 (Ludwig et al., 1998) and EUK-516 (Amann et al., 1990) (Table 2.5). All of them were labeled either with FITC, Cy3 or Cy5.

2.8.1 Microscopic analysis

Hybridized samples were analyzed with a confocal laser scanning microscope (CLSM 510 Axiovert 100 M; Zeiss, Jena, Germany) equipped with an argon laser (laserline 488 nm) and two helium-neon lasers (laserlines 543 and 633 nm), for the excitation of FITC, Cy3, and Cy5, respectively. Plan-Neofluar 100 X /1.3 oil and Apochromat 63 X /1.2 water immersion lenses were used for all analysis and image acquisitions. Monochrome images were taken sequentially at each wavelength to optimize scan conditions and laser

settings. Artificial colors were assigned to the fluorescent images resulting from each excitation wavelength: green for 488 nm, red for 543 nm, and blue for 633 nm. Superimpositions were processed with the Zeiss software package LSM 510, version 3.5.

Table 2.5 Phylogenetic oligonucleotide probes used for FISH analyses

Probe [1]	**Target** [1]	**Position** [1, 2]	**Sequence (5'-3')** [1]	**FA** [1, 3]	**Specificity** [1]
EUK-516	18S-rRNA	502 - 517	ACCAGACTTGCCCTCC	0-50%	Eukaryotes
EUB-338	16S-rRNA	338 - 355	GCTGCCTCCCGTAGGAGT	0-50%	Most bacteria, except *,**
EUB-338 II	16S-rRNA	338 - 355	GCAGCCACCCGTAGGTGT	0-50%	*Planctomycetales**
EUB-338 III	16S-rRNA	338 - 355	GCTGCCACCCGTAGGTGT	0-50%	*Verrucomicrobiales** *
LGC-354-a	16S-rRNA	354 - 371	TGGAAGATTCCCTACTGC	35%	Firmicutes (Gram-positive bacteria with low G+C content)
LGC-354-b	16S-rRNA	354 - 371	CGGAAGATTCCCTACTGC	35%	Firmicutes (Gram-positive bacteria with low G+C content)
LGC-354-c	16S-rRNA	354 - 371	CCGAAGATTCCCTACTGC	35%	Firmicutes (Gram-positive bacteria with low G+C content)
Rh-1247	16S-rRNA	1247-1252	TCGCTGCCCACTGTG	35%	*Rhizobium* sp., *Agrobacterium* sp., *Ochrobactrum* sp., some *Azospirilla* sp., few *Sphingomonas* sp.

[1] Data taken from probe base (http://www.microbial-ecology.net/probebase), (Loy et al., 2003; 2007); [2] Position according to Brosius et al. (1981); [3] % formamide in the hybridization buffer.

2.9 Ultrastructural studies using transmission electron microscopy

For ultrastructural studies, cells were high pressure frozen (HPM 010, BAL-TEC, Liechtenstein) and cryo-substituted in 0.25% glutaraldehyde (Sigma, Taufkirchen, Germany) and 0.1% uranyl acetate (Chemapol, Czech Republic) in acetone for four days using cryo-substitution equipment (FSU, BAL-TEC, Liechtenstein). This was followed by embedding in HM20 (Polysciences Europe, Eppelheim Germany) at -20°C. Sections were post-stained with uranyl acetate and lead citrate in an EM-Stain apparatus (Leica, Wetzlar, Germany) and subsequently observed with an EM 900 transmission electron microscope (Zeiss SMT, Oberkochen, Germany). Micrographs were taken with a SSCCD SM-1k-120 camera (TRS, Dünzelbach, Germany).

2.10 *In vitro* production of indole-3-acetic acid *R. radiobacter*

The Colorimetric assay was used to determine whether the *R. radiobacter* PABac-DSM could produce indole-3-acetic acid (IAA). PABac-DSM was incubated in mineral salt medium (M9 medium) supplemented with 0.5% glucose and 500 µg ml^{-1} tryptophan at 30°C for 24 hrs. Bacteria were removed from cultural broth by centrifugation at 10,000 rpm for 15 min. Two ml of the supernatant were transferred to a fresh tube containing 100 µl of 10 mM orthophosphoric acid and 4 ml of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) (Libbert and Risch, 1969). The mixture was incubated at room temperature for 25 min and the absorbance of the developed pink color was read at 530 nm. *Herbaspirillum frisingense* strain Mb11 (aux^+ reference strain), and *H. hiltneri* strain N3 (aux^- reference strain) were included in the analyses as positive and negative control respectively. The IAA concentration in culture was determined using a calibration curve of pure IAA following linear regression analysis. Bacterial growth was estimated by absorbance at 436 nm.

M9 Medium

Na_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g

A. dist.	880 ml
Autoclave	
20 % Glucose	20 ml
1M $MgSO_4$	2 ml
Tryptophan (500 mg in 100 ml)	100 ml

2.11 Plant materials and growth conditions

Kernels of barley cv. Ingrid and cv. Golden promise were sterilized with 3% sodium hypochloride for two hrs, rinsed in water and germinated for three days. Subsequently, seedling roots were inoculated in a homogenized fungal mycelial solution (1 g ml^{-1}) or bacterial suspension (OD_{600} 1.6) for 1.5 hrs. A bacterial suspension in water as well as in LB medium was used for inoculation. Water or LB medium was used as control.

After inoculating roots, barley seedlings were transferred to pots containing a 2:1 mixture of expanded clay (Seramis®, Masterfoods) and Oil Dri® (Damolin). Plants were grown in a growth chamber at 22°C/18°C day/night cycle, 60 % relative humidity and a photoperiod of 16 h (240 µmol m^{-2} s^{-1} photon flux density), and fertilized once after two weeks with 20 ml of a 0.5% Wuxal top N solution (Schering, N/P/K: 12/4/6) per pot containing three plants. Fungal colonization in roots was checked by staining with fuchsin-lactic acid and wheat germ agglutinin-Alexa Fluor 488 as described in Waller et al. (2005) and Deshmukh et al. (2006).

Arabidopsis thaliana ecotype Colombia (Col-0), mutant lines *npr1-1*, *npr1-3*, *jar1-1* (Staswick et al., 1992), *ein2-1* (Guzman and Ecker 1990), *abi4* (Finkelstein et al., 1998), jin1 (Berger et al., 1996) and *aim1* (Richmond and Bleecker, 1999) were obtained from the Nottingham Arabidopsis Stock Center (Scholl et al., 2000; accessions N3726 (*npr1-1*), N3802 (*npr1-3*), N8072 (*jar1-1*), N8844 (*ein2-1*), N3836 (*abi4*), N517005 (*jin1*), N848762 (*aim1*).

Seeds of all lines of *A. thaliana* were sown on 0.6% agar (Gelrite, Roth, Karlsruhe, Germany) prepared with ½-strength MS salts (Murashige and Skoog, 1962; Sigma-Aldrich, Munich, Germany), 1% sucrose and 0.01% casamino acids (Roth GmbH, Karlsruhe). Petri dishes were incubated at 4°C for 48 hrs and placed in a controlled environment growth chamber (see below). After 14 days, plants were transferred to pots

containing a 1:1 mixture of sand and Oil Dri. After transplanting seedling, either bacterial inoculation or mock treatment was performed. For inoculating with bacteria (PABac), bacterial culture was washed twice with water, resuspended in water and OD_{600} was adjusted to 0.6. One ml of this bacterial suspension was applied to each pot containing one seedling. Water was used as control. After two weeks of transplanting, bacterial suspension was again poured in each pot. Plants were grown in controlled environment growth chambers with an 8 h light (fluorescent cool white, Toshiba FL40SSW/37; 10^4 lux) / 16 hrs dark cycle, at 22°C/18°C and 60% relative humidity and fertilized twice after two and three weeks with 2 ml of a 0.2% Wuxal top N solution.

2.12 Biological activity of endophytes (*Sebacinales* strains and PABac)

Barley cvs. Ingrid and Golden Promise plants were harvested three weeks after inoculation. Shoot length and fresh shoot weight were measured. For the assessment of systemic resistance induction, the youngest leaves were used to perform a detached leaf-segment test for resistance against barley powdery mildew. Leaf segments were kept on agar plates containing 0.4% benzimidazole to inhibit leaf senescence and inoculated with 15 conidia mm^{-2} of *B. graminis* f.sp. *hordei*, race A6. Powdery mildew pustules (or colonies) were counted at seven days after inoculation.

After three weeks of bacterial inoculation of *A. thaliana* lines, powdery mildew challenge was done. *Golovinomyces orontii* (syn. *Erysiphe cichoracearum* USC1) (Adam and Somerville 1996) was propagated on hyper-susceptible *pad4-1 Arabidopsis* plants. Inocula were prepared by rinsing heavily infected *Arabidopsis* leaves with 0.02% Tween-20. For inoculation, a solution containing 3 X 10^4 ml^{-1} conidia was sprayed onto leaves. 100 ml of spore suspension m^{-2} of area was used. Fungal growth was microscopically assessed by determining the amount of *G. orontii* conidia on leaves ten days after inoculation. 5^{th}- 10^{th} leaves were detached from the plants, fresh weight determined and conidia washed from the leaves by vigorous shaking the leaves in a 2 ml reaction tube for 15 min with a defined volume of 0.01% Tween solution and counting the number of conidia microscopically in a counting chamber.

3 Results

3.1 Mutualistic symbiosis between *Sebacinales* and barley

3.1.1 Morphological variation between isolates of *Sebacina vermifera* species complex

Seven *Sebacina vermifera* isolates were selected, which were originally collected from different autotrophic orchids in Australia (Warcup, 1988) or in the case of *P. indica* isolated from woody shrubs in the Indian Thar desert in the mid nineties of the last century (Varma et al., 1998). To study the morphological variations between the different isolates of *S. vermifera* species complex, isolates were grown in different media. All seven cultivable isolates showed variable growth rates in different media. Modified *Aspergillus* medium (CM medium) was best suited for *P. indica* but not for isolates of *S. vermifera* species complex. MYP medium was found to be most effective for isolates of *S. vermifera* species complex (Fig 3.1a and b). Moderate growth was observed on MP

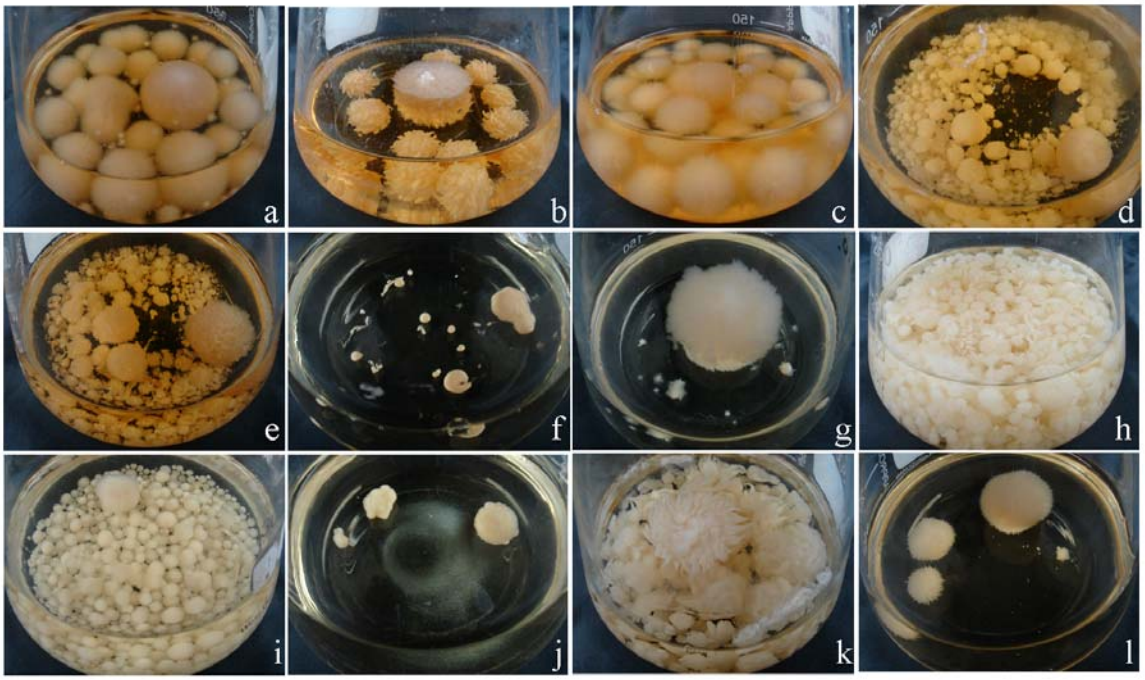

Fig. 3.1a. Axenic cultures of *Sebacinales*. Fungi were grown in MYP-medium (a-e) and CM medium (f-l) for five weeks at 25°C. (a) and (i) *S. v.* MAFF305830; (b) and (j) *S. v.* MAFF305852; (c) and (l) *S. v.* MAFF305828; (d) and (f) *S. v.* MAFF305837; (e) and (g) *S. v.* MAFF305835; (h) *P. indica*; (k) Multinucleate Rhizoctonia DAR29830.

medium (Fig 3.1b). All the isolates varied in their colony morphology, hyphal diameter, chlamydospore formation frequency and number of nuclei in hyphae and chlamydospore (Table 3.1).

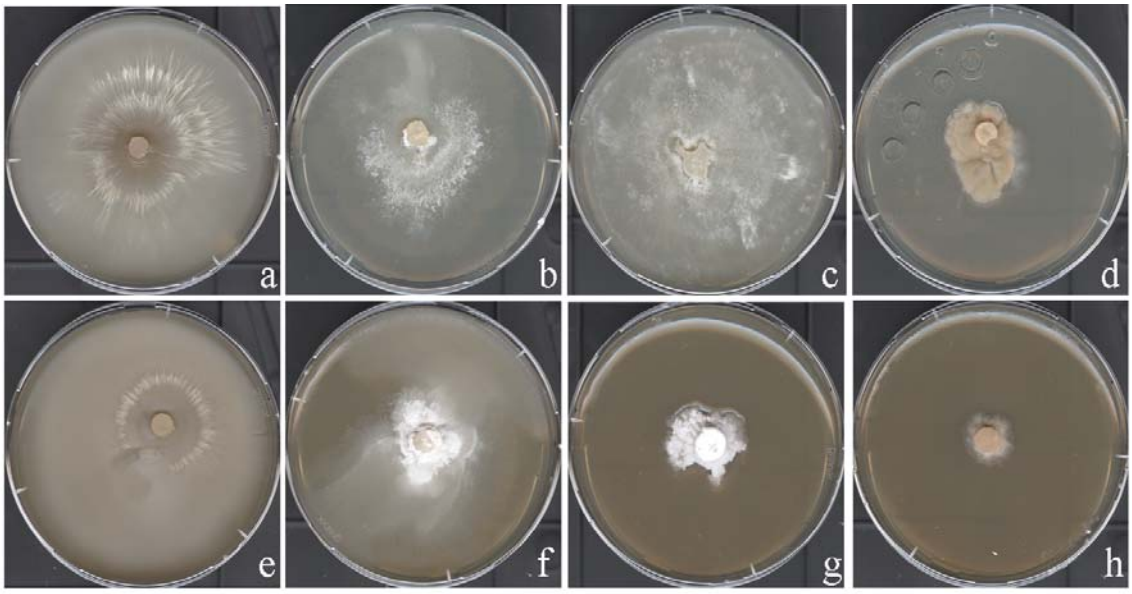

Fig. 3.1b. Axenic cultures of *Sebacinales*. Fungi were grown on MYP-medium (a)-(d) and MP medium (e)-(h) for four weeks at 25°C. (a) and (e) *S. v.* MAFF305828; (b) and (f) *S. v.* MAFF305837; (c) and (g) *S. v.* MAFF305835; (d) and (h) *S. v.* MAFF305838.

3.1.2 Phylogenetic analysis of *S. vermifera* species complex

Phylogenetic relationship between *S. vermifera* isolates was studied on the basis of ITS1-5.8S-ITS2 region (Fig 2.1). PCR with primer pair ITS1 and ITS4 amplified a product covering the ITS1-5.8S-ITS2 region which was found to result in similar sizes for all isolates. The results of the molecular phylogenetic analysis for the ITS sequences (ITS1 and ITS2) of all *Sebacinales* strains tested in this study and a comprehensive set of reference sequences published in Gene-Bank is shown in Fig 3.2. The outcome of the neighbour-joining and bootstrap analysis divided the *Sebacinales* into 3 groups. Group I contains *S. v.* MAFF305830, *S. v.* MAFF305842 and *S. v.* MAFF305828. Group II consists of three subgroups comprising of *S. v.* MAFF305837, *S. v.* MAFF305835 and *S. v.* MAFF305838 while *P. indica* and multinucleate Rhizoctonia DAR29830 formed a separate, less well resolved group (Group III).

Table 3.1 Colony and hyphal characteristics of *Sebacinales* isolates after 14-days incubation at 25°C.

Isolate/Strain	Growth Rate in axenic cultures in MYP medium	Hyphal thickness (µM)[a]	Hyphal cell size (µM) [a]	Spore diameter (µM) [a]	No. of nuclei in [a]	
					Mycelium	Spore
Piriformospora indica DSM11827	+++++	1.5-3.5	Irregularly septate	10-16	Multi-nucleate	8-25
S. vermifera MAFF305830	++++	1.6-3.3	8.0-14.4	4.8-8.0	1-3	1-4
S. vermifera MAFF305842	++++	1.6	3.2-32	6.4-8.0	1-5	2-6
Multinucleate *Rhizoctonia* DAR29830	++++	1.6-3.2	6.4-52.8	3.2-16.0	2-6	2-10
S. vermifera MAFF305828	+++++	1.6-3.2	8.0-12.8	3.2-6.4	1-2	2-6
S. vermifera MAFF305837	++	1.6-4.8	9.6-28.8	8.0	2-4	3-4
S. vermifera MAFF305835	++	1.6-3.2	8.0-12.8	6.4	1-4	2
S. vermifera MAFF305838	+	1.0-1.6	8.0-16.0	8.0	4	2-4

[a] Work done during Master's thesis of Krishnendu Mukherjee under the supervision of Monica Sharma and Frank Waller

Table 3.2 Comparative penetration efficiency of *Sebacinales* in barley roots[a]

Isolate/Strain	Days after inoculation[b]			
	3	6	10	21
Piriformospora indica DSM11827	+++	++++	+++	++
S. vermifera MAFF305830	+++	++++	++++	++
S. vermifera MAFF305842	+	++	++	+
Multinucleate *Rhizoctonia* DAR29830	++	+++	++	+
S. vermifera MAFF305828	+++	++++	++++	++
S. vermifera MAFF305837	++	+++	+++	++
S. vermifera MAFF305835	++	++	++	++
S. vermifera MAFF305838	+++	++++	+++	++

[a] Work done during Master's thesis of Krishnendu Mukherjee under the supervision of Monica Sharma and Frank Waller; [b] ++++ Very good penetration frequency, +++ Good penetration frequency, ++ Average penetration frequency, + Poor penetration frequency

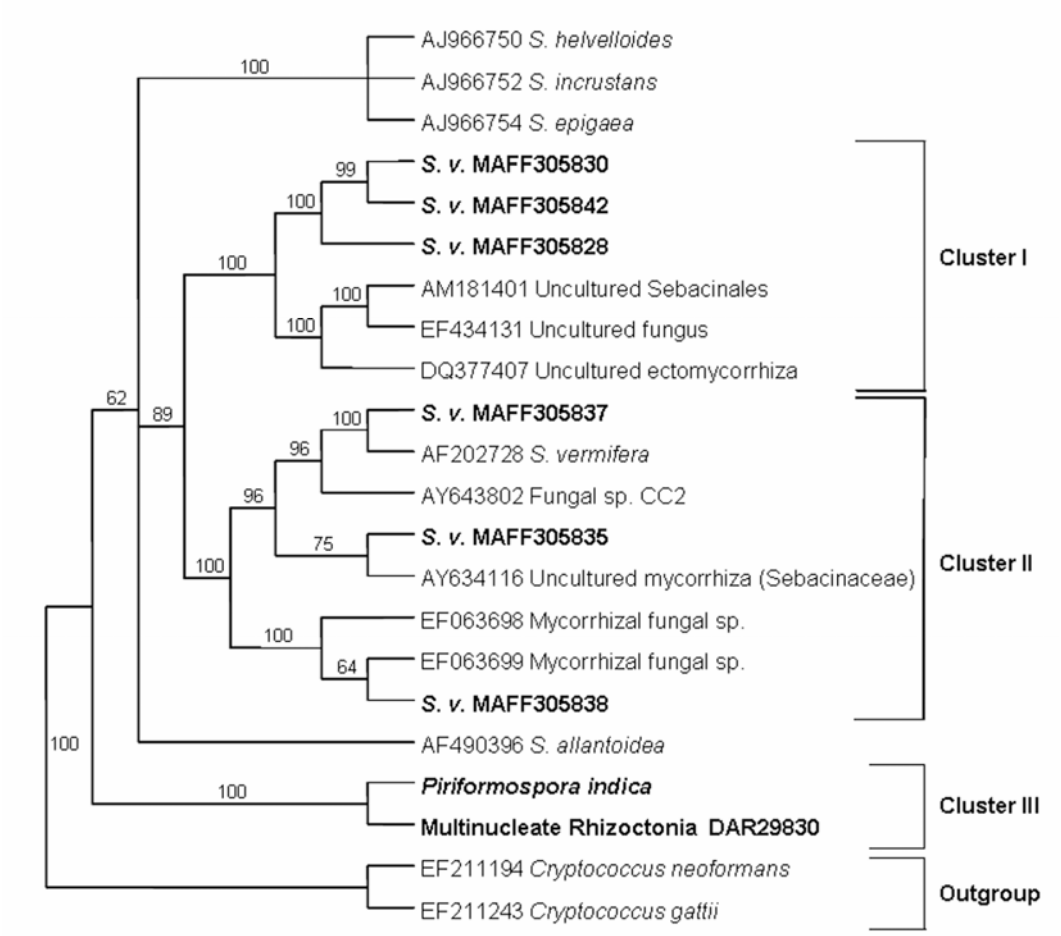

Fig. 3.2. Phylogeny of *Sebacinales* isolates based on nucleotide sequences of ITS1-5.8S-ITS2 region. The tree is computed by UPGMA (Unweighted pair group method with arithmatic mean) and Bootstrap with heuristic search. The tree shown is a 50% majority-rule consensus tree. Bootstrap values are indicated on branches.

3.1.3 Colonization of barley with *Sebacinales*

The isolates were tested for their ability to infest barley roots. Three-week-old axenic cultures were crushed using a blender and used for dip inoculation of three-day-old seedlings of barley cv. Ingrid and cv. Golden Promise. All isolates were able to infest and colonize barley roots to varying degrees (Table 3.2). The isolates formed inter- and intra-cellular hyphae. The frequency of sporulation within roots varied in different *Sebacinales* isolates (Fig. 3.3).

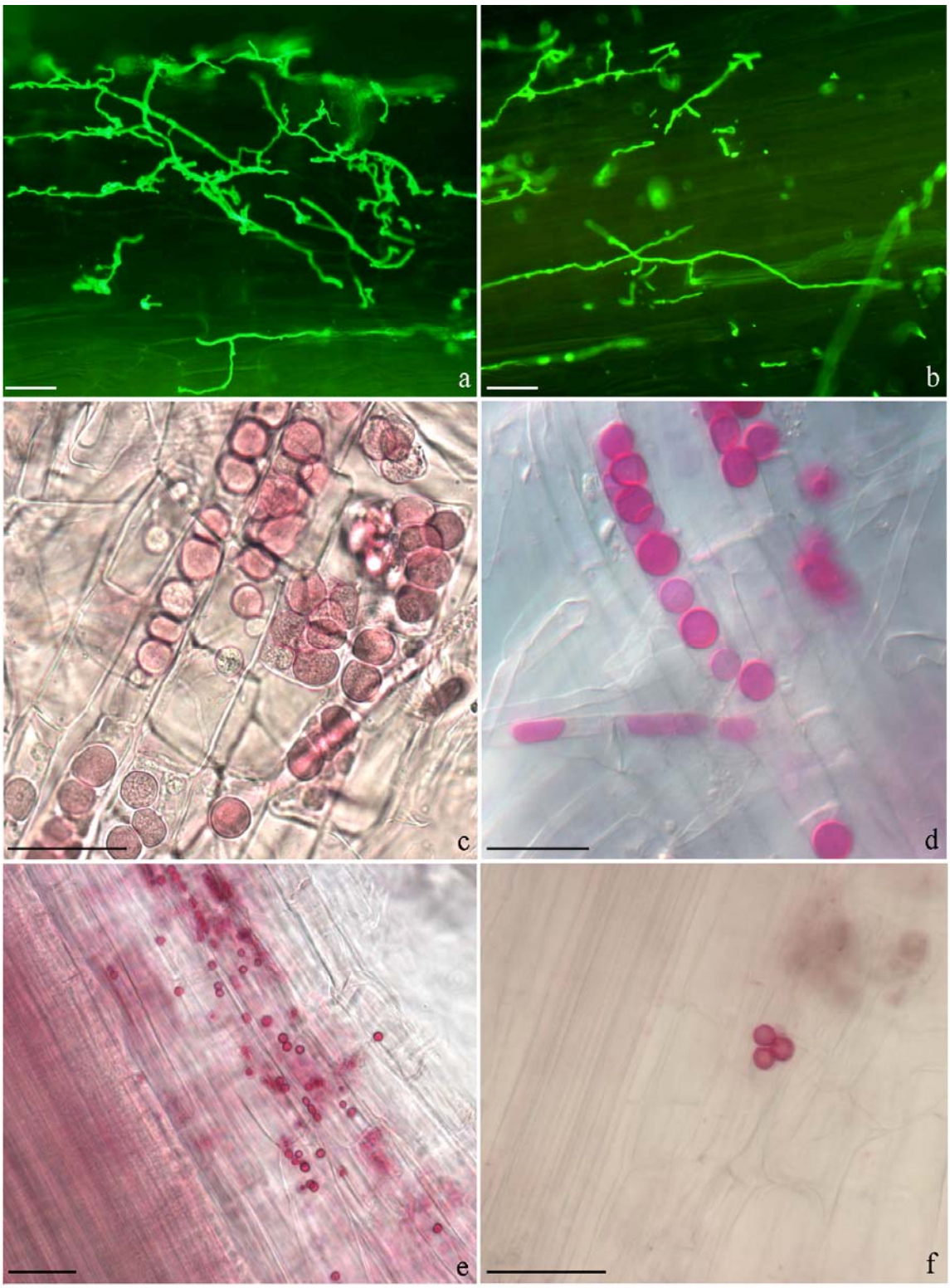

Fig. 3.3. Interaction of *Sebacinales* isolates with barley roots. (a) and (b) Inter and intracellular hyphae in cortical root cells were observed eight-days-after inoculation. Root segments were stained with WGA-AF488; (c)-(f) Chlamydospores in a root cortex cells were observed 21-days-after inoculation. Root segments were stained with fuchsin-lactic acid. (a) *P. indica*; (b) *S. v.* MAFF305830; (c) *P. indica*; (d) Multinucleate Rhizoctonia (e) *S. v.* MAFF305837; (f) *S. v.* MAFF305838. Scale Bar=13 µm.

3.1.4 Biological activity of *Sebacinales* in barley

To address the question whether strains of the *S. vermifera* complex and *P. indica* exhibit comparable biological activities, seedlings of barley cv. Ingrid and cv. Golden Promise were inoculated with different isolates of *S. vermifera* and *P. indica*, respectively, and shoot growth augmentation and systemic resistance induction in leaves against the barley powdery mildew (*Blumeria graminis* f.sp. *hordei*, *Bgh*) were determined. All isolates showed consistent biological activities in both cultivars of barley (Table 3.3 and Table 3.4).

All but two isolates (*S. vermifera* MAFF305842 and *S. vermifera* MAFF305835) caused increased shoot lengths in cv. Ingrid. The maximum increase of 9.8% in shoot length was observed after inoculation with *P. indica* followed by an increase of 7.3% after challenge with *S. vermifera* DAR29830. In contrast, fresh shoot weight was increased by all isolates tested. Maximum increase in fresh shoot weight of 28.2% was observed with *P. indica* followed by 18.3% with *S. vermifera* DAR29830 and 12.3% with *S. vermifera* MAFF305830 (Fig 3.4 a, b).

In cv. Golden Promise, all isolates supported shoot growth. Maximum increase in shoot length (23.2%) and in fresh shoot weight (48.2%) over control plants were observed in *S. vermifera* MAFF305830. Root-colonization with *P. indica* resulted in 13.7% increase in shoot length and 26.5% increase in fresh shoot weight over control plants. *S. vermifera* MAFF305838, *S. vermifera* MAFF305835 and *S. vermifera* DAR29830 were less effective in promoting shoot development (Fig 3.4 d, e).

To determine the potential of *P. indica* and isolates of the *S. vermifera* complex for systemic induction of resistance against *Bgh*, third leaves from endophyte-colonized and non-colonized 21-day-old barley plants (cvs. Ingrid and Golden Promise) were inoculated with conidia of *Bgh*, and powdery mildew pustules were counted. Consistent resistance-inducing activity of *P. indica* and all strains of the *S. vermifera* complex was found in both cultivars though there was considerable variation of the fungal activity among the different isolates.

In cv. Ingrid, *P. indica* was most effective in reducing pathogenic colonization followed by isolates of *S. vermifera* MAFF305842 and *S. vermifera* MAFF305830. *P. indica* significantly decreased powdery mildew pustules (43.1%) compared to control plants. A

Table 3.3 Effect of different *Sebacinales* isolates on shoot biomass and systemic resistance against powdery mildew in barley cv. Ingrid

Isolate/Strain	Shoot length			Shoot fresh weight			Colonisation by *Blumeria graminis* f.sp. *hordei*		
		Length (cm) ± SE	t value		Weight(g) ± SE	t value		No. of colonies per leaf ± SE	t value
P. indica	C	28.49 (± 0.41)	6.19466 E-07	C	0.55 (±0.01)	1.57515 E-11	C	151.08 (± 22.56)	0.021808
	F	31.58 (± 0.44)		F	0.77 (±0.02)		F	86.04 (±15.55)	
Sebacina vermifera MAFF305830	C	28.49 (± 0.41)	0.2055313	C	0.55 (+0.01)	0.0012915	C	151.08 (± 22.56)	0.060914
	F	29.26 (± 0.45)		F	0.63 (±0.02)		F	101.46 (± 12.59)	
S. vermifera MAFF305842	C	28.49 (± 0.41)	0.064832	C	0.55 (±0.01)	0.311704	C	151.08 (± 22.56)	0.032572
	F	27.49 (± 0.34)		F	0.58 (±0.02)		F	92.25 (± 14.28)	
Multinucleate Rhizoctonia DAR29830	C	30.17 (± 0.45)	1.6411E-06	C	0.57 (±0.02)	3.99984 E-07	C	151.08 (± 22.55)	0.021807
	F	32.91 (± 0.33)		F	0.74 (±0.02)		F	86.04 (± 15.55)	
S. vermifera MAFF305828	C	28.49(±0.41)	0.440525	C	0.55 (±0.01)	0.230948	C	151.08 (± 22.56)	0.289933
	F	28.90 (±0.34)		F	0.58 (±0.02)		F	119.58 (± 18.89)	
S. vermifera MAFF305837	C	28.04 (±0.38)	0.811398	C	0.58 (±0.01)	0.330150	C	110.79 (± 15.15)	0.187677
	F	28.16 (±0.25)		F	0.60 (±0.01)		F	83.21 (± 14.23)	
S. vermifera MAFF305835	C	28.49(± 0.41)	0.638357	C	0.55 (±0.01)	0.175621	C	151.08 (± 22.56)	0.137698
	F	28.21(±0.43)		F	0.59 (± 0.02)		F	107.00 (± 18.51)	
S. vermifera MAFF305838	C	25.42 (± 0.54)	1.294498	C	0.53 (± 0.02)	0.753552	C	136.83 (± 22.95)	0.345570
	F	25.75 (± 0.59)		F	0.54 (±0.03)		F	111.17 (± 13.50)	

C, Control; F, Fungal treatment; SE, Standard error values

Table 3.4 Effect of different *Sebacinales* isolates on shoot biomass and systemic resistance against powdery mildew in barley cv. Golden promise

Isolate/Strain	Shoot length			Shoot fresh weight			Colonisation by *Blumeria graminis* f.sp. *hordei*		
	Length (cm) ± SE		t value	Weight(g) ± SE		t value	No. of colonies per leaf ± SE		t value
P. indica	C	21.38 (±0.32)	2.70834E-14	C	0.42 (±0.01)	1.12057 E-14	C	29.68 (± 4.13)	4.13663 E-06
	F	24.76 (±0.26)		F	0.58 (±0.01)		F	8.65 (±1.48)	
S. vermifera MAFF305830	C	19.79 (±0.39)	1.00252E-13	C	0.33 (±0.01)	5.00957 E-12	C	29 (± 5.15)	0.000166
	F	25.79(±0.54)		F	0.63 (±0.04)		F	5.96 (± 2.26)	
S. vermifera MAFF305842	C	19.1 (±0.57)	3.17521E-06	C	0.33 (±0.02)	0.024549	C	27.5 (± 8.26)	0.108674
	F	22.98 (±0.43)		F	0.39 (±0.02)		F	12 (± 4.21)	
Multinucleate Rhizoctonia DAR29830	C	22.55 (±0.44)	0.007371	C	0.52 (±0.01)	0.010897	C	24.29 (± 3.56)	0.009403
	F	24.39 (±0.51)		F	0.58 (±0.02)		F	13.04 (± 2.31)	
S. vermifera MAFF305828	C	21.09 (±0.57)	9.60278E-06	C	0.38 (±0.02)	4.51683 E-05	C	23.97(± 3.95)	0.66178
	F	24.81 (±0.55)		F	0.54 (±0.03)		F	21.36 (± 4.44)	
S. vermifera MAFF305837	C	19.79 (±0.39)	1.84695E-09	C	0.33 (±0.01)	7.09339 E-08	C	29 (± 5.15)	0.004002
	F	23.69 (±0.42)		F	0.48 (±0.02)		F	12.13 (± 2.13)	
S. vermifera MAFF305835	C	21.91 (±0.57)	0.011137	C	0.45 (±0.02)	0.106467	C	21.46 (± 4.68)	0.062626
	F	23.77 (±0.42)		F	0.50 (± 0.02)		F	10.57 (± 2.90)	
S. vermifera MAFF305838	C	20.28 (±0.37)	0.001366	C	0.34 (±0.01)	0.107077	C	44.61 (± 7.22)	0.024492
	F	21.98 (±0.35)		F	0.36 (±0.01)		F	24.58 (± 4.88)	

C, Control; F, Fungal treatment; SE, Standard error values

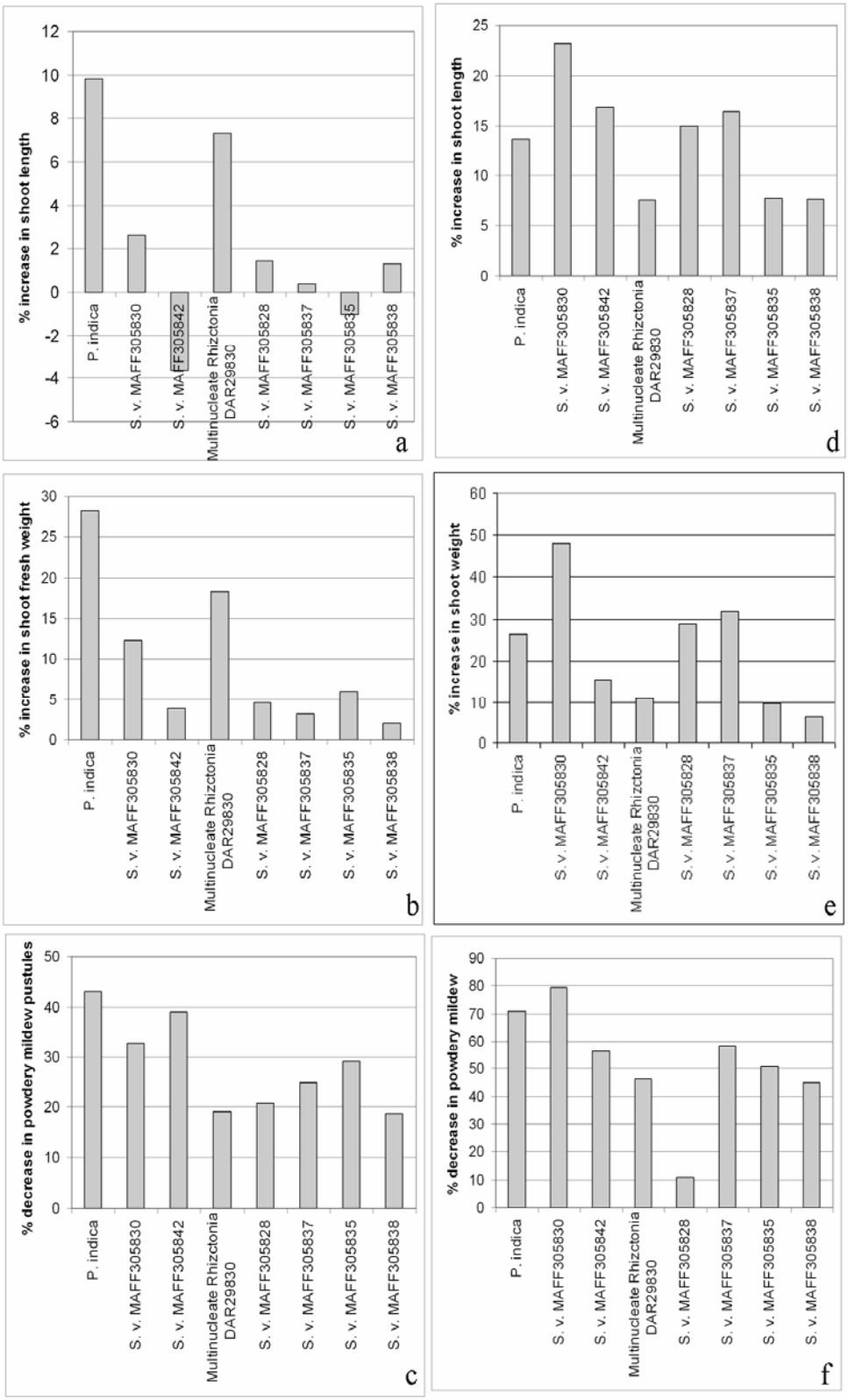

Fig. 3.4. Biological activity conferred by *P. indica* in barley. Plants were harvested 21-days-after fungal infestation and shoot length and shoot fresh biomass were recorded. Relative increase in shoot length and shoot fresh biomass of infested plants over non-infested plants were calculated in cv. Ingrid (a, b) and cv. Golden Promise (d, e). Leaf segment assay was performed and decrease in powdery mildew disease in infested plants over non-infested plants was calculated in cv. Ingrid (c) and cv. Golden Promise (f).

decrease of 38.9% in emerging pustules was observed by *S. vermifera* MAFF305842 followed by 32.9% with *S. vermifera* MAFF305830 (Fig 3.4 c).

In cv. Golden Promise, maximum systemic resistance against powdery mildew was induced by *S. vermifera* MAFF305830 (79.8%) followed by *P. indica* (70.8%) and *S. vermifera* MAFF305837 (58.2%) (Fig 3.4 f). All the isolates of *S. vermifera* species complex were capable of inducing systemic resistance against powdery mildew.

3.2 Bacteria associated with *Sebacinales*

3.2.1 *P. indica* is associated with *Rhizobium radiobacter*

Under standard culture conditions as used for fungal propagation, *P. indica* develops spherical fungal colonies within three to four weeks in transparent CM agar-medium. However, crushing the mycelium with a fine blender and subsequent microscopic examination of the supernatant upon bacterial live-dead staining indicated the presence of rod-shaped bacteria. This initial observation suggested that there is a tight association of *P. indica* with bacteria. To check this notion, we traced the presence of specific bacteria in P. *indica* isolates originating form various laboratories (*P. indica*-JE1, *P. indica*-HA, and *P. indica*-ND) and the original *P. indica* isolate DSM11827 deposited in 1997 immediately after the discovery of the fungus in the Indian Thar desert. Using universal primers, almost full length fragment of the entire bacterial 16S-rRNA gene was amplified from the fungal metagenomic DNA of *P. indica*-DSM11827 as well as in other fungal cultures including *P. indica*-JE1, *P. indica*-HA, and *P. indica*-ND (Fig 3.5). Comparative sequence analysis of the 16S-rRNA gene showed identical sequences for all *P. indica* isolates and designated the bacterium as *α-Proteobacterium* of the genus *Rhizobium* with the highest similarity to the species *R. radiobacter* (synonym *Agrobacterium radiobacter or Agrobacterium tumefaciens*) (Young et al., 2001). These data suggested that *P. indica* contains a single bacterial strain. To confirm this finding, we employed denaturing gradient gel electrophoresis (DGGE) of the semi-nested PCR products of the 16S-rRNA coding gene. DGGE revealed the presence of a single high intensity band in *P. indica*-DSM11827 and *P. indica*-JE1 (Fig 3.6). DNA sequences of the PCR product were identical to a 500 bp region of the 16S-rRNA gene obtained for metagenomic DNA of *P. indica*-DSM11827.

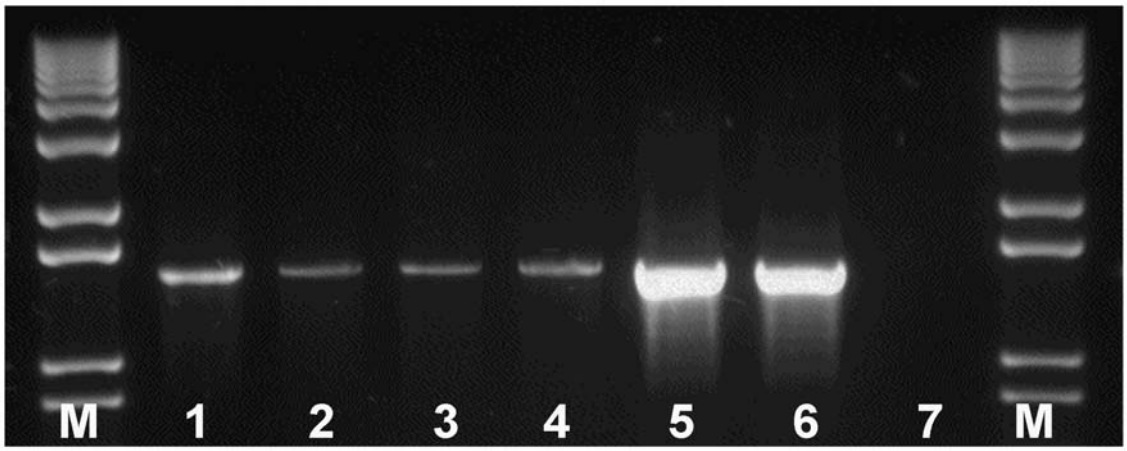

Fig. 3.5. Detection of bacterial 16S-rRNA gene by agarose gel electrophoresis.
Ethidium bromide-stained agarose gel showing PCR products of 16S-rRNA coding regions amplified with universal bacterial primers 27f and 1495r from *P. indica*'s metagenomic DNA, or from bacteria isolated from fungal samples. Lane M, 1 kb plus marker; lane 1, *P. indica*-DSM11827; lane 2, *P. indica*-JE1; lane 3, *P. indica*-HA; lane 4, *P. indica*-ND; lane 5, PABac-DSM isolated *P. indica*-DSM11827; lane 6, PABac-JE isolated from *P. indica*-JE1; lane 7, non-template control.

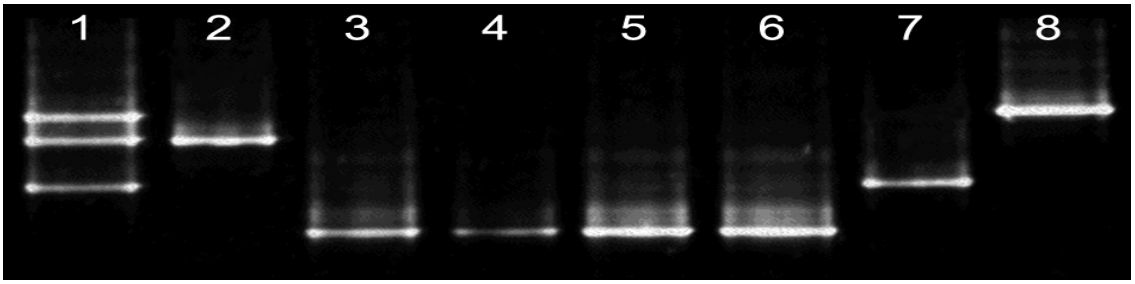

Fig. 3.6. **Detection of *P. indica*-associated bacteria by Denaturing Gradient Gel Electrophoresis (DGGE).** The 16S-rRNA gene fragments of 500 bp were obtained from the metagenome of bacterial communities associated with different isolates of *P. indica*. Lane 1, reference pattern composed with three known bacterial strains (*E. coli*, *H. frisingense* Mb11, *Acinetobacter* sp.); lane 2, *Acinetobacter* sp.; lane 3, *P. indica*-DSM11827; lane 4, *P. indica*-JE1; lane 5, *R. radiobacter* PABac-DSM isolated from *P. indica*-DSM11827; lane 6, *R. radiobacter* PABac-JE isolated from *P. indica*-JE1; lane 7, *E. coli* ; lane 8, *Herbaspirillum frisingense Mb11.* Sequence data from the bands in lanes 3 and 4 showed 100% identity with the 16S-rRNA gene sequences of the bacterial isolates PABac-DSM and PABac-JE in lanes 5 and 6.

For further characterization, isolation of bacteria from the fungal mycelium of *P. indica*-DSM11827 and *P. indica*-JE1 was attempted. Growth of isolated bacteria, named PABac-DSM and PABac-JE, respectively, was observed three days after inoculation of bacteria released from mechanically sheared fungal hyphae in LB medium supplemented

with 0.8% sucrose (Fig 3.7). Microscopic analysis revealed the presence of a pure culture of rod-shaped bacteria of 1-1.5 µm in length. By using the primer pair 27f and 1495r to amplify the 16S-rRNA gene, a PCR-product of the expected size was obtained (Fig 3.5). Cloning, sequencing and comparative 16S-rRNA gene sequence analysis showed that all obtained sequences were identical and were also identical to those sequneces isolated from fungal metagenomic DNA of different *P. indica* isolates. After BLAST search and phylogenetic analysis with ARB software (Fig 3.8), the sequence was identified as belonging to an *α-Proteobacterium* of the genus *Rhizobium* with the highest similarity and 100% homology to the species *R. radiobacter* AJ389909.

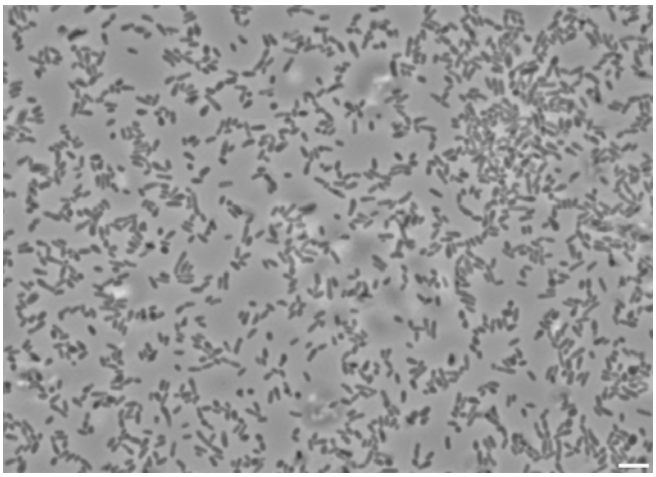

Fig. 3.7. Light microscopy of the pure culture of *R. radiobacter* (PABac-DSM). Rod shaped bacteria observed using epifluorecent microscope (Axioplan 2, Zeiss). (Scale bar = 2 µm).

DGGE profiles of a direct and semi-nested PCR assay using 16S-rRNA gene primers corroborated presence of a single high intensity band in PABac-DSM and PABac-JE which were identical in electrophoretic mobility and proved to have the same sequence as obtained from the metagenomic DNA of *P. indica*-DSM11827 and *P. indica*-JE1 (Fig 3.6).

3.2.2 Quantification of *R. radiobacter* in *P. indica*

Various primers were screened for amplifying *R. radiobacter* PABac-DSM in conventional as well as in real-time quantitative PCR (Table 3.5). *R. radiobacter* PABac-DSM specific primer pair (ITS_Rh), designed in this study, and primer pair for virD2 region were found to be most efficient in amplifying respective bacterial gene sequences on fungal metagenomic DNA and hence were used for the quantification of *R. radiobacter* in *P. indica*. The ratio of bacterial to fungal DNA in different fungal cultures

by real time quantitative PCR showed an average of 0.035 ng of *R. radiobacter* DNA per 100 ng of *P. indica* DNA. Both primer pairs gave similar values.

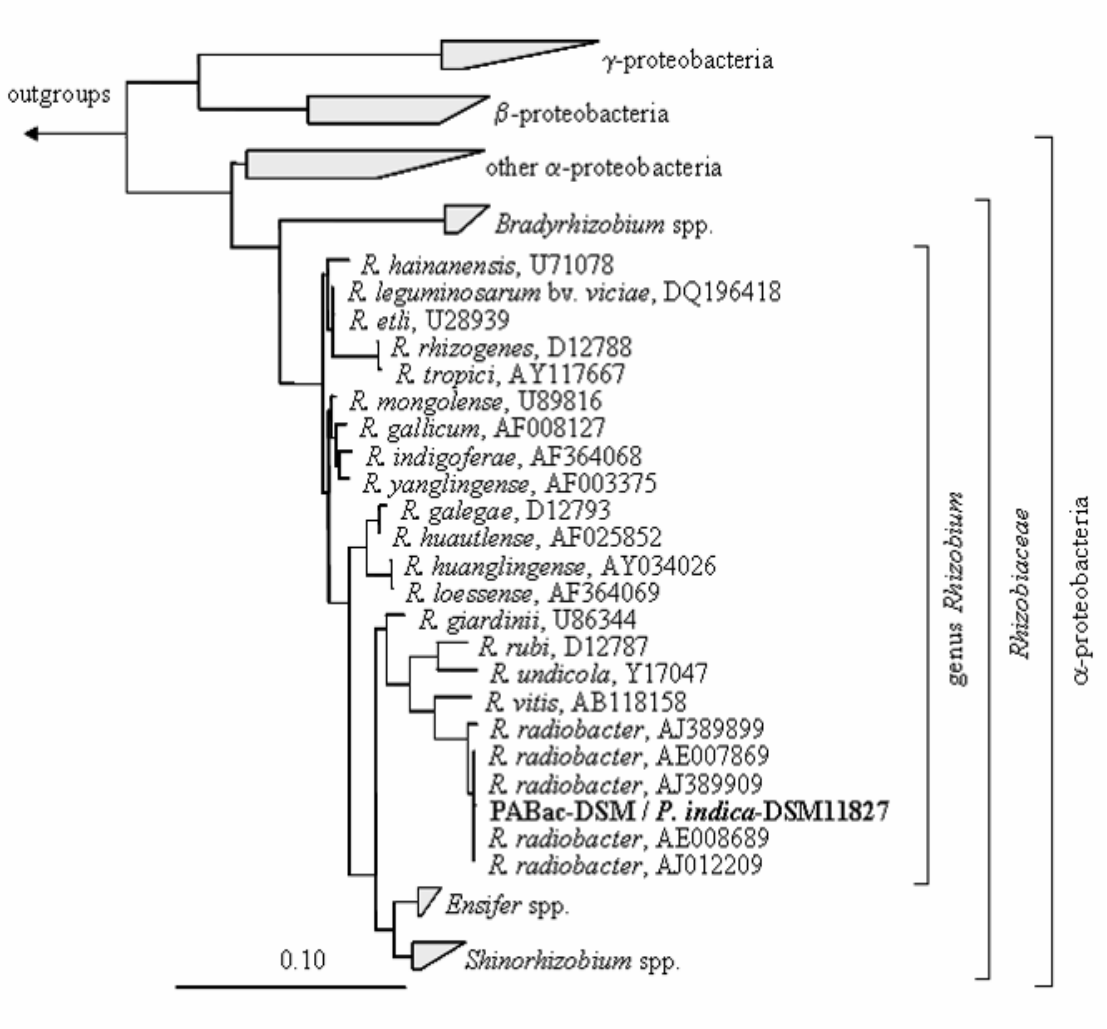

Fig. 3.8. Phylogenetic classification of *P. indica*-associated bacteria.

Phylogenetic tree based on comparative sequence analysis of 16S-rRNA coding genes of *P. indica*-associated bacteria and representatives of related *Rhizobium radiobacter* / *Agrobacterium tumefaciens* group. The bar indicates 10% sequence divergence.

Table 3.5 PCR primers employed to amplifications

Primers	Conventional PCR		Quantitative PCR		Expected size (bp)	No. of bands
	R. radiobacter	*P. indica*	*R. radiobacter*	*P. indica*		
For chromosomal DNA						
27f and 1495r	+	+	nd	nd	1500	Single
27f and R518	+	+	nd	nd	500	Single
9f and 1492r	+	-	nd	nd	900	Single
616F and 630R	+	-	nd	nd	1500	Single
ITSFeub and ITSReub	+	nd	nd	nd	1423	Double
ITS_Rhf and ITS_Rhr	+	+	+	+	266	Slight double
ITS_PiBi F and ITS_PiBi R	+	-	+	+	136	Slight double
ITS_Rhi F and ITS_PiBi R	+	+	+	+	139	Single
ros1 and ros3	+*	-	+	+	550	Double
chvA_F1 and chvA_R1	+	nd	nd	nd	650	Single
chvA_F and chvA_R2	+	nd	nd	nd	777	Single
R16-1 and R23-3R (Rhizibium ITS)	+	nd	nd	nd	1500	Single
On Ti plasmid						
$virD_2$_A and $virD_2$_E	+	nd	+	+	338	Single
$virD_2$_A and $virD_2$_C	+	nd	nd	nd	224	Single
ipt F and *ipt* R	-	nd	nd	nd	427	
nifH1 and nifH2	-	nd	nd	nd	600	
nifH1 and nifH4	+	nd	nd	nd	350	Multiple
nifH1 and nifH5	+	nd	nd	nd	350	Double
fliG F and fliG R	nd	nd	+	+		
rol F and rol R	nd	nd	-	nd		

+, Present; -, Absent; nd, Not determined; * Single band in conventional PCR

3.2.3 Treatments for curing *P. indica* from *R. radiaobacter*

In order to eliminate *R. radiobacter* from *P. indica*-DSM11827, bacteria (*R. radiobacter* PABac-DSM) were first cultivated in LB medium in the presence of various antibiotics while the efficacy of the chemicals was proved by OD_{600} measurement after 48 h (Table 3.6). Among other antibiotics spectinomycin and ciprofloxacin were found to be highly efficient in completely inhibiting the growth of *R. radiobacter* PABac-DSM *in vitro*. However, after axenic culturing of *P. indica*-DSM11827 for 2 months at 25°C in the presence of ciprofloxacin (200µg ml^{-1}) and/or spectinomycin (300µg ml^{-1}) applied succeedingly, bacteria were neither eliminated from the hyphae nor from chlamydospores as evidenced by PCR analysis. In analogy to the strategy followed in arbuscular mycorrhiza (Lumini et al., 2007), *P. indica* was intended to be cured from bacteria by producing successive vegetative generations starting from single chlamydospores. Five generations of single spores (G1, G2, G3, G4, G5) were grown on agar plates containing 300µg ml^{-1} spectinomycin and ten colonies from each generation were tested for bacterial presence by conventional PCR using universal eubacterial primers. However, bacteria were detected in all generations. In a complementing approach, young growing hyphae were transferred on freshly prepared plates containing antibiotics every fourth day for five times. In all seven tested *P. indica* colonies, conventional PCR with universal primes as well as real time quantitative PCR with primers specific for the *Rhizobium / Agrobacterium* intergenic transcribed spacer (ITS) resulted in PCR products of appropriate sizes confirming the presence of *R. radiobacter*. In another attempt, hyphal protoplasts were isolated and subsequently regenerated on plates containing *Aspergillus* minimal medium with 0.3 M sucrose, spectinomycin (300 µg ml^{-1}) and ciprofloxacin (300 µg ml^{-1}) at 30°C and growing mycelium was transferred, every second day for eight consecutive days, to fresh medium plates containing antibiotics. All seven independently regenerated *P. indica* colonies gave positive signals after real-time quantitative PCR analysis of the bacterial ITS region confirming the presence of bacteria.

Table 3.6 Efficacy of antibiotics against *R. radiobacter*. Bacteria (PABac-DSM) were grown in LB medium containing various antibiotics at 25°C. OD_{600} was measured 48 h after inoculation.

Antibiotic	OD_{600} after 48 h
Spectinomycin (300 µg ml^{-1})	0.000
Spectinomycin (200 µg ml^{-1})	0.010
Ciprofloxacin (200 µg ml^{-1})	0.013
Ciprofloxacin (100 µg ml^{-1})	0.017
Cefatoxime (300 µg ml^{-1}) + Ticarcillin (150 µg ml^{-1})	0.019
Oxytertacycline (100 µg ml^{-1})	0.030
Rifampicin (100 µg ml^{-1})	0.032
Cefatoxime (500 µg ml^{-1})	0.050
Oxytertacycline (200 µg ml^{-1})	0.070
Cefatoxime (250 µg ml^{-1})	0.091
Ticarcillin (150 µg ml^{-1})	0.501
Lincomycin (100 µg ml^{-1})	2.160
Carbenicillin (100 µg ml^{-1})	2.243
Gentamicin (100 µg ml^{-1})	2.510
Gentamicin (50 µg ml^{-1})	2.450
Control	2.499

3.2.4 *P. indica* is intimately associated with *R. radiobacter*

In order to locate bacteria associated with *P. indica*, fungal preparations from axenic cultures of *P. indica*-DSM11827 as well as cells of a pure culture of strain PABac-DSM were stained by fluorescence *in situ* hybridization (FISH). The presence of bacterial rRNA in the fungal mycelium was proven by application of a probe specific for eubacteria (EUB-338-mix) (Table 2.5). Concomitantly, a co-localized signal was detected by using the Rh-1247 probe, which is specific for rRNA of bacteria belonging to the *Rhizobium* group (Fig 3.9). In order to exclude the detection of unspecific hybridizations

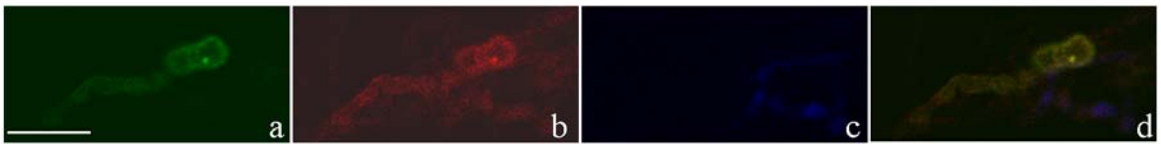

Fig. 3.9. Detection of *P. indica*-associated bacteria by fluorescence *in situ* hybridization (FISH). Fungal mycelium of *P. indica*-DSM11827 was used for FISH analysis. FISH was performed with EUB-338-mix-FITC (green), specific for the domain bacteria (a); and with Rh-1247-Cy3 probe (red), specific for the *Rhizobium* group (b); no fluorescence was observed with EUK-516-Cy5 (blue) specific for eukaryotes (c);. (d) show the superimposed image, composed rgb-images result in a yellow color for the bacteria, indicating co-labeling by EUB-338-mix-FITC and Rh-1247-Cy3. (Scale bar = 10 µm).

EUK-516 probes were introduced that are specific for 18S-rRNA of eukaryotes. These analyses further implicated association of bacteria with mycelium and chlamydospores of *P. indica* and further confirmed the low number of associated bacteria as was already indicated by real-time PCR-based quantification.

3.2.5 *R. radiobacter* produces Indole-3-acetic acid

Many plant-associated and rhizosphere bacteria are known to secrete the auxin indole-3-acetic acid (IAA). To test whether *R. radiobacter* PABac-DSM also showed this characteristic, the bacterium was grown in mineral salt medium supplemented with 0.5%

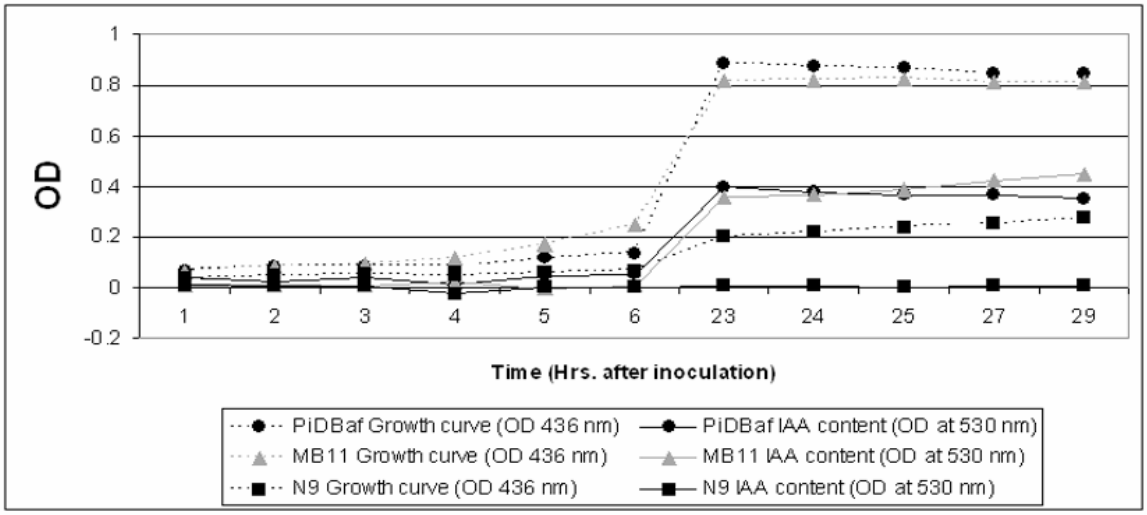

Fig. 3.10. IAA production by *R. radiobacter*. Colorimetric assay using Salkowski's reagent was used to measure IAA production by PABac (*R. radiobacter*). *R. radiobacter* produced substantial amount of IAA. The growth kinetics showed the stability of the metabolically active cells during stationary phase.

glucose and 500 µg ml^{-1} of tryptophan exhibited substantial production of IAA (Fig 3.10). The bacterium produced 40µg ml^{-1} IAA after 24 h at 25°C. However, in the absence of tryptophan in the medium, there was no production of IAA demonstrating tryptophan-dependent IAA production by *R. radiobacter*.

3.2.6 *R. radiobacter* induces growth promotion and disease resistance in barley

Most substantial biological activities of *P. indica* and related *S. vermifera* species in various host plants are growth promotion and systemic induced resistance to fungal pathogens (see above). To assess the biological activity of isolated bacteria, roots of three-day-old barley seedlings (cv. Golden Promise) were dip-inoculated with PABac-DSM (OD_{600} 1.6). Upon three weeks, treated plants showed an increase in shoot length (4.2%) and shoot fresh weight (17.9%) over control plants demonstrating the growth promoting activity of the bacterium (Fig 3.11 a and b). Moreover, the same plants were more resistant to the biotrophic fungal leaf pathogen *Bgh*. Barley leaves showed a decrease in the frequency of powdery mildew pustules of 63.9% over control (plants

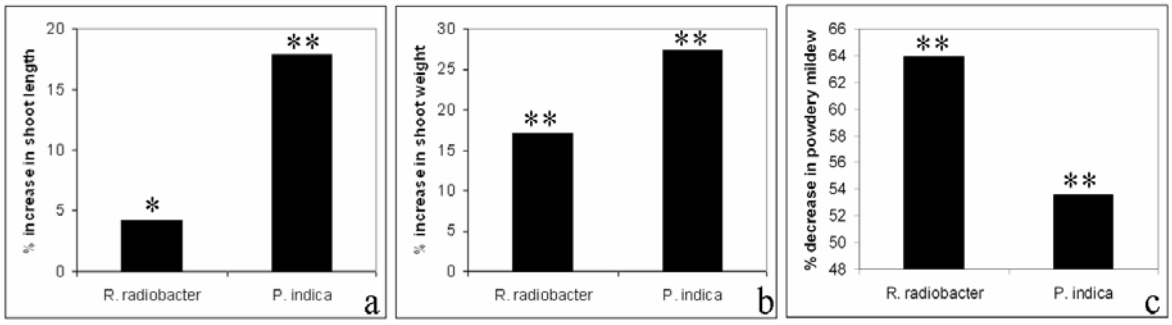

Fig. 3.11. Biological activity conferred by *R. radiobacter* in barley. Increase in shoot length (a) and shoot fresh weight (b) of *R. radiobacter* and *P. indica* treated plants over control (noninfested) plants, determined 3-week-after inoculation. Decrease in powdery mildew disease (c) was calculated from the number of powdery mildew pustules developing on third leaf segments 7 dai with *B. graminis* f.sp. *hordei,* race A6. Values are means of five independent experiments, each consisting of 60 endophyte-inoculated and control plants, respectively. Asterisks denote statistically significant differences between the respective values of endophyte-colonized and noncolonized plants (*****, Student's t test P< 0.01; ** Student's t test P< 0.001).

without bacteria) (Fig 3.11 c). To compare the bacteria- with fungus-mediated activity, barley roots were inoculated with *P. indica* and an increase in fresh shoot biomass (27.4%) and a decrease in the number of powdery mildew pustules (53.6%) as compared to non-colonized plants were found (Fig 3.11).

3.2.7 *R. radiobacter* induces growth promotion and disease resistance in *A. thaliana*

P. indica has been shown to confer growth promotion to *Arabidopsis thaliana* (Peskan - Bergheofer et al., 2004). Recently, the disease resistance potential of *P. indica* has also been demonstrated for *A. thaliana* (E. Stein, A. Molitor, K.-H. Kogel & F. Waller, unpublished data). To answer the question whether *R. radiobacter* exhibits comparable biological activities in *A. thaliana* (ecotype Columbia), 14 day-old seedlings were inoculated with *R. radiobacter* (OD_{600} 0.6) and resistance against powdery mildew fungus *Golovinomyces orontii* was determined. Bacteria-inoculated plants showed a strong reduction in powdery mildew disease (Fig 3.12). The number of powdery mildew conidia per 100 mg of leaf fresh weight was reduced by 20.6% in *R. radiobacter* inoculated plants compared to controls. A strong argument in favor of using *A. thaliana* is the accessibility of a wealth of plant mutants, which can be used to dissect defense pathways potentially involved in the observed systemic resistance phenotype. To elucidate the requirement of known defense pathways for bacterial activity, the development of *G. orontii* on *A. thaliana* mutants inoculated with *R. radiobacter* was investigated. Bacteria-infested plants of mutant lines *npr1-3, ein2-1* and *aim1* were more resistant against *G. orontii* whereas mutant lines *npr1-1*, *jin1*, *jar1-1* and *abi4* were susceptible as to *G. orontii* as control plants non-challenged with bacteria (Fig 3.12). The number of powdery mildew conidia per 100 g fresh leaf at 7 dpi was reduced significantly by 18.8% in *npr1-3* plants, 22.1% in ethylene insensitive mutant *ein2-1* and 22.6% in jasmonate biosynthesis mutant *aim1*, similar to wild type plants (20.6%) indicating that these mutants remained fully responsive to *R. radiobacter*. Consistently, *npr1-1*, in clear contrast to *npr1-3*, showed no reduction of conidia showing that the cytoplasmic function of NPR1 is needed for *R. radiobacter*-mediated resistance responses to *G. orontii*. More number of *G. orontii* conidia could be observed in *jar1-1* and *jin1*. An increase of 13.1%

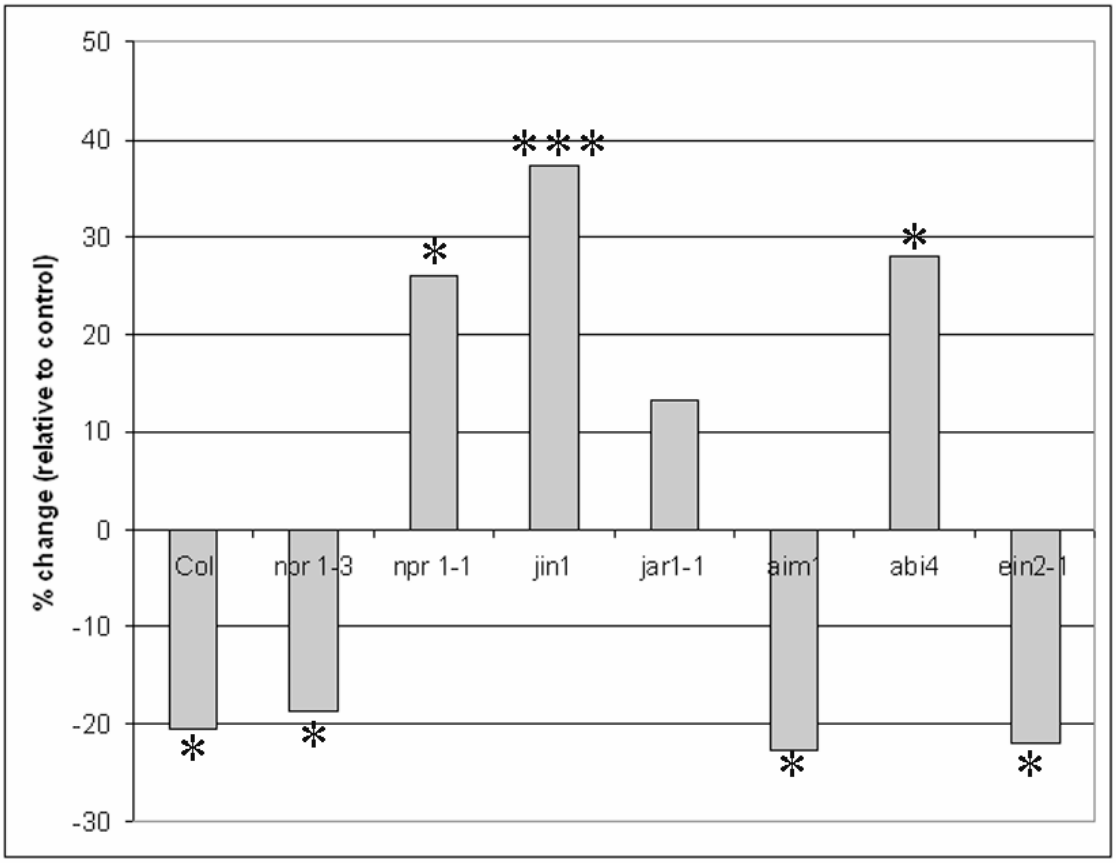

Fig. 3.12. Genotype-dependent enhanced resistance against powdery mildew after colonization with *R. radiobacter*. Leaves of two-week-old plants were inoculated with conidia of the powdery mildew fungus *Golovinomyces orontii*. Ten days post inoculation, leaves were detached, and conidia were washed from the leaves and counted after determination of the leaf fresh weigth. The amount of conidia per 100 mg of leaf fresh weight was calculated for at least ten individually treated plants and amounts for *R. radiobacter* infested plants were calculated relative to control plants. Values are means of three independent experiments. Asterisks denote statistically significant differences between the respective values of endophyte-colonized versus noncolonized plants of the same genotype (*, Student's t test P< 0.5; *** Student's t test P< 0.01).

and 37.2% in powdery mildew conidia was observed in *jar1-1* and *jin1 R. radiobacter*-inoculated plants respectively. Arabidopsis abscisic acid-insensitive mutant *abi4* also showed increase in powdery mildew conidia (27.9%).

3.2.8 Bacterial associations are common in *Sebacinales*

The hypothesis was followed that species of the order *Sebacinales* commonly contain endosymbiotic bacteria. Therefore, all strains tested in this study for biological activity were investigated for their association with bacteria. After PCR and sequencing analyses using bacterial universal primers we provided evidence that all *S. vermifera* isolates contained bacteria (Table 3.7).

Table 3.7 *Sebacinales* analyzed for bacterial presence

Isolate	Associated bacteria
Piriformospora indica DSM11827	*Rhizobium radiobacter*
S. vermifera MAFF305838	*Paenibacillus* sp.
S. vermifera MAFF305828	*Acinetobacter* sp.
S. vermifera MAFF305835	*Rhodococcus* sp.
S. vermifera MAFF305837	P
S. vermifera MAFF305830	P
S. vermifera MAFF305842	P
Multinucleate *Rhizoctonia* DAR29830	P

P, Bacteria present

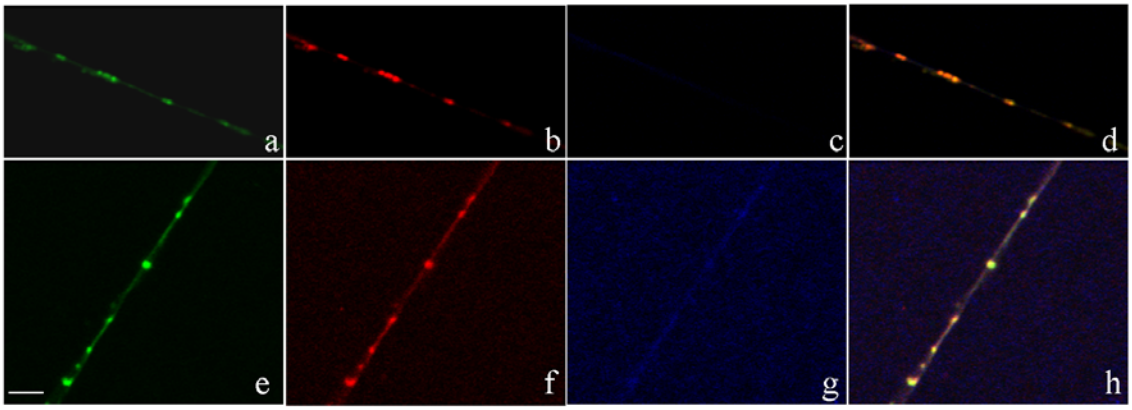

Fig. 3.13. Detection of *S. vermifera* MAFF305838-associated bacteria by fluorescence *in situ* hybridization (FISH). Fungal mycelium of *S. vermifera* MAFF305838 was used for FISH analysis. FISH was performed with EUB-338-mix-FITC (green), specific for the domain bacteria (a) and (e); and with LGC-mix-Cy3 (red), specific for the Firmicutes group to which *Paenibacillus* sp. belongs (b) and (f); no fluorescence was observed with EUK-516-Cy5 (blue) specific for eukaryotes (c) and (g);. (d) and (h) show the superimposed image, composed rgb-images result in a yellow color for the bacteria, indicating co-labeling by EUB-338-mix-FITC and LGC-mix-Cy3. (Scale bar = 4 µm).

A more detailed analysis of the symbiotic association showed an intimate association between a *Paenibacillus* sp. and *S. vermifera* MAFF305838. The endocellular location of the bacterium was demonstrated by FISH and confocal laser-scanning microscopy on axenically grown fungal cultures (Fig 3.13). Spherical bacteria of 0.5-1 µm size were detected with the probe EUB-338-mix, indicative for almost all bacteria (Fig 3.13 a, e), and co-localized with probe LGC-354-mix (Fig 3.13 b, f), specific for the Firmicutes group to which *Paenibacillus* belongs (Table 2.5). The number of bacteria varied from one to three per hyphal cell. To exclude unspecific probe labeling, the samples were hybridized with EUK-516 probe (specific for 18S-rRNA of eukaryotes) which gave no signals (Fig 3.13 c, g). To further substantiate the presence of endobacteria, transmission electron microscopy of *S. vermifera* MAFF305838 was done. The mycelium of fungal material grown in liquid culture was frozen at high pressure and cryo-substituted in glutaraldehyde and uranyl acetate. Examination of the samples confirmed the presence of endobacteria within the cytosol of intact cells of *S. vermifera* MAFF305838 (Fig 3.14).

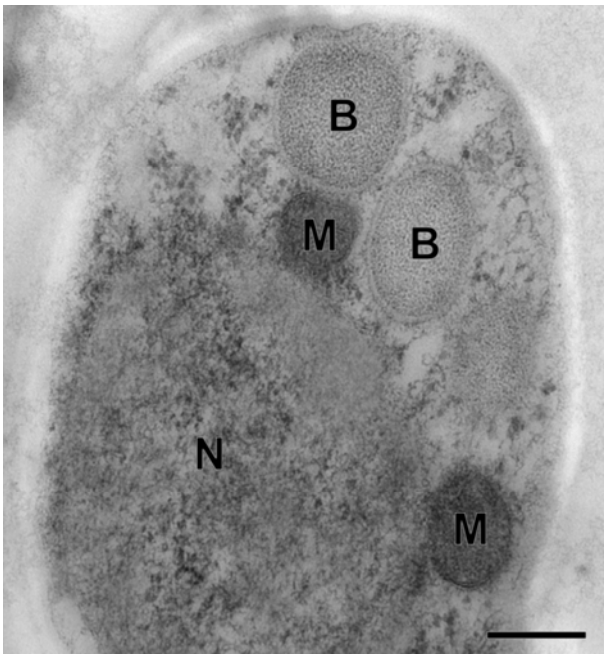

Fig. 3.14. Transmission electron micrograph showing endocellular location of *Paenibacillus* sp. in the hyphal cell of *S.v.*MAFF305838. Fungal mycelium was frozen at high pressure and cryo-substituted in cryo-substitution equipment for four days followed by embedding and staining. Two endocellular bacteria (B), a nucleus (N) and mitochondria (M) are visible in one hyphal cell. (Scale bar = 200 nm).

4 Discussion

4.1 Morphological, physiological and phylogenetic analyses of members of the *Sebacinales*

Mycorrhizal taxon of *Sebacinales* is the most basal group with known mycorrhizal members. This order includes mycobionts of ectomycorrhizae, orchid mycorrhizae, ericoid mycorrhizae, and jungermannioid mycorrhizae, and is distributed worldwide (Weiss et al., 2004). The order is subdivided into two groups, one of which includes the cultivable *Sebacina vermifera* isolates along with *Piriformospora indica* (Selosse et al., 2007) whereas other group consists of ectomycorrhizae and ectendomycorrhizae species. The basidiomycete *P. indica* forms mutualistic symbioses with a broad spectrum of land plants including barley in which the endophyte colonizes the plant root, proliferates by inter- and intracellular growth and produces chlamydospores in dead root tissue (Deshmukh et al., 2006). After establishment of the symbiosis the fungus confers improved growth, disease resistance and abiotic stress tolerance to the host plant (Waller et al., 2005). In the present study, seven isolates/strains of the *S. vermifera* species complex, which have been isolated from different autotrophic orchids at different locations throughout the world, were obtained and their axenic cultures were established. Based on the nucLSU sequences these strains of the *S. vermifera* species complex are closely related to *P. indica* (Deshmukh et al., 2006). To gain more knowledge about the phylogenetic position of *Sebacinales*, the internal transcribed spacer (ITS1-5.8S-ITS2) regions from eight strains of *Sebacinales* (*S. vermifera* strains and *P. indica*) were sequenced and comparative sequence analyses with sequences retrieved from the NCBI databank were made. The sequences were grouped into three clusters (Fig 3.2). Cluster I and Cluster II were well supported and consisted of three isolates of *S. vermifera* species together with uncultured fungi. Cluster III, which contains *P. indica* and the Multinucleate Rhizoctonia DAR29830 sequences displayed a lower bootstrap support. Among the available ITS sequences, most of them are without any taxonomic identity and are named as ‘uncultured mycorrhiza’ or ‘uncultured *Sebacinales*’. The fewer number of similar sequences in public databases makes it difficult to better resolve the position of *P. indica* within the *Sebacinales*. All the isolates varied in the growth rate on

different media. All the *S. vermifera* strains had smaller hyphal diameter and hyphal cell size and less number of nuclei in hyphae as well as in chlamydospore as compared to *P. indica.* Taken together, morphological, physiological as well as phylogenetic analyses showed that the strains of *Sebacinales* are distinct. The next question was whether these isolates were also distinct in biological activity in different cultivars of barley encompassing growth accruement and induction of systemic resistance against powdery mildew disease caused by *Blumeria graminis* f.sp. *hordei* (*Bgh*). Although variation was found between isolates belonging to the different phylogenetic clusters, all tested fungi yielded comparable increases in plant biomass and systemic resistance against *Bgh* in two different barley cultivars (cvs. Ingrid, Golden Promise) (Fig 3.4) as previously reported for *P. indica* (Waller et al., 2005). These findings are reminiscent of positive effects reported for *S. vermifera* isolate (Multinucleate Rhizoctonia DAR29820) and *P. indica* in tobacco (Barazani et al., 2005). Here, inoculation of *Nicotiana attenuata* seeds with either *Sebacinale* isolate resulted in a stimulation of seed germination, an increase in growth and in stalk elongation. *Sebacinales* inoculated plants flowered earlier, produced more flowers and yielded in more seed capsules compared to non-inoculated plants. Based on their findings, the authors postulated that *Sebacinale* fungi may interfere with defense signaling and allow plants to increase growth rates at the expense of herbivore resistance mediated by TPIs (trypsin proteinase inhibitors). The same working group further reported that *S. vermifera* promotes the growth and fitness of *N. attenuata* by inhibiting ethylene signaling (Barazani et al., 2007). In contrast, the mechanism of disease resistance induced by *P. indica* against various root and shoot diseases, is not yet known. Waller et al. (2005) determined the expression levels of salicylate (SA) - and jasmonate (JA) - induced genes in leaves of *P. indica* infested barley plants. Both JA-induced protein-23 (JIP-23) and SA responsive pathogenesis-related 5 (PR5) mRNAs were not consistently stronger expressed in *P. indica*-infested plants. Therefore, constitutively elevated SA or JA levels are unlikely to be required for the observed systemic resistance. The elements of the antioxidative system were greatly enhanced in leaves of *P. indica*-infested plants (Waller et al., 2005). Further transcriptome analyses in leaves of *P. indica*-infested and non-infested barley using the Affymetrix Barley 1 GeneChip revealed a low number of differentially regulated genes in response to *P. indica* root colonization

(Waller et al., 2008). Two genes i.e., pathogenesis-related gene *HvPr17b* and the molecular chaperone *HvHsp70*, were detected among the identified systemically induced transcripts. The expression of *HvHsp70* in leaves of barley plants infested with three *S. vermifera* strains (*S. v.* MAFF 305830, *S. v.* MAFF305828 and *S. v.* MAFF305835) was quantified and similar expression levels as reported for *P. indica* were found for all three isolates. Hence, *HvHsp70* was reported as a useful marker gene due to its expression in systemic tissue after root colonization with endophytic fungi of the order *Sebacinales* (Waller et al., 2008). Additionally, *S. vermifera* colonized barley showed low levels of pathogenesis-related gene 1b (*Pr1b*) expression, similar to *P. indica*.

4.2 Association of *Sebacinales* with bacteria

Both extracellular as well as intercellular associations between bacteria and fungi are complex and have been reported in many fungi including mycorrhizal and pathogenic species. Mechanical rupturing of *P. indica* mycelium indicated initial sign of bacterial presence in several occasions. It was second part of the present study to isolate, characterize and determine the putative roles of the bacterial strain associated with *P. indica*. The most frequently approach used to detect the presence of bacteria is the identification of the bacterial small subunit ribosomal RNA (16S-rRNA) gene sequences. Using universal primers, the bacterial 16S-rRNA gene was amplified from the fungal metagenome by PCR (Fig 3.5). Clonning, sequencing and phylogenetic analysis of 16S-rRNA gene sequences revealed that *P. indica* contains a single bacterial strain, *Rhizobium radiobacter* (synonym *Agrobacterium radiobacter or Agrobacterium tumefaciens*) (Young et al., 2001) (Fig 3.8). The presence of the identical bacterial strain was eventually proven in the original isolate *P. indica*-DSM11827 deposited in the 1997 to DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Braunschweig, Germany by A. Varma and in all fungal cultures derived thereof (i.e., *P. indica*-JE1, *P. indica*-HA, and *P. indica*-ND). *P. indica*-associated bacteria were isolated from the fungus and their axenic culture, named as PABac-DSM (from *P. indica*-DSM11827) and PABac-JE (from *P. indica*-JE1), were established. 16s-rRNA gene was amplified from the bacterial cultures using eubacterial primers and was sequenced. All bacterial 16S-rRNA gene sequences retrieved during this investigation were aligned and were found to

be identical to each other which suggested the association of single bacterial species with *P. indica*. To confirm the notion, denaturing gradient gel electrophoresis (DGGE) analysis was employed. DGGE analysis is a classically established molecular method which offered valuable insights into the genetic diversity of the microbial populations in multiple samples (Muyzer, 1999). DGGE of a semi-nested PCR assay using 16S-rRNA gene primers led to the detection of a single dominant band for *P. indica*-DSM11827 and *P. indica*-JE1 as well as for PABac-DSM and PABac-JE (Fig 3.6), further emphasizing that only one distinct bacterial species was associated with *P. indica*. Additionally bacterial presence was also detected in three other isolates of *S. vermifera* (*S. v.* MAFF305838, *S. v.* MAFF305828, *S. v.* MAFF305835). Bacterial species belonging to three different genera, namely *Paenibacillus*, *Acinetobacter* and *Rhodococcus* were detected in association with these three phylogenetically and morphologically distinct members of the *S. vermifera* species complex (Table 3.7). Association between bacteria and fungi has been reported (de Boer et al., 2005). Although many mycorrhizosphere-bacteria have been reported in close contact with mycorrhizal fungi, either in the fruit bodies (Sbrana et al., 2000), or with the mycorrhizal mats or hyphae in soil (Filippi et al., 1995; Mogge et al., 2000), or attached to the spores (Walley and Germida, 1997) or to the hyphae (Nurmiaho-Lassila et al., 1997), the importance of interactions between bacteria and fungi have been so far very poorly documented (Frey-Klett and Garbaye, 2005; Lumini et al., 2006). The mycorrhizal fungal-bacteria association is a complex interaction that influences dramatically the biology of the fungus and the nutrition of the plant (Frey-Klett et al., 2005). These bacteria could have positive or negative effect on mycorrhizal fungi and on plants (Frey-Klett et al., 1997). The study of bacteria with a positive effect has led to the concept of mycorrhization helper bacteria (MHB) (Duponnois and Garbaye, 1991). Garbaye (1994) defined MHB for bacteria associating with mycorrhizal fungi and promoting the establishment of mycorrhizal symbiosis. However, the term MHB is more generic and is used more generally for all bacteria associated with mycorrhizal fungi. The term is now dissected in two working definitions as ‘mycorrhization helper bacteria’ and ‘mycorrhizal helper bacteria’, with MHB as abbreviation (Frey-Klett et al., 2007). The authors have suggested the term ‘mycorrhization’ to strictly refer to the bacteria that help mycorrhiza formation, and the

term ‘mycorrhiza’ to refer to those bacteria that interact positively with the functioning of the symbiotic organ. MHB modify fungal differentiation and development in a wide variety of ways which include inhibition or promotion of germination, and alterations to foraging behaviour, hyphal branching (fungal architecture), growth, survival, reproduction, exudate composition and production of antibacterial metabolites (Rainey et al., 1990; Duponnois and Plenchette, 2003; Frey-Klett and Garbaye, 2005; Aspray et al., 2006; Riedlinger et al., 2006; Frey-Klett et al., 2007). MHB have also been shown to induce changes in the transcriptome of mycorrhizal fungi (Schrey et al., 2005; Deveau et al., 2007). Some of these bacterial-induced effects on fungi in return do have beneficial effects on MHB (de Boer et al., 2005). The accumulation of nutrients in fungal structures (hyphae and developing sporocarps and/or spores) and better fungal associated dispersal may benefit MHB (Leveau and Preston, 2008). A close association between disease helper bacteria and phytopathogenic *Stagnospora nodorum* fungal hyphae has been reported and these helper bacteria were proved to promote fungal pathogenicity (Dewey et al., 1999; Newton and Toth, 1999).

Phylogenetic sequence analysis of bacterial 16S-rRNA gene obtained from PCR on *P. indica* metagenomic DNA had identified the bacterium as *Rhizobium radiobacter* (syn. *Agrobacterium radiobacter* (Young et al., 2001), a member alpha-proteobacteria and of *Rhizobaceae* (Fig 3.8). *Agrobacterium* sp., *Rhizobium* sp. and *Bradyrhizobium* sp. have been reported as MHB in arbuscular mycorrhizal fungi (AMF) of *Glomus fasciculatum, G. mosseae, G. intraradices* and *Pleurotus ostreatus* (Azcon et al., 1991; Requena et al., 1997; Fester et al., 1999; Xie et al., 1995; Jayasinghearachchi and Seneviratne, 2004) which have lead to increase in mycorrhization and nitrogen fixation. There is some argument regarding the taxonomy of *Agrobacterium* / *Rhizobium* group. This is a complex bacterial group which consists of beneficial nitrogen fixing bacteria and pathogeneic bacteria. The nature of effects afforded by bacteria on host plants, nitrogen fixation or pathogenicity, is plasmid borne. Recently, the genus *Agrobacterium* is included to genus *Rhizobium* (Young et al., 2001) which has changed the taxonomic structure of the family *Rhizobiaceae*, although many bacteriologists do not agree with the new reclassification because there are molecular and phenotypic data which support the separation of both genera (Farrand et al., 2003). In this study, we had followed the

nomenclature proposed by Young et al., 2001. *R. radiobacter* (syn. *A. tumefaciens*) is well known as a soil-borne bacterium that produces tumors (galls) near the top (crown) of dicotyledonous plants and can devastate crops worldwide. The genome of the bacterium consists of one circular (2.8 Mega-basepairs (Mbp), 59.4% GC content) and one linear chromosome (2.1 Mbp, 59.3% GC content) and two plasmids, one of which is At plasmid (0.54 Mbp, 57.3% GC content) and other one is the conjugative Ti plasmid (0.21 Mbp, 56.7% GC content) (Goodner et al., 2005). Tumor induction is dependent on the bacterial virulence plasmid (Ti), which contains *vir* genes and genes encoding for the phytohormones auxin and cytokinin whereas the nodulating strains from the genera *Rhizobium* and *Sinorhizobium* carry plasmids (pSym) containing *nod* and *nif* genes responsible for the nodulation and nitrogen fixation, respectively. In the present study, PABac-DSM did not trigger any harmful effect on barley, but instead stimulated growth and fitness of plants. The *virD2* gene was detected in PABac-DSM by conventional and quantitative PCR analyses indicated that the Ti plasmid was carried by this strain. However the *isopentenyltransferase* (*ipt*) gene, which is associated with cytokinin biosynthesis, could not be detected. Hence, the lack of the *ipt* gene may explain the non-pathogenic nature of this bacterial strain. Concordantly, a non-pathogenic strain of *A. radiobacter* containing *virD2* but not *ipt* was previously described by Haas et al. (1995). These authors speculated that the strain arose from a pathogenic progenitor through a deletion in the T-DNA. Moreover, coexistence of symbiosis- and pathogenicity-determining genes has been shown to occur in strains of *Rhizobium rhizogenes* enabling this bacterium to induce nodules or tumors in plants (Velazquez et al., 2005).

A cyto-histochemical approach, using fluorescence *in situ* hybridization (FISH) analysis in combination with the *Rhizobium*-specific probe Rhi-1247, was adopted to identify and locate *R. radiobacter* associated with *P. indica*. The FISH analysis uses fluorescence oligonucleotide probes for the detection of specific RNA and DNA targets. The methodology has been used successfully to detect and identify environmental non-culturable prokaryotes (Amann et al., 1995) and bacterial endosymbionts of insects (Fukatsu et al., 1998) and fungi (Bianciotto et al., 2000; Bertaux et al., 2003, 2005). By using eubacterial probes (EUB-338-mix) and *Rhizobium*-specific probe Rhi-1247, *R. radiobacter* could be detected in and around the fungal hyphal wall (Fig 3.9). In general

the number of bacteria per hypha was very low, which is consistent with the low number of endobacteria (2-20 per cell) present in the ectomycorrhizal fungus *Laccaria bicolor* (Bertaux et al., 2003, 2005). Real-time quantitative PCR analyses using *Rhizobium* specific ITS and virD2 primer pairs further supported the microscopic data revealing a ratio of 0.02-0.035 ng of bacterial DNA per 100 ng of *P. indica* DNA. A combined strategy that included transmission electron microscopy and FISH with Firmicute-specific probes indicated the endocellular nature of *Paenibacillus* sp. in *S. vermifera* MAFF305838 with roughly one to three bacteria per fungal cell (Fig 3.13, Fig 3.14). Detection of endobacteria with FISH indicated the viability of bacteria as only viable bacteria, containing enough ribosomes, are detectable with FISH (Christensen et al., 1999). In agreement with this correlation between bacterial viability and detection, labeling of the bacterial structures obtained with eubacterial probes and group-specific probes for respective bacteria indicated presence of physiologically active bacteria in *Sebacinales* fungi. Non-hybridization of probes specific for eukaryotes ruled out the false detection of bacteria. Symbiotic associations between endocellular bacteria and eukaryotic cells are widespread among animals (e.g. *Buchnera* and *Wolbachia* spp. in insects,) and plants (e.g. *Nostoc* spp. with *Gunnera*; *Burkholderia* spp. with *Rubiaceae*; rhizobia with legumes) (de Boer et al., 2005). In the fungal kingdom, it is a budding area of research. Endocellular bacteria have been reported in some *Glomeromycota* species (both in arbuscular mycorrhizal fungi (AMF) and *Geosiphon pyriforme*), ectomycorrhizal basidiomycete (e.g., *Laccaria bicolor*, *Tuber borchii*, *Pinus strobus-Endogone flamicorona*, *Picea abies*-'Type F' ectomycorrhizae and *Pinus sylvestris-Suillus bovines*) and pathogenic Zygomycetes *Rhizopus microsporos* (Buscot, 1994; Bianciotto et al., 1996b; Barbieri et al., 2000; Scheussler and Kluge, 2001; Bertaux et al., 2003, 2005; Partida-Martinez and Hertweck, 2005; Lumini et al., 2006). Using transmission electron microscopy (TEM) and FISH analysis, intra-cytoplasmic bacterium was detected in AMF *Gigaspora margarita* (Bianciotto et al., 1996b). Phytopathogenic fungus *R. microsporos* harbours an intracellular bacteria in its cytosol which were localized by using Live /Dead bacterial staining kit and TEM (Partida-Martinez and Hertweck, 2005; Partida-Martinez et al., 2007a). Using the FISH analysis, endocellular bacterium *Paenibacillus* sp. was detected in the axenic and non-axenic cultures of ectomycorrhizal fungus (ECM)

Laccaria bicolor S238N (Bertaux et al., 2003, 2005). In the present study, there was no evidence for a focal accumulation of *R. radiobacter* or *Paenibacillus* sp. in fungal hyphae. In contrast, such an accumulation was found in the *Rhizopus microsporus* - *Burkholderia rhizoxinica* sp. nov. and /or *Burkholderia endofungorum* sp. nov. interaction in which the bacteria were predominantly accumulating at hyphal tips, the region with best supply of nutrients and the place for formation of sporangia. In the latter example, endobacteria were required for triggering fungal sporulation as bacteria-free host strains were strictly incapable of developing sporangia in the cultivation medium (Partida-Martinez et al., 2007b).

In the present study, bacteria belonging to different genera were found to be associated with different isolates of *S. vemifera* species complex indicating the specificity of the associations (Table 3.7). Numerous studies have shown that the bacterial communities associated with fungal hyphae, fungal spores, or with the mycorrhizosphere of mycorrhizal plants display fungi-specific differences in composition (de Boer et al., 2005; Frey-Klett and Garbaye, 2005; Roesti et al., 2005). Nutrients in fungal exudates, chemotaxis, attachment to fungal hyphae, tolerance to fungal antimicrobial chemicals and bacterial ability to alter fungal membrane permeability to increase or modify nutrient efflux are all likely to be important traits for fungal associated bacteria (de Boer et al., 2005; Leveau and Preston, 2008).

R. radiobacter could be isolated from crushed *P. indica* mycelium and multiplied in liquid cultures meaning the bacterium is not entirely dependent on the fungus. That the association bases on a critical balance between bacterium and fungus was suggested by an experiment in which the *R. radiobacter* was added in abundance to fungal suspension cultures. Here, the bacteria overgrew and entangled the hyphae and the fungus eventually died. However in liquid culture, incubation of crushed hyphae, containing hyphae and bacteria released from mechanical shearing, resulted in clearance of the medium after two days. This finding suggests a certain affinity between fungal hyphae and bacteria. Further analysis will show how specific this absorption is. Attempts to isolate *Paenibacillus* sp. from *S. vermifera* MAFF305838 were not successful so far. This is in agreement with non-cultivability of *Candidatus* Glomeribacter gigasporarum, endobacterium of *G. margarita* (Bianciotto et al., 2003), but these endobacteria can stay alive for four weeks

(Jargeat et al., 2004). Genome size of *Ca*. Glomeribacter gigasporarum is very small, approximately 1.4 Mbp with a 750 kilobase-pairs (kbp) chromosome and a 600 to 650 kbp plasmid. Such small genome sizes are typically found in endocellular bacteria living permanently in their host (Jargeat et al., 2004). However, *Burkholderia rhizoxinica* and /or *B. endofungorum*, endobacterium of *Rhizopus microsporus* are cultivable and were shown to biosynthesize polyketide metabolite rhizoxin, the causal agent of rice seedling blight (Partida-Martinez and Hertweck, 2005). Symbiont-free strain of *R. microsporus* does not produce any detectable amounts of rhizoxin (Partida-Martinez et al., 2007a).

Bianciotto et al. (2004) demonstrated for the AMF *G. margarita* a continuous vertical transmission of its endobacteria from one generation to another guaranteeing the stable nature of the association. Comparably, the bacteria (PABac-DSM) was found to be present in developing chlamydospores in *P. indica*-DSM11827 and detected in fungal colonies regenerated from single spores or from hyphal tip cells.

Different strategies to obtain bacteria-free fungus failed, which further argues against a temporal or loose association. Neither cultivation of hyphae in axenic culture under high antibiotic concentrations, nor successive *in vitro* single-spore isolation steps, nor the exposure of fungal protoplast to antibiotics in the regenerating medium resulted in bacteria-free *P. indica*. These findings suggest that the bacterium is either protected inside the fungus or its absence is meeting in reduced fungal competitive fitness as reported for a strain of *G. margarita* cured from its endocellular bacteria (*Ca.* Glomeribacter gigasporarum) (Lumini et al., 2007). Curing of endosymbiont from *R. microsporus* halts the vegetative reproduction of the host. Formation of sporangia and spores is restored only upon reintroduction of endobacteria (Partida-Martinez et al., 2007b).

P. indica has been shown to promote the growth, development, seed production and resistance to various abiotic and biotic stresses in barley (Waller et al., 2005) and *Arabidopsis* (Shahollari et al., 2005, 2007). It was certainly of interest to clarify the impact of each symbiosis partner of *P. indica- R. radiobacter* system on observed beneficial effects in colonized plants. Biological potential of *R. radiobacter* (PABac-DSM) in barley had been examined in this study. The bacterium qualitatively and quantitatively induced symbiosis phenotypes comparable with those induced by *P.*

indica, e.g. growth augmentation and systemic resistance to powdery mildew (Fig 3.11). Furthermore recently, *P. indica* has been shown to induce resistance against the Arabidopsis powdery mildew *Golovinomyces orontii* (E. Stein, A. Molitor, unpublished data). *R. radiobacter* (PABac-DSM) was also tested for its activity in *A. thaliana* in this study. The bacterium showed a significant reduction in the powdery mildew disease (Fig 3.12). Taken together, the data suggested that *P. indica*-associated *R. radiobacter* has biological activity similar to that observed for *P. indica*. Consistently, the potential of specific strains of *R. radiobacter* for improvement of plant performance in integrated production systems has been reported earlier. *R. radiobacter* strain 204 increased barley root and shoot length as well as improved crop yield in barley and wheat leading to its commercial distribution as biofertilizer in Russia (Humphry et al., 2007).

Many strains of plant growth-promoting rhizobacteria (PGPR) have been reported to activate plant defense through induced systemic resistance (ISR) (Kloepper et al., 1992; van Loon et al., 1998) and protect the plants against various diseases. ISR mediated by PGPRs could be independent of SA (salicylic acid) accumulation (Pieterse et al., 1996; Press et al., 1997; Ryu et al., 2003) or dependent on SA pathway (De Meyer and Hofte, 1997; De Meyer et al., 1999). van Loon et al. (1998) and Pieterse et al. (2002) proposed a model pathway describing ISR caused by PGPR is dependent on jasmonic acid (JA), ethylene (ET), and the regulatory gene *NPR1* (Non-expresser of Pathogenesis Related Genes1), while it is independent of SA and does not result in accumulation of PR-proteins (Pathogenesis related-proteins). Recent analysis in our institute showed a requirement of the jasmonate defense pathway for *P. indica*-mediated disease resistance. This notion has been concluded from the observation that the jasmonate-insenitive mutant *jar1-1* and the jasmonate biosynthesis mutant *opr3* were non-responsive to *P. indica* (E. Stein, A. Molitor, K.-H. Kogel & F. Waller, unpublished data). To elucidate the requirement of known defense pathways for *R. radiobacter* (PABac) activity, the development of powdery mildew (*G. orontii*) on *Arabidopsis* mutants inoculated with *R. radiobacter* was compared. Arabidopsis mutant lines *npr1-1*, *jin1*, *jar1-1* and *abi4* showed a loss in protection of plants against powdery mildew disease which suggests the resistance induction in the plant is mediated via induced systemic resistance (ISR) mechanism (Fig 3.12). ISR mechanism is abolished in *npr1-1, jar1-1* and *jin1* whereas it

is functional in *npr1-3*. *npr1-3* mutant inoculated with *R. radiobacter* showed a reduction in powdery mildew disease suggesting that *npr1-3* in not required for resistance mediated by *R. radiobacter*. NPR1 (also referred as NIM1 (Non-Inducible Immunity1)) is a key regulatory protein in defense signaling pathways. It is required downstream of SA to activate the expression of PR genes (Cao et al., 1997; Ryals et al., 1997) and also for ISR (Dong, 2004). NPR1 is part of the crosstalk control between signaling pathways (Spoel et al., 2003). Nuclear localization of NPR1 is essential for resistance induction. *npr1-3* mutant is compromised in SA-mediated gene expression but contain the nuclear-localization signal. *npr1-1* is a null mutant in which genes for JA and ethylene signaling are affected (Dong, 2004). The loss of *R. radiobacter* mediated resistance in *npr1-1* observed in this study shows the requirement of cytosolic function of NPR1 and not nuclear localization of NPR1.

The plant hormones SA, JA and ET have been repeatedly reported to participate in the regulation of defense responses in plants (Pieterse et al., 2002). To investigate the role of JA in *R. radiobacter* mediated resistance against powdery mildew in *A. thaliana,* JA response mutants *jin1* (*jasmonate-insensitive 1*) and *jar1-1* (*jasmonate-resistant 1*) were tested to for their ability to express ISR. Both these mutants were not protected from powdery mildew after inoculation with *R. radiobacter*. The inability of these jasmonate defective mutants to build up resistance against powdery mildew after inoculation with *R. radiobacter* indicates that *R. radiobacter* mediated ISR requires JA. In mutant *jar1-1*, protection induced by methyl jasmonate (MeJA), a natural precursor of ET, is blocked (Staswick et al., 1998). Further, JA biosynthesis mutant *aim1* was tested. In this mutant, fatty acid ß-oxidation is disrupted and wounding-induced expression of JA-responsive genes is present (Delker et al., 2007). *R. radiobacter* inoculation of *aim1* showed enhanced resistance against powdery mildew which indicated the non-involvement of fatty acid ß-oxidation enzymes in JA-biosynthesis in *R. radiobacter* mediated ISR.

Ethylene-insensitive *Arabidopsis* mutant line *ein2* is blocked in ethylene mediated signaling responses (Alonso et al., 1999). *R. radiobacter* inoculated *ein2* plants showed an increase in resistance against powdery mildew disease hence showing that ET signaling is not required for *R. radiobacter* mediated ISR.

The phytohormone abscisic acid (ABA) is involved in various plant responses of growth and development (e.g., seed germination, seedling growth, flowering, stomatal opening) and is an important hormone in mediating plant adaptation to various environmental challenges, mainly including several abiotic stresses (drought, salt and cold) (Zhu et al., 2007). Many reports on signaling crosstalk between biotic and abiotic stress are emerging (Timmusk and Wagner, 1999; Xiong and Yang, 2003; Mauch-Mani and Mauch, 2005). Some reports are showing the antagonism between biotic and abiotic stress responses (Xiong and Yang, 2003) whereas other describe the common components shared by biotic and abiotic stress responses (Park et al, 2001; Mengiste et al, 2003; Chini et al, 2004). The role of ABA in plant disease resistance is not well defined (Mauch-Mani and Mauch, 2005). ABA may play a key role in host plant susceptibility as several fungal pathogens are known to produce ABA (Crocoll et al., 1991). In addition, ABA is a key factor in the suppression of disease resistance to *Magnaporthe grisea* (Koga et al., 2004). ABA-deficient mutant *aba1-1* of *Arabidopsis* and *sitiens* of tomato increased the plant resistance to infection by *Hyaloperonospora parasitica* and *Botrytis cinerea* respectively (Audenaert et al., 2002; Mohr and Cahill, 2003). Recently, Torres-Zabala et al. (2007) demonstrated that the virulence factors of *Pseudomonas syringae* manipulate the components of the ABA biosynthetic and response machinery which facilitate the bacterial growth and hence increase susceptibility of plants. The authors further reported that bacterial colonization was reduced in an ABA biosynthetic mutant. In the present study, *abi4* mutant (Arabidopsis ABA-insensitive) was tested for elucidating involvement of ABA in ISR. This mutant has pleiotropic defects in seed development, including decreased sensitivity to ABA inhibition of germination and altered seed-specific gene expression (Finkelstein et al., 1998). *abi4* plants were more susceptible to powdery mildew after inoculation with *R. radiobacter* which suggests that ABA may play a role in the ISR mediated by *R. radiobacter*.

Together these data show that *R. radiobacter* induces a type of resistance reminiscent of jasmonate-dependent induced systemic resistance (ISR) provided by non-pathogenic rhizobacteria.

A recent report by Sirrenberg et al. (2007) demonstrated production of indole acetic acid (IAA) in liquid culture of *P. indica*, which might be the basis for its growth promotion

activity. Since bacteria-free fungus is not yet available, it remains vague whether the fungus itself, the bacterium or even both partners produced the hormone. We demonstrate here that *R. radiobacter* (PABac-DSM) produces IAA in the presence of tryptophan (Fig 3.10). Despite the fact that Salkowski's reagent also detects indole pyruvic acid and indole acetamide in addition to IAA (Glickmann and Dessaux, 1995), the method is fairly accurate since IAA is usually known as the main excreted microbial auxin. IAA is best known for its role in plant signal transduction (Quint and Gray, 2006). However, this hormone can act as a signal molecule in bacteria and fungi (Leveau and Preston, 2008) and induce adhesion and filamentation of *Saccharomyces cerevisiae* (Prusty et al., 2004). Importantly, IAA has been implicated in plant-microbe compatibility (Robert-Seilaniantz et al., 2007). This could be accomplished by suppression of defense reactions otherwise elicited by fungal MAMPs (Microbe-Associated Molecular Patterns). Repression of auxin receptors in *Arabidopsis* by microRNAs after recognition of flagellin, an integral MAMP of the flagella of phytopathogenic bacterium *Pseudomonas syringae*, has been shown (Navarro et al., 2006). As a result of receptor repression, plant immunity response was activated. Accordingly, SA-mediated repression of auxin-related genes including auxin receptors (e.g. TIR1) has been suggested to be part of the plant defense machinery (Wang et al., 2007). Notably, in barley and *Arabidopsis*, root colonization by *P. indica* leads to suppression of PR genes (Deshmukh and Kogel, 2007). Thus, it is tempting to speculate that bacteria-derived auxin contributes to successful root colonization by sebacinoid fungi.

In addition, MHB have been reported to influence the plant defense. Lehr et al. (2007) have shown that MHB *Streptomyces* sp. AcH 505 suppresses the plant (Norway spruce) defense by downregulating peroxidase activity and pathogenesis-related peroxidase gene (*Spi2*) expression, both of which have been linked to the defense response of Norway spruce towards phytopathogens (Asiegbu et al., 1999; Nagy et al., 2004). Suppression of plant defense caused faster colonization of the roots by fungal pathogen *Heterobasidion abietinum* after MHB pre-inoculation. It has also been suggested that the bacteria could release lipases or produce plant cell wall-degrading enzymes, making it easier for fungi to penetrate the cuticle of the leaves (Dewey et al., 1999). This is in agreement with Cui et al. (2005) who had observed that the *Pseudomonas syringae*-derived virulence factor

coronatine, that resembles the phytohormone jasmonic acid, made *Arabidopsis thaliana* more susceptible to secondary infections by the same bacterium. Taken all these considerations together, it could be speculated that *R. radiobacter* can help in suppressing host defense and thereby facilitating *P. indica* colonization.

5 Summary / Zusammenfassung

The order *Sebacinales* is the most basal order of *Hymenomycetes* (Basidiomycetes) which contains an amazing diversity of mycorrhiza. Frequent detection of *Sebacinales* in ecological studies using various molecular techniques suggests their possible role in shaping plant communities. The root-colonizing mutualistic fungus *Piriformospora indica* is axenically cultivable and represent the model organism for symbiotic species of the order *Sebacinales*. In the present work, axenic cultures of seven isolates of *Sebacina vermifera* have been established and their phylogenetic relationship was characterized by sequencing and comparing nuclear genes coding for the internal transcribed spacer (ITS) region. Despite their sequence similarity for the large ribosomal subunit (nucLSU), they showed difference in the ITS region. Isolates were clustered into three different groups based on their ITS sequences. Morphological variation in terms of colony morphology and growth, hyphal and spore size and number of nuclei per hyphal cell and spore was also observed for the isolates. The isolates were further evaluated in barley for their biological potential with respect to growth promotion and ability to induce systemic resistance against the biotrophic leaf pathogen *Blumeria graminis* f. sp. *hordei* (powdery mildew). All isolates conferred beneficial effects (growth augmentation and induced resistance) on two cultivars of barley i.e., cvs. Ingrid and Golden Promise. Hence, the data support the working hypothesis that the mutualistic symbiosis of crop plants and *Sebacinales* has a great potential for sustainable agriculture.

While culturing *P. indica*, initial signs of bacterial presence were observed several times after mechanical rupturing of fungal mycelium. Using universal primers, bacterial 16S-rRNA gene from the metagenomic DNA of *P. indica* and seven isolates of *S. vermifera* was amplified. Cloning, sequencing and phylogenetic analysis of 16S-rRNA gene sequences revealed the association of *P. indica* with *Rhizobium radiobacter,* while 3 isolates *S. vermifera* complex showed association with 3 different bacteria belonging to *Paenibacillus* sp., *Acinetobacter* sp. and *Rhodococcus* sp. *R. radiobacter* (PABac) was isolated from *P. indica* and its axenic culture was established. All bacterial sequences from *P. indica* and PABac were identical within the amplified region of the bacterial 16S-rRNA gene displaying the presence of a unique bacterial isolate. This notion was

confirmed by detecting single dominant band in denaturing gradient gel electrophoresis (DGGE) analysis of 16S-rRNA gene. The *Sebacinales* associated bacteria were localized using fluorescence *in situ* hybridization (FISH). *R. radiobacter* could be detected close to and in the hyphal walls of *P. indica*. *Paenibacillus* sp. was localized within the cytosol of *S. vermifera* MAFF305838. Endocellular nature of *Paenibacillus* sp. was further confirmed by transmission electron microscopy (TEM).Vertical transmission of *R. radiobacter* was proved by single spore cultures of *P. indica.* Real time quantitative PCR showed an average of 0.035 ng of *R. radiobacter* DNA per 100 ng of *P. indica* DNA. Various attempts to cure *P. indica* from bacteria (by growing single spore cultures, young mycelium and regenerated hyphal protoplast on medium containing antibiotic for five generations) were not successful which indeed suggests towards strong association between *P. indica - R. radiobacter*. Initial biochemical characterization of *R. radiobacter* revealed its ability to produce auxin from tryptophan. Biological activities (growth augmentation and induction of systemic resistance against powdery mildew) of *R. radiobacter* in host plants (*Hordeum vulgare* and *Arabidopsis thaliana*) were demonstrated. In addition, using *Arabidopsis* mutants defective in various defence pathways clarified that *R. radiobacter* induces a type of resistance reminiscent of jasmonate-dependent induced systemic resistance (ISR) provided by non-pathogenic rhizobacteria. Thus, the *Sebacinales*-associated bacteria might be involved in the growth response of the host plant as well as in the evasion of the host defense response by the mutualistic fungi. Together, these findings suggest that *Sebacinales* species undergo complex symbioses involving bacteria and requires reconsideration of the role played by the fungus in its symbiotic interaction with the plant.

Zusammenfassung

Sebacinales stellen die ursprünglichste Ordnung der *Hymenomyceten* (Basidiomyceten) und beinhalten eine erstaunliche Vielfalt an Mycorrhiza ausbildenden Vertretern. Molekulare Analysen verschiedener Pflanzenwurzeln weisen zudem auf eine starke Verbreitung dieser Ordnung hin. Das häufige Auftreten in Assoziation mit Pflanzen lässt auf eine Funktion beim Aufbau von Pflanzengemeinschaften schließen. Ein

Modellorganismus aus der Ordnung der *Sebacinales* ist *Piriformospora indica*, ein axenisch kultivierbarer mutualistischer Wurzelendophyt. In der hier vorliegenden Arbeit wurde die axenische Kultivierung von sieben *Sebacina vermifera* Isolaten etabliert und deren phylogenetische Verwandtschaft durch Sequenzanalysen und den Vergleich nuklearer Gensequenzen aufgeklärt.

Während die Sequenzen der großen ribosomalen Untereinheiten der Isolate eine große Ähnlichkeit aufweisen, zeigen sich Unterschiede in der Region, die für die „internal transcribed spacer region“ (ITS) codiert. Auf Grundlage der Unterschiede in dieser Region konnten die Isolate in drei verschiedene Gruppen zusammengefasst werden. Neben den Sequenzanalysen wurden die sieben Isolate hinsichtlich Koloniemorphologie, Wachstum, Gröβe der Hyphen und Sporen, sowie Anzahl an Zellkernen pro Hyphenzelle und Spore untersucht. Des Weiteren wurde das Potential der einzelnen Isolate untersucht, ein verstärktes Wachstum und eine erhöhte Resistenz gegen den biotrophen Mehltaupilz *Blumeria graminis f. sp. hordei* in Gerste zu induzieren. In den Gerstekultivaren cvs. Ingrid und Golden Promise zeigten alle Isolate positive biologische Effekte (Größenwachstum als auch induzierte Resistenz). Diese Ergebnisse unterstützen die Hypothese, dass der mutualistische Symbiont in Getreidepflanzen ein großes Potential für nachhaltige Landwirtschaft birgt.

Bei der Kultivierung und anschließenden mechanischen Zerkleinerung von *P. indica* Mycel wurde mehrfach das Auftreten von Bakterien beobachtet. Unter Verwendung universeller Primer für die bakterielle 16S-rRNA konnten mittels PCR-Reaktionen Amplifikate aus metagenomischer *P. indica* DNA als auch den *S. vermifera* Isolaten gewonnen werden. Eine sich anschließende Klonierung, Sequenzierung und phylogenetsiche Analyse der gefundenen 16S-rRNA-Gensequenzen deutete auf eine Assoziation von *P. indica* mit dem Bakterium *Rhizobium radiobacter*. In drei der *S. vermifera* Isolate wurde auf diese Weise eine Assoziationen mit Bakterien der Gattung *Paenibacillus* sp., *Acinetobacter* sp. bzw. *Rhodococcus* sp. gefunden.

R. radiobacter (PABac) wurde aus *P. indica* isoliert und axenisch kultivert. Alle aus *P. indica* isolierten bakteriellen Sequenzen waren mit der bakteriellen 16S-rRNA von PABac identisch, was auf das Vorliegen eines einzelnen Bakterienstammes in Assoziation mit dem Pilz hinweist. Diese Vermutung wurde dadurch bestätigt, dass auch

in einer denaturierenden Gradientengelelektrophorese (DGGE) nur eine einzelne 16S-rRNA Bande gefunden werden konnte. Eine Lokalisierung der mit den Sebacinalen assoziierten Bakterien erfolgte mittels *in situ* Hybridisierung (FISH). Mit dieser Methode konnte *R. radiobacter* in der Nähe von und in den Zellwänden der Hyphen von *P. indica* detektiert werden. *Paenibacillus* sp. war im Cytosol von *S. vermifera* MAFF305838 nachweisbar. Das endozelluläre Vorkommen von *Paenibacillus* sp. wurde durch Transmissions-Elektronenmikroskopie (TEM) weiter bestätigt. Vertikale Transmission von *R. radiobacter* wurde durch Einzelsporkultivierung von *P. indica* gezeigt. Die Quantifizierung des Verhältnisses von Pilz zu Bakterium mittels quantitativer real time PCR zeigte im Durschnitt 0.035 ng *R. radiobacter* DNA pro 100 ng *P. indica* DNA. Verschiedene Versuche, durch die Anzucht von Einzelsporen, jungem Mycel oder regenerierten Hyphenprotoplasten auf antibiotikahaltigem Medium bakterienfreien Pilz zu erhalten, schlugen fehl. Dies weist auf eine starke Assoziation zwischen *P.indica* und *R. radiobacter* hin. Eine anfängliche biochemische Charakterisierung von *R. radiobacter* zeigte dessen Fähigkeit, Auxin aus Trypthophan zu produzieren.

Zudem zeigte *R. radiobacter* biologische Aktivität (Wachstumsinduktion und systemische Resistenz gegen Mehltau) in den Wirtspflanzen *Hordeum vulgare* und *Arabidopsis thaliana*. Ein Test von Arabidopsis-Mutanten mit Defekten in verschiedenen Abwehrsignalwegen zeigte, dass die von *R. radiobacter* induzierte Resistenz der jasmonatabhängigen induzierten systemischen Resistenz (ISR) nicht-pathogener Rhizobakterien ähnelt. Zusammenfassend kann daher angenommen werden, dass die mit den Sebacinalen assoziierten Bakterien sowohl bei der Wachstumsinduktion in der Wirtspflanze, als auch bei der veränderten Pathogenresistenz durch den mutualistischen Pilz beteiligt sind. Alles in allem deuten die Ergebnisse dieser Arbeit darauf hin, dass *Sebacinales* eine komplexe Symbiose unter Einbezug von Bakterien mit Pflanzen eingehen, was eine neue Beurteilung der Rolle des Pilzes in der Interaktion mit der Pflanze sinnvoll erscheinen lässt.

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List of abbreviation

AM	Arbuscular mycorrhizae
AMF	Arbuscular mycorrhizal fungi
Bgh	*Blumeria graminis* f.sp. *hordei*
BI-1	BAX inhibitor-1
BLOs	Bacteria-like organisms
CLSM	Confocal laser scanning microscope
cv	Cultivar
DGGE	Denaturing gradient gel electrophoresis
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ECM	Ectomycorrhizae
FISH	Fluorescence *in situ* hybridization
GFP	Green fluorescent protein
hrs	Hours
Hsp	Heat-shock protein
Hv	*Hordeum vulgare*
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance
ITS	Inter transcribed spacer region
kbp	Kilo base-pairs
Mbp	Mega base pairs
MHB	Mycorrhization / Mycorrhizal helper bacteria
NF	Nod-factors
ng	nanogram
nucLSU	Nuclear large ribosomal RNA subunit
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
PR	Pathogenesis related
rDNA	Ribosomal deoxy-ribonucleic acid
rRNA	Ribosomal ribonucleic acid
VAM	Vesicular-arbuscular mycorrhizae

Declaration

Ich erkläre:
Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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