Institut für Biochemie und Biologie

# Characterization of tomato root-endophytic fungi and analysis of their effects on plant development, on fruit yield and quality and on interaction with the pathogen *Verticillium dahliae*

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**Eidesstattliche Erklärung**

Diese Dissertation ist das Ergebnis experimenteller Arbeit, die vom April 2006 bis Dezember 2009 im Leibniz-Institut für Gemüse- und Zierpflanzenbau – IGZ (Goßbeeren) und im Institut für Biochemie und Biologie der Universität Potsdam durchgeführt wurde. Ich erkläre, dass die vorliegende Arbeit an keiner anderen Hochschule eingerecht sowie selbständig und nur mit den angegebenen Mitteln angefertigt habe.

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Berlin, July 2010. Diana Rocío Andrade Linares

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Es gibt Menschen, die kämpfen einen Tag,
und sie sind gut.
Es gibt andere, die kämpfen ein Jahr
und sind besser.
Es gibt Menschen, die kämpfen viele Jahre
und sind sehr gut.
Aber es gibt Menschen,
die kämpfen ein Leben lang.
Das sind die Unersetzlichen.

*Bertolt Brecht*

Contents

# Chapter 1

# General Introduction

# 1. General Introduction

"The living together of unlike organisms" is known since de Bary (1879) as symbiosis. In natural ecosystems plants are potential hosts for a broad spectrum of microbes (usually bacteria and fungi) that can live on the surface as epiphytes or colonize plant tissues as endophytes. The term symbiosis (from the Greek: *syn* "with" and *biosis* "living") can be applied to different kind of associations. They can be roughly categorized into mutualism, commensalism and parasitism according to benefits for both partners (mutualistic symbiosis), microbial benefit not affecting the host plant (commensalistic symbiosis) or negative impact on host fitness (pathogenic or parasitic interaction) (Paszkowski, 2006). The borders between these categories are, however, not fixed. For example, mycorrhizal interactions, the most known and wide-spread terrestrial plant-fungus symbioses, are usual mutualistic (Smith and Read, 2008). Depending on environmental conditions, however, the fungus can have negative impact on plant development (Dickson *et al.,* 1999). Also, genetic factors can switch this interaction (Redman *et al*., 1999; Ruiz-Lozano *et al*., 1999). On the other hand, pathogenic fungi can colonize their hosts without causing any symptoms (Photita *et al*., 2004; Slippers and Wingfield, 2007). In addition to this, a large spectrum of endophytic fungi represent a continuum of plant-fungal associations, ranging from positive over neutral to negative interactions (Johnson *et al*., 1997; Brundrett, 2004; Schulz and Boyle, 2005). These fungal endophytes are represented by diverse taxonomic groups (Arnold *et al*., 2007; Rodriguez *et al*., 2009). Like the mycorrhizal fungi, they have been recognized to be ancient (Krings *et al*., 2007) are mainly characterized by the production of important secondary metabolites (Strobel, 2004) and have significant effects on plant fitness and ecology (Brundrett, 2004; Saikkonen *et al*., 2004; Rodriguez *et al*., 2008).

## 1.1 Fungal endophytes

The term endophyte comes from the Greek *endo* "within" and *phyton* "plant" that denotes a broad spectrum of plant endosymbionts from bacteria to insects colonizing inside any organ of the plant with variable life styles (Schulz and Boyle, 2005). Fungal endophytes are fungi that grow internally in living plant tissues for at least part of their life cycle (Fig. 1) without causing disease symptoms (Petrini, 1991; Wilson, 1995; Saikkonen *et al*., 1998; Stone *et al*., 2000). They include a large spectrum from latent pathogens to mutualistic symbionts (Carroll, 1998; Schulz and Boyle, 2005). Although asymptomatic fungal colonization inside plant

tissues has been known since the 19th century (e.g. Guerin, 1898), this kind of fungi was begun to be more studied not before the presence of these endophytes was related to a syndrome called toxicosis suffered by cattle fed in pastures of colonized grass (Bacon *et al*., 1977).

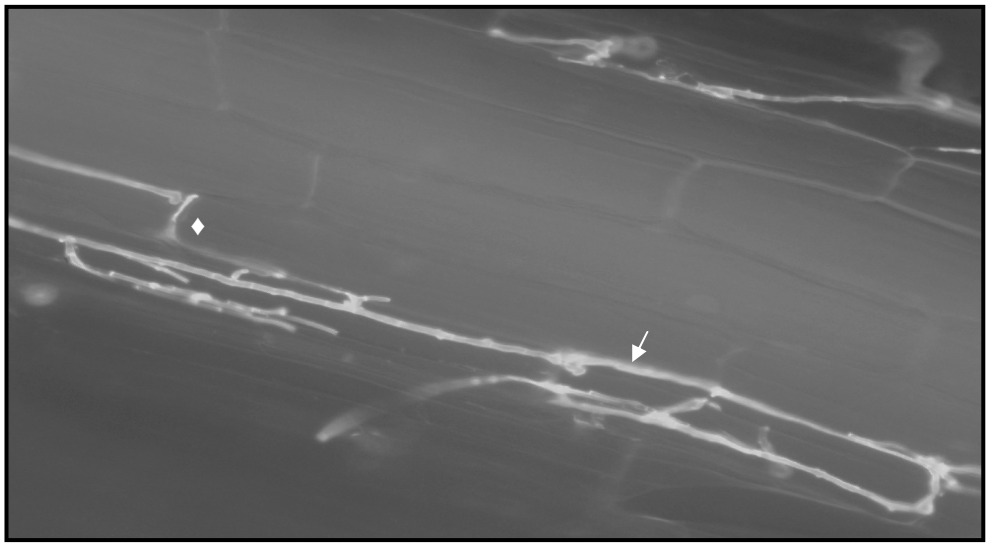

Figure 1. Tomato root colonization by DSE48. Observation by confocal laser scanning microscopy (CLSM) (400x) of fungal hyphae growing inter (→) and intracellularly (♦) in root tissues visualized by WGA-Alexa fluor dye. For more detail see chapter 2.

The procedures most commonly used for recovering endophytes from plant tissues are based on surface disinfection of apparently healthy plant organ samples to kill epiphytic fungi (Bills, 1996). Fragments of disinfected plant samples are subsequently placed on different culture media. After incubation, when endophytic hyphae emerge from the plant tissue and start growing in the agar medium, the isolation of the fungus can be carried out (Fig. 2). Fungi that emerge from these samples can be identified by means of phenotypic (morphological) or genotypic (molecular) characters (Weiss *et al*., 2004; Arnold and Lutzoni, 2007; Higgins *et al*., 2007).

Obligated biotrophs or fungi not growing well in the agar medium have been detected by molecular techniques (Neubert *et al*., 2006; Duong *et al.*; 2006; Gallery *et al*., 2007). Sequencing of ribosomal DNA and internal transcribed spacers (ITS) has improved taxonomic studies of sterile isolates obtained in endophyte surveys (Guo *et al*., 2000; Wirsel *et al*., 2001; Promputtha *et al*., 2005; Crozier *et al*., 2006; Higgins *et al*., 2007).

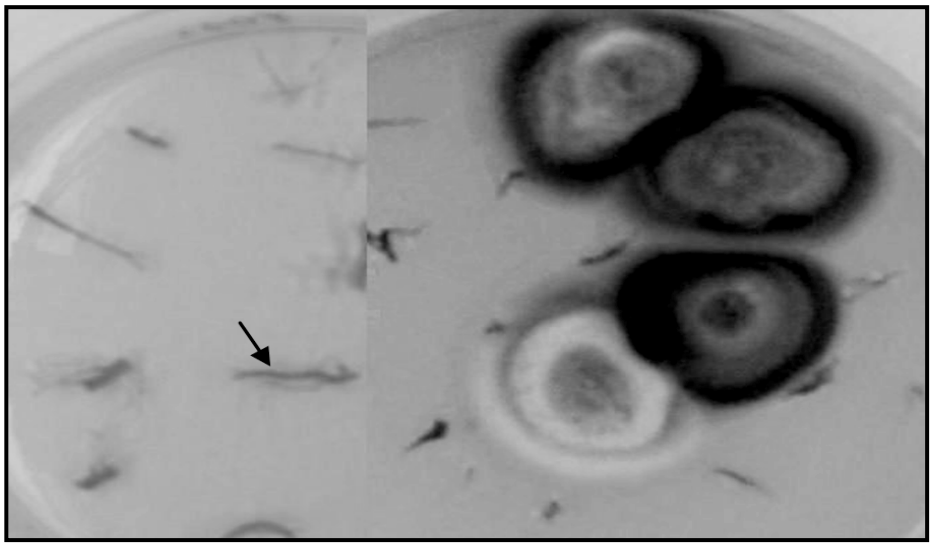

Figure 2. Agar plates with disinfected root fragments (→). At the right side the fragments show fungal growth of endophytes after 4 weeks of incubation. For more information see chapter 2 and 3.

Fungal endophytes can be isolated from leaves, stems and/or roots. They seem to be saprophytic and therefore potentially cultivable on agar and, they are highly diverse within the phyla Ascomycota and Basidiomycota representing distinct functional groups (Carroll, 1988; Weiss *et al*., 2004; Arnold and Lutzoni, 2007; Rodriguez *et al*., 2009; Selosse *et al.,* 2009). These fungi can show positive effects on the plant host like growth promotion and enhancement of resistance and tolerance to biotic and abiotic stresses (Fernando and Currah, 1996; Varma *et al*., 1999; Mandyam and Jumpponen, 2005; Waller *et al*., 2005; Kavroulakis *et al*., 2007; Rodriguez *et al*., 2008; Shoresh *et al.,* 2010).

### 1.1.1 Diversity of fungal endophytes

Fungal endophytes have been recognized in two major groups. “Clavicipitaceous endophytes (CEs)” (Ascomycota, Clavicipitaceae) colonize shoots and rhizomes of a narrow host range of cool- and warm-season grasses (*Poaceae*), while “nonclavicepataceous endophytes (NCEs)” have been isolated from shoots and/or roots of almost all sampled plants and are phylogenetically diverse, most of them belonging to Ascomycota (Petrini, 1996; Saikkonen *et al*., 1998; Schulz and Boyle, 2005; Rodriguez *et al*., 2009). CEs and NCEs have been categorized into four distinct functional groups or classes according to host range, colonized plant tissue, biodiversity, transmission, fitness benefits and colonization pattern (Rodriguez *et al*., 2009).

The CEs (class 1 endophytes) belong to the fungal teleomorphic genera *Epichloë* and *Balansia* corresponding to the anamorphs *Neotyphodium* and *Ephelis*, respectively. They form systemic intercellular infections with a hyphal gradient along the plant axis and are therefore primarily transmitted vertically by seeds (Saikkonen *et al*., 1998; Schulz and Boyle, 2005; Kuldau and Bacon, 2008). The NCEs have been divided into three functional groups (class 2, 3 and 4; Rodriguez *et al*., 2009). Class 2 endophytes extensively colonize both above-and below-ground tissues, but with limited biodiversity in individual plant hosts. They are transmitted vertically and horizontally and confer non-habitat- and habitat-adapted benefits to the plant host. Endophytic fungi in this class belong to a few members of the Agaricomycotina and Pucciniomycotina (Basidiomycota) (Rodriguez *et al*., 2009) and also to Pezizomycotina (Ascomycota) such as *Phoma* sp. and *Arthrobotrys* spp. (Newsham, 1994; Lopez-Llorca *et al*., 2006) and *Fusarium culmorum*, *Colletrichum spp*. and *Curvularia protuberata* in roots, rhizomes, stems and leaves (Rodriguez *et al*., 2008). NCEs class 3 present a localized colonization restricted to shoots, but with high biodiversity in individual tissues. They have been isolated from tropical forest to boreal and Arctic plant populations (Arnold *et al*., 2003; Higgins *et al*., 2007). They are horizontally transmitted by wind, rain and insects (Arnold, 2008; Feldman *et al*., 2008). The foliage isolates usually correspond among the Ascomycota to the Sordariomycetes, Dothidiomycetes, Pezizomycetes, Leotiomycetes and Eurotiomycetes, while basidiomycetous isolates belonging to Agaricomycotina, Pucciniomycotina and Ustilaginomycotina have been less frequently isolated from foliage than from woody tissues. This could be, however, underestimated because of the low recovering on agar cultures (Arnold *et al.,* 2007; Higgins *et al*., 2007; Vega *et al*., 2010). Their benefits to the plant seem to be more complex and non-habitat-adapted (Rodriguez *et al*., 2009). Class 4 endophytic fungi colonize only roots. They have a broad host range and belong to different phylogenetic groups among Ascomycota and among non-mycorrhizal members of the order *Sebacinales* (Basidiomycota) (Jumpponen and Trappe, 1998; Weiss *et al*., 2004; Addy *et al*., 2005; Selosse *et al*., 2009; Schäfer and Kogel, 2009). This kind of endophytes will be described in detail below (see 1.3).

### 1.1.2 Secondary metabolites produced by endophytes

Endophytes may *in vitro* and *in planta* produce new bioactive substances because of a continual metabolic interaction with their hosts and the environment and, this metabolic interaction is important for the symbiosis of both partners (Schulz *et al*., 2002; Schulz and Boyle, 2005; Suryanarayanan *et al*., 2009). Endophytic fungi exhibit a higher proportion of

new substances with biological activity than soil isolates and, the spectrum varies depending on the conditions (habitat, plant and substrate; Gloer, 1997; Schulz *et al*., 2002). These secondary metabolites have been isolated and characterized according to their ecological role and their potential in industry and medicine (Tan and Zou, 2001; Strobel and Daisy, 2003). Some examples of important metabolites that can be produced by endophytic fungi cultivated in synthetic medium and /or by the endophyte-colonized plant host are shown in table 1.

Table 1. Bioactive compounds produced by fungal endophytes (mostly, reviewed by Tan and Zou, 2001; Strobel, 2003).

| Activity | Compound | Endophyte | Plant host | Reference |
|---|---|---|---|---|
| Anti-cancer;anti-oomycetes | Taxol | *Taxomyces andreanae* | *Taxus brevifolia* | Stierle *et al*., 1993; Strobel *et al*., 1993 |
| | Taxol | *Pestalotiopsis microspora* | *Taxus wallichiana* | Strobel *et al*., 1996 |
| Anti-cancer;anti biotic | Torreyanic acid | *Pestalotiopsis microspora* | *Torreya taxifolia* | Lee *et al*., 1996 |
| Anti-cancer | Cytochalasins | *Rhinocladiella* sp. | *Tripterigeum wilfordii* | Wagenaar *et al*., 2000 |
| Anti-cancer;anti-fungal | Botryorhodine A-D (depsidones) | *Botryosphaeria rhodina* | *Bidens pilosa* | Abdou *et al*., 2010 |
| Antifungal | Cryptocandin | *Cryptosporiopsis quercina* | *T. wilfordii*. | Strobel *et al*., 1999 |
| | Ambuic acid | *Pestalotiopsis* spp. *Monochaetia* sp. | *Taxodium disticum Wollemia nobilis* | Li *et al*., 2001a |
| Anti-fungal;antio xidant activities | Isopestacin | *Pestalotiopsis microspora* | *Terminalia morobensis* | Strobel *et al*., 2002 |
| Anti-fungal;anti-bacterial | 6-isoprenylindole-3-carboxylic acid | *Colletotrichum* sp. | *Artemisia annua* | Lu *et al*., 2000 |
| | Colletotric acid | *Colletotrichum gloeosporioides* | *Artemisia mongolica* | Zou *et al*., 2000 |
| | VOCs | *Muscodor albus* | *Cinnamomum zeylanicum* | Worapong *et al*., 2001; Strobel *et al*., 2001 |
| Insecticide; toxic to mammals | Ergovaline Loline Peramine Lolitrem | *Neotyphodium coenophialum N. lolli Epichloe festucae E. typhina* | *Lolium* spp. *L. perenne Festuca* spp. *F. arundinacea* | Schardl and Phillips, 1997; Spiering *et al*., 2005 |
| Immuno-suppressive | Subglutinols A and B | *Fusarium subglutinans* | *T. wilfordii* | Hu *et al*., 2001 |
| | Collutellin A | *Colletotrichum dematium* | *Pteromischum* sp. (*Araceae*) | Ren et al., 2008 |
| Phyto-hormone | IAA | *Colletotrichum* sp. | *A. annua* | Lu *et al*., 2000 |
| | | *Piriformospora indica* | *Arabidopsis thaliana* | Sirrenberg *et al*., 2007 |

IAA: Indole-3-acetic acid.

Table 1. (continued)

| Activity | Compound | Endophyte | Plant host | Reference |
|---|---|---|---|---|
| Phyto-hormone | auxin-like metabolites | *Trichoderma* spp. | *Lycopersicum esculentum*<br>*Brassica napus*<br>*Pisum sativum* | Vinale *et al*., 2008 |
| | indole-β-carboxylic acid | *Botryosphaeria rhodina* | *Bidens pilosa* | Abdou *et al*., 2010 |
| | Cytokinins | *Hypoxilon serpens* | *Nicotiana sp.* | Petrini *et al*., 1992 |
| | Gibberellins | *Gliomastix murorum* | *Elymus mollis*<br>*Oryza sativa*<br>*Atriplex gemelinii* | Khan *et al*., 2009 |

Fungal endophytes produce diverse metabolites in various chemical substance classes such as cytochalasines, steroids, chinones, phenols, isocoumarins, terpenoids, xanthones, enniatines, tetralones and benzopyranones which show antibacterial, antifungal, antimalarial, antiviral and anticancer activities (Li *et al*., 2005; Gunatilaka, 2006; Suryanarayanan *et al*., 2009). Certain endophytes produce a mixture of volatile organic compounds (VOCs) which consist of various alcohols, esters, ketones, acids and lipids with synergistic activities against pathogenic bacteria and fungi (Strobel, 2006; Mitchell *et al*., 2010). Secondary metabolites with antagonistic activity towards pathogenic fungi can be also produced by endophytic fungi in axenic cultures which eases their analysis and application (Kim *et al*., 2007; Vinale *et al.*, 2010).

Medicinal plants had been used to isolate and characterize directly metabolites with biological activity. The discovery of fungal endophytes inside these plants with capacity of produce the same compounds shifted, however, the focus of new drug sources from plants to fungi. In this way the substances could be produced by fermentation processes which reduced the production costs (Strobel, 2003; Suryanarayanan *et al*., 2009). This was e. g. the case for taxol, a diterpene initially extracted from yew tree (*Taxus brevifolia*) and subsequently turned out to be also produced by fungal endophytes (Strobel *et al*., 1996; Li *et al*., 1998). Being synthesized in both, the host and the fungus, raised the question, if the corresponding genes were horizontally transferred between endophyte and plant (Stierle *et al*., 1993).

### 1.1.3 Benefits for the plant host

The outcome of the symbiosis between fungal endophytes and plants seems to be regulated by the physiological and genetic status of both partners and the environmental conditions (Redman *et al*., 2002; Rodriguez *et al*., 2008). Endophytes become parasites and pathogens can turn into endophytes (Johnson *et al.*, 1997; Redman *et al*., 2001; Schulz and Boyle, 2005).

It is hypothesized that mutualism presents a balanced antagonism in which defense responses of the plants and the nutrient demand of the endophyte are in an equilibrium allowing benefits for both symbionts (Schulz and Boyle, 2005; Kogel *et al*., 2006). If interactions are beneficial for the plant, very often increased plant biomass can be observed (Fig. 3) finally leading to higher yield (Waller *et al.*, 2005; Achatz *et al*., 2010). Growth promotion can be based on the regulation of plant development by the direct production of phytohormones or the control over their synthesis and perception in the plant (Table 1). In addition, siderophores and/or enzymes release in the environment can support the uptake of mineral nutrients (Bartholdy *et al*., 2001; Malla *et al*., 2004; Sherameti *et al*., 2005; Khan *et al*., 2009). Finally, increased tolerance to salinity, drought or high temperatures (Waller *et al*., 2005; Rodriguez *et al*., 2008; Redman *et al*., 2002) as well as higher resistance to different foliar and root pathogens (Arnold *et al*., 2003; Waller *et al*., 2005; Jäschke *et al*., 2010) have been reported. The latter can be based on the induction of plant defense mechanisms (see below) known as plant priming (Conrath *et al*., 2006) or the production of substances toxic against microorganisms and insects (see above). In return, the niche inside plant tissues protects the endophyte and can even supply its demand for nutrients (Mandyam and Jumpponen, 2005; Violi, 2007).

Rodriguez *et al*. (2008) demonstrated a new ecological interaction between some endophytes in stressed habitats and their plant hosts which was defined as habitat-adapted symbiosis. Fungal endophytes (*Fusarium culmorum* and *Curvularia protuberata*) isolated from natives grass species from coastal and geothermal habitats conferred salt and drought tolerance. This phenomenon was not observed if plants were colonized by another fungal isolate of the same species recovered from habitats without stress conditions. This capacity of tolerance was also evident in tomato and rice colonized by the same isolates indicating that these associations were not a co-evolutionary relationship between specific plants and endophytes. Interestingly, plants from different habitat such as tomato and rice were drought tolerant after being colonized by these endophytes. This tolerance was not correlated with increased osmolyte production but a decrease in water consumption or in reactive oxygen sensitivity and generation was observed. Other endophytes such as *Colletotrichum magna*, which is related to pathogenic species of *Colletotrichum* and is adapted to agricultural habitat-specific stresses, are able to confer not only disease resistance but also drought tolerance to plant hosts (Redman *et al*., 2001; Rodriguez *et al*., 2008).

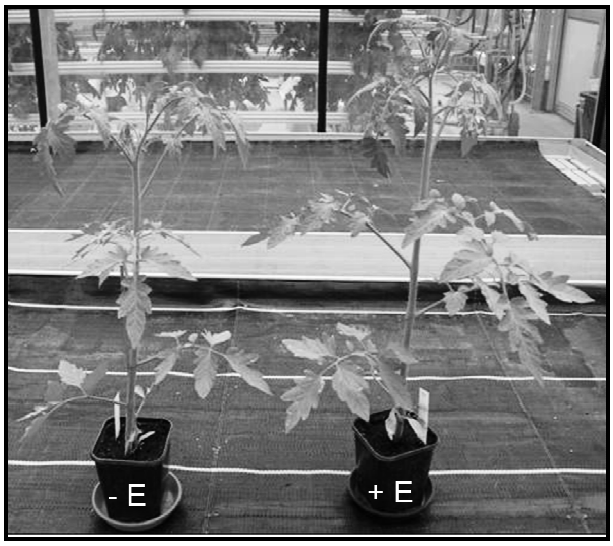


Figure 3. Tomato plants cv. Hildares colonized (+ E) or not (- E) by a fungal endophyte under green house conditions. After 5 weeks plant biomass and development are increased in endophyte-colonized plant. Detailed information about this topic is given in chapter 4.

### 1.1.4 Implications for plant evolution

The symbioses between endophytes and plants have two interesting characteristics. At first, they are highly diverse showing no specificity for the plant host and secondly, they mostly occur in stressful environments (Rodriguez *et al*., 2009). It could be therefore hypothesized that endophytes have an important role in the adaptation of the plants to particular ecosystems in which they have evolved (Zilber-Rosenberg and Rosenberg, 2008; Rodriguez *et al*., 2008). The interaction of plants with a rich endophytic consortium may influence plant gene expression and metabolism. Such intergenomic epigenetic mechanisms would contribute to a successful plant response to abiotic and biotic stressors. Hence, in spite of lack of specificity and co-evolution as explained above, endophytes influence plant adaptation and evolution (Barrow *et al*., 2008).

## 1.2 Root fungal endophytes

The NCEs class 4 is conformed by fungal endophytes that specifically colonize root tissues (Rodriguez *et al*., 2009). They form symbiotic associations with a very wide range of plant species but their biodiversity is less known (Rodriguez *et al*., 2009). The Sebacinales fungi (Basidiomycota) show a diversity of mycorrhizal interactions forming ectomycorrhizae on tree roots (Urban *et al*., 2003), ectendomycorrhizae on Ericaceae (Setaro *et al*., 2006; Selosse *et al*. 2007), endomycorrhizae on Orchidaceae and Ranunculaceae (McKendrik *et al*., 2002;

Kottke *et al*., 2003). *Sebacina vermifera*, *Piriformospora indica* and multinucleate *Rhizoctonia* are non-mycorrhizal mutualistic members of this order in the subgroup B and can be axenically cultivated (Verma *et al*., 1998; Weiss *et al*., 2004; Waller *et al*., 2005; Selose *et al*., 2009). *P. indica* might be considered as a model root endophyte that confers considerable benefits to plant hosts (Chapter 5). Among the ascomyceteous root endophytes, the DSEs known before as "mycelium radicus astrovirens" (Merlin, 1922) have been frequently observed especially in roots of gymnosperms of the family *Pinaceae* due to the easy visualization of their dark pigmented hyphae (Addy *et al*., 2005). The presence of other fungal root endophytes of the Phylum Ascomycota is probably overlooked due to their fine hyaline hyphae. Some of them have been described for their positive impact on the host. In the following, the interaction of plants with *Piriformospora indica*, with some species of *Trichoderma* and with the group of dark septate endophytes will be described as examples for class 4 NCEs.

### 1.2.1 *Piriformospora indica*

*Piriformospra indica* is a basidiomycete (Hymenomycetes). Clamp connections or any sexual basidiospore have not been observed up to now, but the fungus produces pear-shaped chlamydospores at hyaline hyphae (Fig. 4) with dolipore septa. This fungus was isolated from a *Glomus mosseae* spore in the rizosphere of the shrubs *Zyzyphus nummularia* (*Rhamnaceae*) and *Prosopis juliflora* (*Fabaceae*) of the Indian Thar desert (Verma *et al*., 1998).

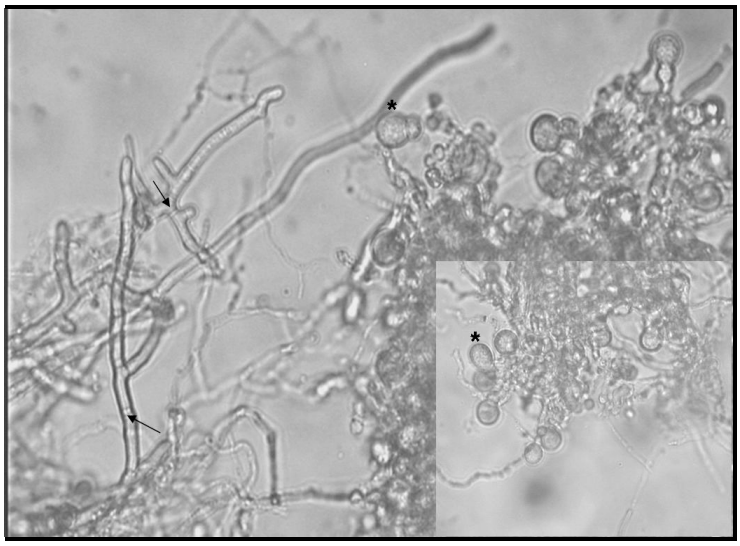

Figure 4. Growth of *P. indica* in liquid medium (400 x (right below 200 x)). Hyline septate mycelium (→) is observed when the fungus is incubated during 3 weeks in a complete medium. Chlamydospores (*) and hyphae can by filtered for the production of inoculum. More details are given in chapter 5.

*P. indica* significantly increases plant biomass, flowering and yield (Varma et al. 1999, Rai *et al*., 2001; Barazani *et al*., 2005; Waller *et al*., 2005; Shahollari *et al*. 2007). Additionally, the fungus confers resistance against root and shoot pathogens such as *Fusarium* spp., *Cochliobolus sativus*, *Rhizoctonia solani*, *Blumeria graminis* and *Verticillium dahliae* and also increases abiotic stress tolerance (Waller *et al*., 2005; Deshmukh and Kogel, 2007; Serfling *et al*., 2007; Waller *et al*., 2007; Fahkro *et al*., 2010).

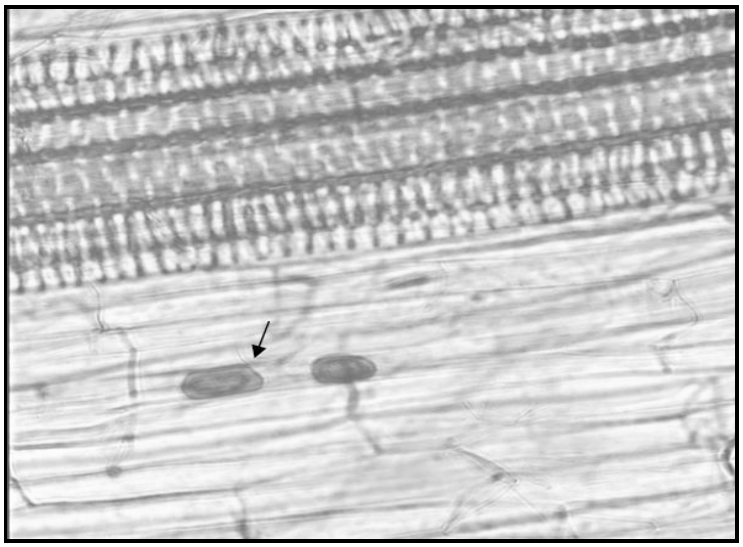

Figure 5. Tomato root stained with trypan blue (400 x). A root fragment is colonized by *P. indica* and chlamydospores (→) in cortical cells are seen.

The root colonization of hyphae germinating from chlamydospores starts with the penetration of the epidermal cells through cell wall or between intercellular spaces and shows different patterns according to the age and the type of the tissues (Schäfer *et al*., 2007). Single cells of the differentiation zone can be fully packed with fungal hyphae before adjacent cells are penetrated, cells of the root hair zone are rapidly colonized by single hyphae and root tips are colonized only intercellularly. The colonization increases in older tissues where cell death is induced by the fungus and new chlamydospores are formed (Fig 5) (Deshmukh *et al.*, 2006). After inoculation of barley, defense-related genes were not induced, but genes involved in senescence were earlier expressed in colonized roots compared to controls (Waller *et al.*, 2008). Additionally, two genes were systematically induced in leaves encoding the pathogenesis-related protein HvPR17b and the chaperone HvHSP70 (Waller *et al*., 2008). More information about this fungus will be presented in chapter 5 of this thesis.

### 1.2.2 *Trichoderma* spp.

*Trichoderma* species (asexual stage or anamorph) have been linked to the genus *Hypocrea* (sexual stage or teleomorph) based on sequence data (Kullnig-Gradinger *et al*., 2002). This genus belongs to the order *Hypocreales* in the phylum Ascomycota. *Trichoderma* produces fast-growing colonies, asexual clear green conidia held in watery drops and phialides (conidiogenic cells) held in a penicillate (Fig. 6) (Samuels, 2006).

*Trichoderma* isolates have shown antagonistic activity against pathogens by mycoparasitism, production of antibiotics and competition in soil (Chet *et al*., 1998; Hoitink and Boehm, 1999). However, additional attributes were observed as for instance increase of plant growth and induction of disease resistance. Maize plants colonized by *Trichoderma harzianum* T22 increased yield and growth up under low levels of nitrogen fertilizer (Harman, 2000). Another strain of the same species promoted growth of crack willow (*Salix fragilis*) in both clean and metal-contaminated soil (Adams and De-Lij, 2007).

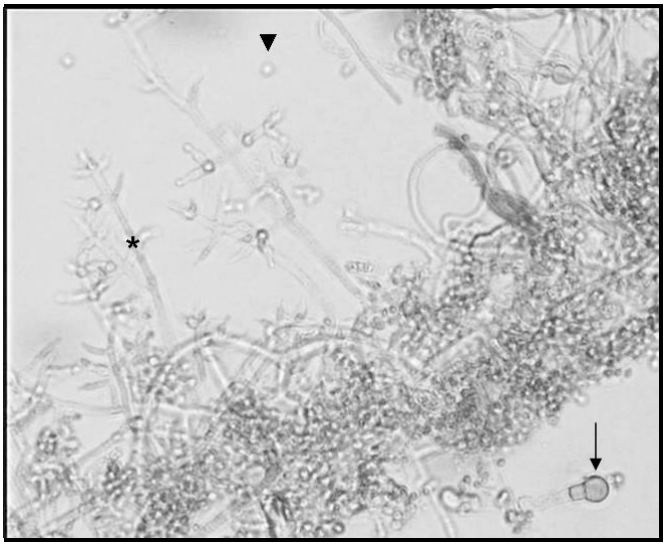


Figure 6. Microscopic observation of the *Trichoderma* endophyte E67 isolated from tomato roots. Hyaline round conidia (▼), divergent branched conidiophores and phialides(*) and chlamydosphores(→) were produced on PDA after 9 days incubation. More information will be presented in chapter 2.

Not only strains from different species as *T. asperellum, T. hamatum, T. harzianum and T. virens* are able to colonize roots, but also a single *Trichoderma* strain can also interact with different plant hosts and induces resistance against different diseases such as Phytophthora blight on cucumber, Botrytis blight on begonia and Botryosphaeria dieback on ericaceous plants (Harman *et al*., 2004; Khan *et al*., 2004; Horst *et al*., 2005; Hoitink *et al*., 2006; Harman and Shoresh, 2007). *Trichoderma* hyphae have been shown to grow around roots, to form appresoria-like structures, to penetrate through epidermal and cortical cell layers and to

induce deposition of cell wall material and production of phenolic compounds by the surrounding plant cells (Yedidia *et al*., 1999).

### 1.2.3 Dark septate endophytes

Dark septate endophytes (DSEs) are usually found in boreal and alpine habitats and produce mitosporic conidia or sterile dark mycelia forming microsclerotia in roots that can be also colonized by ecto- and endomycorrizal fungi (Jumpponen and Trappe, 1998; Mandyam and Jumponen, 2005; Wagg *et al*., 2008). The accumulation of melanin in hyphae is characteristic of this diverse group and probably reflecting a common response to shaped environmental pressures (Butler and Day, 1998; Robinson, 2001). Fungi isolates from roots correspond to different anamorph genera from different orders among the Ascomycota, such as *Phialocephala, Scytalidium, Oidiodendron, Trichocladium, Cadophora, Exophiala, Heteroconium, Chloridium, Leptodontidium,* and *Cryptosporiopsis* (Addy *et al*., 2005).

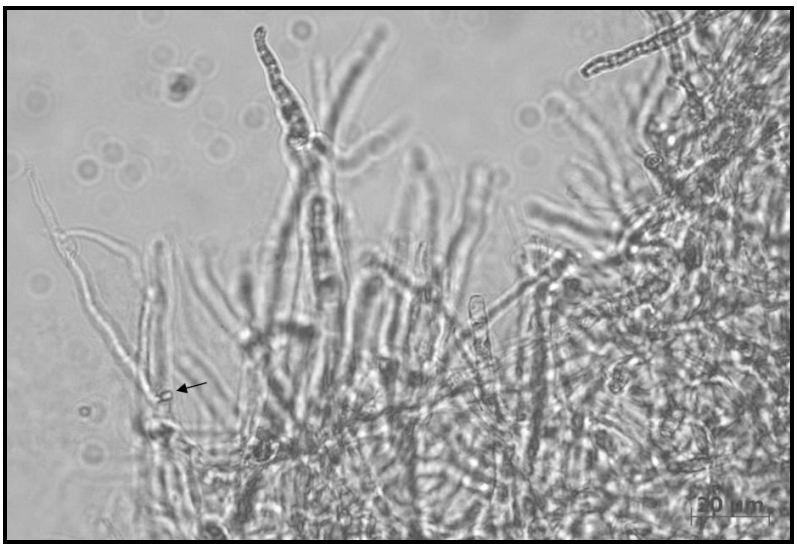

Figure 7. Dark septate hyphae of DSE135 isolated from tomato root and identified as *Leptodontidium orchidicola*. This isolate presents poor production of conidia which are directly formed at hyphae (→).

Colonization patterns by DSEs seem to be different according to the plant host and the cultivation system (Peterson *et al*., 2008). It has been best described for *Phialocephala fortinii*. The hyphae grow on the root surface forming a network. Single hyphae colonize parallel to the main axis of the root and between epidermal and cortical cells. Intracellular colonization is visualized as formation of microsclerotia (O´Donell *et al*., 1993).

DSEs are able to hydrolyze major detrital C, N and P polymers into usable subunits and therefore could provide N and P to the host plant (Jumpponen *et al.* 1998; Caldwell *et al*.,

2000). The fungus *Aspergillus ustus* which is heavily colonizing the roots of fourwing saltbush takes up and transports P from tricalcium phosphate and rock phosphate resources (Barrow and Osuna, 2002). Some investigations revealed positive effects on plant growth and yield (Shivanna *et al*., 1994; Wu and Guo, 2008). The interaction of the DSE EF-37 (related to *Mycocentrospora*) with a Chinese medicinal plant showed for example a positive effect on plant biomass, on the number of root hairs and the levels of the flavonoid rutin (Wu *et al*., 2010). Upson *et al*. (2009) showed an interrelation between plant growth promotion and the use of organic resources. DSE-colonized roots of *Deschampsia antarctica* possessed enhanced development and increased shoot and root nitrogen and phosphorus contents only when they had been supplied with organic nitrogen.

## 1.3 The model crop tomato

Tomato (*Solanum lycopersicum* L.) is an eudicotiledonous plant that belongs to the family *Solanaceae* together with other economically important crops such as pepper, eggplant and potato. It is the most important grown fresh market vegetable world wide with more than 5 million hectares harvested in China, United States of America, India, Turkey and Egypt as the five first producers, respectively (htpp://faostat.fao.org). Tomato was classified by Miller (1754) as *Lycopersicon esculentum* but later it was renamed as *Solanum lycopersicum* (Child, 1990; Peralta *et al*., 2008). The tomato genome is diploid composed of 12 chromosomes with a total of 10,600 markers localized on its physical map and about 14.5 million sequenced bases (http://solgenomics.net/).

The centre of origin of the genus *Solanum* section *Lycopersicum* (formerly genus *Lycopersicon*) is the Andean region that includes parts of Colombia, Ecuador, Peru, Bolivia and Chile (Rick, 1973; Taylor, 1986). The tomato was cultivated and consumed by the "Azteca" culture in the 16$^{th}$ century before the arrival of the Spanish. The name in the native language of Mexico was *tomatl* which was later transferred as *tomate* into the Spanish language in 1532 (Corominas, 1990). In spite of tomato was domesticated in Mexico, it was cultivated in some zones of Europe, Africa and Asia before it was re-introduced in the rest of the American continent through the maritime trade of Spain (Esquinas-Alcázar and Nuez, 1995).

The high level of consumption of tomato makes it one of the main sources of minerals and vitamins. The nutritional quality is mainly determined by its lycopene and vitamin C and E

contents. The ripened tomatoes present high levels of carotenoids, of which carotenes make up between 90 and 95%. Lycopene is with 90% the most abundant carotene in the red tomato fruit (Minoggio *et al*., 2003; Guil-Guerrero and Rebolloso-Fuentes 2009). Lycopene has important health properties since it reduces the risk of several types of cancer and heart attacks (Dorgan *et al*., 1998; Clinton, 2005). β-carotene is a provitamin A and its deficiency can cause xerophtalmia, blindness and premature death (Fawzi *et a*l., 1993; Mayne, 1996; West *et al*., 1999). Tomato cultivars show high variations in their carotenoid contents. β-carotene content varies from 1.15 to 3.7 mg/kg fresh weight and the total carotenoid content from 18.5 to 60.7 mg/kg fw (Abushita *et al*., 1997). In wild species such as *S. pimpinellifolium*, the average values for lycopene content were as much as five times higher than those found in the cultivated tomato (Fernández-Ruiz *et al*., 2002), making these wild accessions a promising source of variability for increasing the lycopene content in tomato breeding programs.

Currently, various tomato genomic projects are been carried out facilitating the advance towards new tools and methodologies (Gidoni *et al*., 2003; Fei *et al*., 2004; Mueller *et al*., 2005; http://bti.cornell.edu/CGEP/CGEP.html). The large-scale gene expression profiling and the EcoTILLING techniques are two examples which are used to identify genes and alleles of interest. A great number of molecular markers (RFLP, AFLP, RAPD, CAPs, SSR) are mapped onto the genetic map of tomato (Foolad and Sharma, 2005). The most recently developed molecular markers are single nucleotide polymorphisms, SNPs (Yang *et al*., 2004; Yamamoto *et al*., 2005) and conserved ortholog sets (COS). Such COS markers are ESTs of single or low copy genes also identified in the *Arabidopsis* genome (Fulton *et al*., 2002). The International Solanaceae Genome project (SOL), a Genomic Network, is a comparative resource for the plants of the *Solanaceae* family, which includes important crops such as tomato, potato, eggplant and pepper (http://www.sgn.cornell.edu/about/). Additionally, the identification of QTLs (Quantitative Trait Loci) has been carried out for the study of quantitative characters highly influenced by environment (Bretó *et al*., 1996; Monforte *et al*., 1996). Organoleptic and nutritional quality of fruits and the resistance to biotic and abiotic stresses are the great breeding objectives these new methodologies are currently dealing with (Causse *et al*., 2003).

### 1.3.1 Tomato cultivation

Tomato is considered a strict autogamous plant with natural self-pollination. The percentage of allogamy varies depending on the cultivation type, the presence of pollinating insects,

frequency and intensity of winds, relative humidity and other factors. Tomato plants are usually germinated in greenhouses during the late winter to be subsequently transplanted into the field, but in cooler climates they are adapted for indoor growing (Hu *et al*., 2007). Cultivation can be carried out in soils, substrates or in hydroponic systems using mineral nutrient solutions (Gualberto *et al*., 2002).

Different characteristics of tomato plants are required for optimal production of tomatoes for fresh consumption. These are an open growth habit, high yield, earliness, external and internal quality of fruits, long shelf life, adaptation to different cultivation systems and resistance to biotic and abiotic stresses. Particular features are required for greenhouse production such as not excessively compact plants and adaptation to long harvesting periods and to low temperature and light intensity. Pollination occurs between 15.5°C /13°C and 32°C /24°C (day/night temperatures). At lower or higher temperatures flowers will drop without setting fruit. The time require for an early tomato variety is approximately from 50 to 65 days from planting to market while for a late tomato variety from 85 to 95 days (Jones, 1999).

Many cultivated plant varieties showing a determinate or indeterminate growth habit, and differences in fruit flavor, shape and color. Some examples are

- beefsteak tomatoes (Big Beef F1, Evergreen, Giant Belgium) being larger than the traditional round tomatoes, 180–250 g in weight and containing five or more locules,
- salad tomatoes (Yellow Perfection, Green Zebra, Moneymaker) with an average fruit weight of 70–100 g and diameter 4.7–6.7 cm,
- cherry tomatoes (Red Pear, Santa F1, Sun Cherry F1) being very sweet and smaller than the classic tomatoes with a weight varying between 10 and 20 g and 1.6–2.5 cm diameter,
- plum tomatoes (Amish Paste, Costoluto Genovese) and paste tomatoes (San Marzano) that present a typical oval shape.

Production of tomato in temperate regions is usually done under green house conditions in hydroponic plant cultivation systems (Schwarz *et al*., 1996; Savvas *et al*., 2003). Plants are grown using nutrient solution which can either be re-circulated in a closed system or drained after one use in an open system. Since hydroponic plants have access to unlimited nutrition and water, they can grow up to ten times faster than soil grown counterparts (Geraldson, 1982).

### 1.3.2 Tomato breeding

The cultivated tomato is moderately to highly sensitive to different abiotic and biotic stresses, including drought, salinity, extreme temperatures, nutrient imbalances, excessive moisture, environmental pollution and diseases. However, there are several wild species that represent a rich source of useful genetic variation (Rick 1976, 1983). Such variation has been extensively utilized in tomato breeding programs for improving desirable agricultural characteristics and to develop stress-tolerant plants (Fooland, 2005).

The *in vitro* culture techniques have facilitated the hybridization between sexually incompatible wild and cultivated relatives as well as the introgression of resistance and tolerance genes. In addition, heterosis as improved characteristics or functions in a hybrid offspring, has been found in tomato for characteristics such as yield, resistance and tolerance and as well as for quality of the fruit (Esquinas-Alcazar and Nuez, 1995). Thus, tomato breeding between different commercial and wild tomato relatives produces hybrids with tolerance to humidity, salinity, cold and chilling and drought. For instance, the vegetative and fruit development of tomato below 13$^{o}$C is decreased by reduction in quality and quantity of the pollen. Thus, one strategy to get fertile pollen at low temperatures is the crossing with wild species, which grow at high altitudes, such as *Solanum peruvianum* and *S. habrochaites* (Chetelat *et al*., 2009).

Moreover, resistance to fungal, bacteria and viral diseases has been also transferred. Most varietal types, especially hybrids cultivated in greenhouses, have introgressed a high number of genes of resistance mainly to fungi (*Ve,I, I-2, Fol 1,2, Frl, Asc, Cf*-series, *Sm, Ph-2* and *Lt*) bacteria (*Pto*), nematodes (*Mi*) and viruses (*Tm-22*, *Sw-5* and *Ty-1*) (Meyers *et al*., 1999; Laterrot, 2000). Additionally, some characteristics of the fruit have been improved such as pigmentation, sugar content and ripening (Esquinas-Alcazar and Nuez, 1995; Chaib *et al*., 2006). The identification of QTLs associated with characters of interest and marker-assisted selection is another alternative that is being studied extensively (Villalta *et al*., 2008; Finkers *et al*., 2007; Lin *et al*., 2010) and may support the tomato breeding programs.

### 1.3.3 Tomato diseases and control

The responses of plants to biotic stressors or pathogens can be classified as susceptibility, tolerance or resistance. If the plant is susceptible for infection, attack by a pathogen will be followed by its further development on or inside of plant tissues. The expression of such a successful infection is the occurrence of symptoms. If the plant is resistant, defense mechanisms act to avoid invasion and pathogen development is limited. In consequence, the

expression of symptoms if any occur is low. Tolerance describes the phenomenon that the pathogen colonizes the plant but reduced symptom levels are expressed and yield in case of crops is similar to healthy plants (Robb, 2007).

Table 2. Principal diseases of tomato plants and their control
( Jones, 1999;; http://www.extension.iastate.edu/publications/pm1266.pdf; Agrios, 2005).

| Disease | Microorganisms | Control |
|---|---|---|
| | **Fungi** | |
| Late blight | *Phytophthora infestans* | Approved fungicides; resistant varieties; Crop rotation; soil solarization. |
| Verticillium wilt | *Verticillium albo-atrum* | |
| | *Verticillium dahliae* | |
| Anthracnose | *Colletotrichum coccodes* | |
| | *Colletotrichum dematium* | |
| | *Colletotrichum gloeosporioides* | |
| Early blight | *Alternaria solani* | |
| Cercospora leaf mold | *Pseudocercospora fuligena* | |
| Fusarium wilt | *Fusarium oxysporum f.sp. lycopersici* | |
| Fusarium crown and root rot | *Fusarium oxysporum f.sp. radicis-lycopersici* | |
| Powdery mildew | *Oidiopsis sicula* | |
| Pythium damping-off and fruit rot | *Pythium aphanidermatum* | |
| | *Pythium arrhenomanes* | |
| | *Pythium debaryanum* | |
| | *Pythium myriotylum* | |
| | *Pythium ultimum* | |
| Rhizoctonia damping-off and fruit rot | *Rhizoctonia solani* | |
| Gray leaf spot | *Stemphylium botryosum f.sp. lycopersici* | |
| | *Stemphylium lycopersici* | |
| Septoria leaf spot | *Septoria lycopersici* | |
| Leaf mold | *Fulvia fulva* (*Cladosporium fulvum*) | Stake and prune to provide air movement |
| | **Bacteria** | Approved bactericides; hot-water-treated seed; avoid planting in affected fields for 3 years. |
| Bacterial spot | *Xanthomonas campestris* pv. *vesicatoria* | |
| Bacterial wilt | *Ralstonia solanacearum* | |
| Bacterial speck | *Pseudomonas syringae* pv. *Tomato* | |
| Bacterial canker | *Clavibacter michiganensis* | |
| | **Virus** | |
| Common mosaic of tomato | Tobacco mosaic virus (TMV) | Avoidance of contact by smokers; control of aphid carrier with insecticides; stylet oil; resistant varieties; prevention and eradication; disease-free seed and plant material. |
| Tomato mosaic | Tomato mosaic virus (ToMV) | |
| Tomato fern leaf | Cucumber mosaic virus (CMV) | |
| Curly top | Curly top virus | |
| Tomato bushy stunt | Tomato bushy stunt virus | |
| Tomato etch | Tobacco etch virus | |
| Potato virus Y | Potato virus Y | |
| Tomato necrosis | Alfalfa mosaic virus | |
| Tomato spotted wilt | Tomato spotted wilt virus | |
| Tomato mosaic | Pepino Mosaic Virus (PepMV) | |
| | **Nematodes** | |
| Root-knot | *Meloidogyne* spp. | Control methods before planting; resistant varieties; crop rotation; alternate flooding and drying; soil solarization; methylbromide-chloropicrin. |
| Sting | *Belonolaimus longicaudatus* | |
| Reniform | *Rotylenchus reniformis* | |
| root lesion | *Pratylenchus* spp. | |
| false root-knot | *Nacobbus* spp. | |
| potato cyst nematodes | *Globodera* spp. | |
| Stunt | *Tylenchorhynchus* spp. | |

Tomato plants are exposed to different pathogens and parasites including bacteria, fungi, viruses, nematodes and insects (Table 2). Concerning fungal diseases, tomato producers are confronted with great losses in the production caused by plant pathogens especially such as the soil-borne fungi *Fusarium oxysporum*, *Verticillium dahliae* and the Oomycete *Phytophthora infestans* (Brayford, 1996; Shattock, 2002; Fradin and Thomma, 2006). Different types of chemical control are usually employed to reduce yield losses. For instance, the efficacy of chloropicrin was evaluated as a possible alternative fumigant to methyl bromide against the fungal pathogens, but soil type and organic matter content influences the efficacy of the treatment (Gullino *et al*., 2002). In hydroponic system, seven fungicides were tested for their inhibitory activities against *F. oxysporum* and prochloraz and carbendazim turned out to be most effective (Song *et al*., 2004). However, high input of fungicides causes health and environmental problems (Soares and Porto, 2009) and leads over time to resistance of the pathogens (Stammler *et al*. 2006). The use of tomato resistant cultivars is one alternative for controlling such diseases but new races of pathogens have appeared which overcame these resistances (e.g. Parlevliet, 2002).

A third possibility to confine pathogens is the use of different microorganisms that either have antagonistic activity against the pathogen or are able to induce plant systemic resistance. The antagonistic activity can involve different modes of action that produce a detrimental effect on pathogen growth. These are not necessarily mutually exclusive meaning that one microorganism is able to express several of such modes of action (Whipps, 2001). Some of these modes of action include pathogenic bacterial endosymbionts of fungi, antimicrobial compounds (antibiosis), competition for iron by siderophore production, parasitism and extracellular enzyme production, competition in the rhizosphere for nutrients, space or infection sites (reviewed by Whipps, 2001; by Kobayashi and Crouch, 2009). Antagonistic activities in the rhizosphere depend on the natural resident microbiota and the environment (Whipps, 2001). Hence, repeated applications of *Penicillium oxalicum* were needed to ensure efficient biocontrol of *Fusarium oxysporum* f. sp. *lycopersici* (FORL) in tomato (De Cal and Melgarejo, 2001)

Biological agents, which act as inducers of plant systemic resistance, promote defense responses in the plant and additionally have other benefits on physiological and abiotic stresses (reviewed by Shoresh *et al*., 2010). This is possibly due to overlapping signaling networks for the expression of genes regulated by biotic and abiotic stresses (Torres and Dagl, 2005; Fujita *et al*., 2006; AbuQamar *et al*., 2009). Positive effects were first evident with plant growth promoting rhizobacteria (PGPR) (Van Loon *et al*., 1998). PGPRs have not only

the ability to increase plant growth but some of them (e.g. *Bacillus* spp. and *Pseudomonas* spp.) can also induce resistance in different plant hosts against pathogens such as *F. oxysporum* and *Phytium aphanidermatum* (reviewed by Whipps, 2001). That was latter known as priming which has been suggested as a potentiating cellular defense responses of the plants for a further attack by pathogens (Conrath *et al*., 2006). For instance, the control of the Oomycete *Phytophthora infestans* that causes late blight disease in tomato was tested using preinoculation of different PGPR. They induced an effective defense by callose formation against late blight and in addition promoted the growth of tomato seedlings (An *et al*., article in press).

Besides bacteria, also fungi can induce plant systemic resistance. They colonize roots and also induce a primed state in the plant which in consequence responds better or more rapidly to biotic or abiotic stressors (Conrath *et al*., 2006). Species of *Trichoderma*, DSE, *P. indica*, hypovirulent species of *Rhizoctonia solani* or non-pathogenic strains of binucleate *Rhizoctonia* sp. and of *Fusarium* are some examples of fungi that show this effect on plant hosts (Yedidia *et al*., 1999; Narisawa *et al*., 2000; Waller *et al*., 2005; Kavroulakis *et al*., 2007). For example, *Trichoderma koningiopsis* reduced significantly the stem colonization by the pathogen FORL in tomato and *T. koningiopsis*-treated tomato plants grown in a split root system showed induction of genes involved in the jasmonic and ethylene transduction pathways (Moreno *et al*., 2009). The mycoparasite *Pythium oligandrum* establishes close contact with fungal pathogens and inhibit their growth (Rey *et al*., 2005). Additionally, the fungus is able to colonize root tissues without inducing extensive cell damage but structural barriers at sites of potential fungal penetration. Hence, *P. oligandrum*-colonized tomato roots showed resistance to the pathogen *Fusarium oxysporum* (Benhamou *et al*., 1997). Combinations of different biological agents have turned out to be especially effective. An inoculum made of fluorescent *Pseudomonas, T. harzianum* and *Glomus intraradices* provided a better control of *Fusarium* wilt of tomato than single isolate treatments and also increased yield by 20% (Srivastava *et al*., 2010). *F. oxysporum* strain *Fo*47 and *Trichoderma harzianum* were combined with the oomycete *Phytium oligandrum* to improve its efficacy in tomato plants. Although the oomycete was inhibited on agar by *F. oxysporum* and *T. harzianum*, the combination provided optimal plant protection against *Botrytis cinerea* infection of leaves (Le Floch *et al*., 2009). The different beneficial activities of biological agents can be of each other. Particular mutants of *Trichoderma virens* were deficient in mycoparasitism against *Rhizoctonia solani* but still able to induce phytoalexins in cotton and control of *R. solani* (Howell *et al*., 2000).

The mechanisms underlying induced resistance have been subdivided in two categories. Systemic acquired resistance (SAR) is mediated by salicylic acid (SA), induced by a local infection that leads to the production of Pathogen Related (PR) proteins such as PR1, PR2, chitinase and phenylalanine ammonia-lyase (PAL). It mainly affects the interaction with biotrophic pathogens (Durrant and Dong, 2004). In contrast, induced systemic resistance (ISR) is developed in response to the colonization of PGPRs and fungal endophytes, involves jasmonic acid (JA) and ethylene (ET) signaling and mainly directed against necrotrophs (Van Loon *et al*., 1998; Shoresh *et al*., 2010). In SAR, SA induces defense gene expression via *nonexpresor-of-PR-genes-1* (NPR1) which encodes a protein with nuclear localization and protein-protein interaction domains that is active depending on its cytosolic redox stage (Mou *et al*., 2003). Interestingly, NPR1 is also required for ISR signaling in which PR genes are not activated (Pieterse and van Loon, 1999). Arabidopsis inoculated with *Pseudomonas fluorescens* WCS417r showed differential expression locally in roots, but alteration in gene expression in leaves was not observed (Verhagen *et al*., 2004). Additionally, there is no alteration in the production of JA or ET (Pieterse *et al*., 2000), but plants are more sensitive to plant hormones and respond faster or stronger to the subsequent pathogen attack with the activation of genes encoding plant defensins (PDF1.2) and thionins (THI2.1) (Epple *et al*., 1995; Penninckx *et al*., 1996; Verhagen *et al*., 2004).

ABA is involved in plant responses to abiotic stresses and developmental processes, but also in modulating pathogen defense (Finkelstein *et al*., 2002; Mauch-Mani and Mauch, 2005). Basal or elevated ABA levels suppress PAL activity, a key enzyme in the early steps of the phenylpropanoid biosynthetic pathway leading to phytoalexin production (McDonald and Cahill, 1999). In addition, ABA is involved in the suppression of synthesis of the plant defense hormone SA. A tomato mutant for ABA production, *sitiens*, showed hypersensitivity to benzothiadiazole, an analog of SA and high induction of PAL activity after the pathogen attack (Audenaert *et al*., 2002). *Sitiens* shows increased accumulation of PR1 both prior to and quickly after inoculation with *Botrytis cinerea,* extensive ROS accumulation and enhanced peroxidase activity as a result of ABA deficiency. In contrast, this hormone seems to induce JA biosynthesis and callose deposition (reviewed by Asselbergh *et al*., 2008).

The primary defense response against pathogens in the early stages of infection is an oxidative burst which produces a rapid generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide ($H_2O_2$). These processes are part of the hypersensitive reaction (HR) that induces rapid and localized death of plant tissue at the site of infection (Tenhaken *et al.*, 1995; De Gara *et al.*, 2003). ROS-scavenging enzymes, such as superoxide

dismutase (SOD), peroxidases (POXs) and catalase (CAT) are induced for detoxifying ROS generated in this localized response (Mittler, 2002). ROS are, however, also involved in regulating the mutualistic interaction between the fungal endophyte *Epichloë festucae* and its host *Lolium perenne*. (Tanaka *et al*, 2006). It was, moreover, observed that the induced resistance of barley leaves against powdery mildew systemically induced by *P. indica* – colonization of the roots is correlated with antioxidant enzyme activity (Waller *et al*., 2005)

### 1.3.4 *Verticillium dahliae* a vascular fungal pathogen of tomato

*Verticillium* spp. are soil-borne fungal pathogens (Ascomycete) which are able to infect a broad range of plant species (Bhat and Subbarao, 1999; cited in Klosterman *et al.*, 2009). The most studied isolates belong to the species *V. dahliae* and *V. albo-atrum*, which worldwide cause great losses (Pegg and Brady, 2002). *V. dahliae* Kleb. is able to infect more than 200 plant species and produces microsclerotia that live for more than 10 years in soil. This makes its control very difficult and produces long lasting problems in crop production (Klosterman *et al.*, 2009). The fungus presents six main vegetative compatibility groups (VCGs) and some of them exhibit host specificity and differential pathogenicity on other hosts. VCG1 is principally associated with cotton and VGC4 generally with Solanaceae. Strains of VCG4A are, however, prevalent and damaging on potato, while VGC2A isolates are more aggressive in tomato (Bhat and Subbarao, 1999; Tsror *et al*., 2001). *V. dahliae* has further been classified according to its virulence on either tomato cultivars lacking the *Ve* resistance gene (*V. dahliae* race 1) or on all cultivars whether or not this gene is present (*V. dahliae* race 2) (Korolev *et al*., 2007).

Microesclerotia of *V. dahliae* germinate in soil in presence of a host and hyphae extend toward the roots (Fitzell *et al.*, 1980). The fungus enters the root and grows inter- and intracellularly reaching the trapping sites of vascular tissues and, xylem parenchyma cells surrounding the trapping sites begin to respond. If there is vascular coating, colonization of adjacent vessels can be avoided and the plant is resistant or tolerant to the pathogen. Further responses involve the production of phytoalexins, cell wall degrading enzymes and other PR-proteins (Dayf *et al.*, 1997). Susceptibility involves little or no coating and the hyphae penetrate the pit membrane into the adjacent vessel element (Chen *et al*., 2004). Conidia are produced in xylem vessels and carried upward with the transpiration stream, where they acropetally spread until the whole plant is affected (Pegg and Brady, 2002). Secondary infection cycles are occurring after germination of these conidia (Gold and Robb, 1995) and

vascular infection results in gradual chlorosis and necrosis of leaves, browning of vascular bundles, wilting and stunting of the plant (Gold *et al.*, 1996). The environmental conditions such as temperature, light intensity and soil moisture levels can influence symptom development (Robb, 2007). The fungus produces toxins, elicitor and cell wall degrading enzymes. Its ability to escape in this way from primary trapping sites and to further colonize the plant determines its aggressiveness (Fradin and Thomma, 2006; Novo *et al.*, 2006).

## 1.4 Research focus and organization of the thesis

This thesis focuses on the interaction of root fungal endophytes with the important vegetable crop *Solanum lycopersicum*. For the first time, native root endophytic fungi are isolated from tomato crops and characterized concerning taxonomy and root colonization patterns. The interaction of three selected endophytes with tomato plants concerning their impact on vegetative growth, yield and fruit quality was studied and plant protective effects were evaluated. In addition, experiments are described which evaluate the impact of the model endophyte *Piriformospora indica* on growth and yield in tomato and on nutrient uptake in barley. These results are presented in the next four chapters as follows:

Chapter 2: **Dark septate and other ascomycetous endophytes from roots of cultivated *Solanum lycopersicum* in Colombia: taxonomic identification and characterization of their endophytic colonization.** Taking into account the benefits of root fungal endophytes to plant hosts and the great potential of such biological agents for crop production, this work was aimed to isolate new endophytic fungi from tomato crops and to characterize them for their taxonomic position and colonization pattern. All experiments were carried out by me under supervision of the co-authors. The corresponding manuscript is under revision in the Journal Mycologia.

Chapter 3: **Screening of tomato endophytic fungi for potential biological agents**. This part presents the initial screening of fourteen selected root fungal endophytes in order to evaluate their ability to colonize tomato plants in pot experiments under green house conditions. Additionally, their mutualistic or parasitic interactions with the colonized plants were estimated to select those without any negative effect on plants for further experiments. All work was carried out by me under supervision of the co-authors and it is in press in the IOBC Bulletin.

Chapter 4: **Effects of dark septate endophytes on tomato plant performance**. Based on plant growth promoting effects on young tomato plants under green house conditions and stable colonization pattern, three new dark septate endophytes (DSEs) were selected to evaluate their influence on vegetative tomato growth characters in young and older plants, their capacity to protect tomatoes against the pathogen *Verticillium dahliae* and their impact on fruit yield and quality. All experiments were carried out by me under supervision of the co-authors. This work was submitted to the Journal Mycorrhiza.

Chapter 5: This part of the thesis corresponds to the results on the interaction of the model root endophyte *P. indica* and two important crop plants (tomato and barley). Therefore, this chapter has been subdivided into three parts:

- 5.1. **Impact of *Piriformospora indica* on tomato**. This part summarizes the work concerning the impact of *P. indica* on tomato plants which has been carried out at the Leibniz-Institute of Vegetable and Ornamental Crops - IGZ. In this work three out five experiments were done by me. This work is in press in Oelmüller R, Kost G, Tripati S, Varma A (eds.), Sebacinales. Springer-Verlag, Heidelberg.

- 5.2. **Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens.** The present work was carried out to analyze the ability of *P. indica* to reduce the disease caused by the pathogen *V. dahliae* and for estimating tomato interaction with the Pepino mosaic virus (PepMV) in hydroponic systems. The experiment on *V. dahliae* was carried out by me. This work was published in Mycorrhiza: 20(3):191-200, 2010.

- 5.3. **Root colonization by *Piriformospora indica* enhances grain yield in barley under diverse nutrient regimes by accelerating plant development.** This work presents the results of a collaborative work between the IGZ and the Institute of Phytopathology and Applied Zoology at the Justus Liebig University (Giessen). The work was carried out to test different possibilities how *P. indica* enhances yield in barley. Inoculation experiments with *P. indica* and the mycorrhizal fungus *Glomus mosseae* were conducted by me to test whether *P. indica* increases total P and N content. This work was published in Plant and Soil: 333:59-70, 2010.

In chapter 6, all results are discussed together. This chapter is followed by the reference list related to the general introduction and discussion and appendices of the chapter 2, 3, 4 and 5.

# Chapter 2

## Dark septate and other ascomycetous endophytes from roots of cultivated *Solanum lycopersicum* in Colombia: taxonomic identification and characterization of their endophytic colonization.

# Dark septate and other ascomycetous endophytes from roots of cultivated *Solanum lycopersicum* in Colombia: taxonomic identification and characterization of their endophytic colonization.

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- All experiments were carried out by me under supervision of the co-authors. Supplemental Material (Appendix 1 - 5)

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**Abstract**

Plants can be internally colonized by endophytes without causing visible disease symptoms. Tomato (*Solanum lycopersicum* L.) roots from four different crop sites in Colombia were surface sterilised and 51 isolates of endophytic fungi were obtained and conserved for further analysis. Based on microscopical observations and growth characteristics, 20 fungal isolates corresponded to the genus *Fusarium*, six presented asexual conidia different to *Fusarium*, eight showed sterile mycelia, seven of them with dark septate hyphae and 17 did not continue to grow on plates after being recovered from conservation. Growth on different media, detailed morphological characterisation and ITS region sequencing of the six sporulating and eight sterile endophytes revealed that they belong to different ordes among the phylum Ascomycota and that the sterile dark septate endophytes did not correspond to the well-known *Phialocephala* group. Interaction of nine isolates with tomato plantlets were *in vitro* assessed. No impact on shoot development was revealed, but three isolates caused brown spots in roots. Colonization patterns as analysed by confocal microscopy were different between the isolates and ranged from epidermal to cortical penetration. All together, eleven new isolates from root-endophytic fungi were obtained whereof seven showed features of dark septate endophytes. Six isolates belonged to five morphotypes of putative new unknown species.

*Key words:* Ascomycota, dark septate endophyte, plant-fungal interactions, root colonization, tomato (*Solanum lycopersicum* L.)

## INTRODUCTION

Fungal endophytes colonize internal plant tissues without causing disease symptoms or overt tissue damage in their hosts (Schulz and Boyle 2005; Kogel et al 2006). This group comprises mycorrhizal fungi together with the non-mycorrhizal fungi, of which the latter occur in above-ground plant tissues and in roots (Smith and Read 1997; Arnold et al 2001; Brundrett 2004; Saikkonen et al 1998; Bacon and White 2000; Saikkonen 2007). Numerous non-mycorrhizal endophytes, usually Ascomycota, have been isolated from gymnosperms to angiosperm monocots and eudicots in a wide diversity of habitats (Faeth and Hammon 1997; Grünig et al 2002; Seena and Sridhar 2004; Raviraja 2005; Gilbert and Strong 2007; Larran et al 2007). Non-mycorrhizal endophytes were previously recognized in two major groups (Petrini 1996) according to their plant hosts, their taxonomy and their ecological functions. The "clavicipitaceous group" (Ascomycota, Clavicipitaceae) which colonize some grasses (Kuldau and Bacon 2008) and the nonclavicipitaceous endophytes being a highly diverse group with broad plant host range (Rodriguez et al 2009).

Endophytes establish associations that may have no obvious negative impact on the plant, and in some cases may be mutualistic. Mutualism can be based on reciprocal exchange of nutrients, protection against biotic and abiotic stress and/or beneficial influence on development (Rodriguez et al 2009). In some interactions, these fungi can exert positive effects on the plant based on the delivery of particular compounds, such as auxins (Vadassery et al 2008), polyketides, terpenoids (e.g. taxol) and derivatives of indol (Strobel et al 2004; Zhang et al 2006), or by the induction of plant defense mechanisms (Waller et al 2005; Waller et al 2006; Deshmukh and Kogel 2007; Kavroulakis et al 2007; Stein et al 2008). In return, the niche inside plant tissues protects the microorganisms and can even supply their demand for nutrients (Jumpponen and Trappe 1998; Mandyam and Jumpponen 2005; Violi 2007).

Non-clavicipitaceous endophytes include fungi that colonize specifically plant roots (Rodriguez et al 2009; Selosse et al 2009) including the heterogeneous complex of dark septate endophytes (DSE) which are conidial or sterile inhabitants of diverse plant species from arctic to temperate habitats. DSE produce melanized septate hyphae, microsclerotia in the roots and can influence plant growth both positively and negatively (Jumpponen 2001; Mandyam and Jumpponen 2005). Much information is available for non-mycorrhizal endophytes in roots of forest trees and shrubs, in alpine and subalpine

plants and in terrestrial orchids (Read and Haselwandter 1981; Currah et al 1990; Stoyke et al 1992; Ahlich and Sieber 1996). However, little is known about such fungi colonizing crops grown in agri- and horticulturally used sites.

The current study was aimed to isolate new endophytic fungi from Colombian crops and to characterize them for their taxonomic position and colonization pattern. Tomato (*Solanum lycopersicum L.*) was chosen because it is one of the most commonly grown fresh market vegetables world wide (http://faostat.fao.org/faostat).

## MATERIALS AND METHODS

*Isolation of fungal endophytes.*—Four to six-month old tomato plants without disease symptoms were collected from four different semi-opened plastic greenhouses in which the tomato plants were transplanted directly to soil after growth in seedbeds. Three sites are located in the Cundinamarca department (Cota in the central savanna province at 4°49'N 74°06'W (CT); Tena in the Tequendama province at 4°39'N 74°22'W (TN) and Madrid - Tibaitata in the western savanna province at 4°50'N 74°18'W (M)) and one in the Boyaca department (Villa de Leyva in the Ricaurte province at 5°38'N 73°32'W (VL)). Mean annual temperatures were 13.5 C (between 10 C and 24 C) in the savanna area (CT and MD), 21 C (between 7 C and 32 C) in TN and 18 C (between 2 C and 30 C) in VL. CT and VL present commercial production systems with the use of pesticides and mineral fertilizers, tomato plants in TN are cultivated with a semi-commercial management with lower application of pesticides and mineral fertlizers and, MD is an experimental site with organic management without pesticide application and some reported cases of the presence of *Botrytis* sp. and *Alternaria* sp. In general the soils are classified as Andic Eutrudepts (Cundinamarca region) and Fluventic Ustropept (in the valley area of VL, Boyaca). They have a low pH (pH 5.0 – 6.3; Cundinamarca/pH 4.0 – 5.7; Boyaca) and a high content of organic matter (>10%). The soils of Cundinamarca show relatively high concentrations of phosphorous (>100ppm) (Gomez et al 2006; Galindo and Clavijo 2007; Jiménez et al 2008). Three tomato plants were sampled in each site and their total root system without symptoms were intensively washed with tap water, disinfected with 70% ethanol for one minute and random samples were directly transferred to 5% of a commercial sodium hypochlorite solution (2.5% NaOCl) for five (secondary roots) or ten minutes (primary roots). After washing three times with sterile distilled water, thirty fragments of approximately 1-2 cm

were cut per each disinfected root system and the effectiveness of surface sterilization was verified on potato dextrose agar (PDA; Oxoid, Lenexa, USA) using the imprint technique (Schulz et al 1999). Subsequently, each root fragment were subdivided into three pieces and each one was transferred on PDA, sabouraud dextrose agar (SDA; Oxoid, Lenexa, USA) and 2% malt extract agar (MEA; Oxoid, Lenexa, USA) supplemented with 0.5 mg $ml^{-1}$ chloramphenicol (Quemicetina; CarloErba, Colombia) and incubated at 25 C. Each fragment was checked weekly for eight weeks for mycelia emerging from the cut ends. Emergent mycelia were sub-cultured to new PDA plates and incubated in darkness at 25 C to recover pure fungal colonies. Pure isolates were maintained on PDA covered with sterile oil at 4 C and deposited in the culture collection of the Laboratory of Mycology and Plant Pathology (LAMFU, Andes University) and the Leibniz- Institute of Vegetable and Ornamental Crops (IGZ, Grossbeeren).

Oil-conserved fungal isolates were recovered on MEA (Roth, Karlsruhe, Germany) for further analysis. After recovering on agar, 17 isolates showed limited growth and were therefore not suitable for morphological characterization and analysis of colonization patterns. According to the morphological features of hyphae and spores, the remaining 34 isolates were roughly categorized in three major groups: dark septate or sterile mycelia, *Fusarium*-like isolates and other sporulating colonies. Non-fusaria isolates were selected in this study for further analyses because our intent was to focus on new root endophytes not related to putative pathogens.

*DNA extraction, PCR and phylogenetic analyses.*—Total DNA was extracted from hyphae of the non-fusaria endophytes grown in liquid complete medium (CM, Pontecorvo et al 1953) using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) and quantified by measuring absorbance at 260/280 nm. One ng of DNA was used as template for a 50 µl PCR reaction containing 0.5 µM ITS1 and ITS4 primers (White et al 1990), 0.2 mM dNTPs and 0.025 u of *Taq* polymerase (PeQLab, Erlangen, Germany) in a buffer system supplied by the producer of the enzyme. DNA was initially denatured (95°C for 4 min), followed by 35 cycles of amplification (94 C for 30 sec, 57 C for 30 sec, 72 C for 1 min), and a final 10 min extension at 72 C. After control of amplification on 2% agarose gels, PCR products were purified on spin columns according to the manufacturer's protocol (Roche, Mannheim, Germany) and cloned into the pGEMTeasy vector (Promega, Mannheim, Germany). Both strands of the inserts were sequenced and edited by

Eurofins MWG Operon (Ebersberg, Germany). ITS sequences from the endophytes and from related genera of the NCBI and Broad institute databases were aligned by MUSCLE (Edgar 2004) and alignments were processed using the program GENEDOC (Nicholas et al 1997). All positions were removed where the sequences of the endophytes under investigation showed gaps in the alignment. Alignments are deposited at TREEBASE under the accession numbers 19819. Phylogenies of sequences were reconstructed by maximum-likelihood carrying out 1.000 replicates with the software PUZZLE (Version 5.2; Strimmer and von Haeseler 1996) using the HKY model of substitution (Hasegawa et al 1985) or the programs SEQBOOT, DNAML and CONSENSE of the program package PHYLIP (version 3.69; Felsenstein 1993). Results were displayed as dendrograms using the program TREEVIEW (Version 1.5.3; Page 1996).

*Morphological characterization.* —Pure colonies of the selected endophytes were grown on different culture media such as PDA (Merck KGaA, Darmstadt, Germany), corn meal agar (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), commercial extract from 8 vegetables (V8; Gemüsesaft, Penny, Germany), 2% MEA (Carl Roth GmbH, Karlsruhe, Germany) and 1.5 % water agar (Agar-agar, Merck KGaA, Darmstadt, Germany) at 22 C or 25 C. Growth rate of the colonies was measured. Hyphal characteristics, conidiogenous cells and type of spores or conidia were studied by light microscopy with an AxiosKop 2 plus microscope (ZEISS, Germany) at magnifications of 200, 400, and 630. Identification of putative endophytes was carried out based on these characters at least to genus level (Raper and Thom 1949; Ellis 1976; Domsch et al 1980).

*Tomato seedling colonization assay.*— Based on a protocol for co-cultivation experiments (Shahollari et al 2007), disinfected tomato seeds (cv. Hildares) were germinated on liquid MS medium (Murashige and Skoog 1962) at 22 C with a photoperiod of 16/8 h. (d/n) to produce more roots to be colonized. After 7 days, the seedlings were transferred to Plant Nutrition culture Medium (PNM; Shahollari et al 2007) and WA (0.8%) in square plates (six seedlings per plates, two plates per fungal isolate). After 24 h, three fungal plugs of 5 mm in diameter were placed between each two seedlings at a distance of 1 cm from the roots. The fungi were previously cultivated on PDA for 1-2 weeks at 25 C.

For visualization of hyphae in tomato roots, fragments were sampled at the first fungal contact and 2 and 4 weeks after inoculation and stained with WGA-

AF 488 (Molecular Probes, Karlsruhe, Germany) according to Deshmukh et al (2006). Root fragments were fixed in 4% paraformaldehyde in PBS (pH 7.4) and 0.05% Tween 20 (w/v) at room temperature for 2 - 4 h. Fixed root samples were boiled for 2 min in 10% (w/v) KOH and after rinsing with PBS (pH 7.4), stained for 15 min with 5 µg/ml Congo Red (Merck, Darmstadt, Germany) and with 10 µg/ml WGA-AF 488 for 1 – 2 h.

A confocal laser scanning microscope (TCS SP2, Leica, Germany) was used for detecting the fluorescence signal from stained hyphae. The instrument was equipped with an argon/krypton laser, and the following objectives: 63x plan apo water and 20x plan apo water. Two different filter settings were used: (i) for the dye Alexa 488, excitation wave length: 488 nm, beam splitter: DD 488/568 (double dichroic, reflects at 488 and 568 nm), barrier filter: BP 530 (band pass, 515-545 nm); (ii) for the Congo Red, excitation wave length: 568 nm, beam splitter: DD 488/568, barrier filter: BP 590 (long pass > 590 nm). During image acquisition each line was scanned 4 times and averaged. Image analysis was performed with the Leica Confocal Software of TCS SP2 (version 2.61. build 1537).

**RESULTS**

*Isolation and selection of fungal endophytes.*—51 fungal isolates were initially obtained from 30 root fragments of three tomato plants from four sites in Colombia (TABLE I). Isolation frequency showed a clear difference between the sites. While it was 7% and 6% in Villa de Leyva and in Cota, hyphae grow out from 24% and 20% of the root fragments from tomato plants in Madrid and Tena. Among the 51 isolates, 17 do not show growth in culture after recovering from oil and were therefore not further characterized (NG in TABLE I). 34 isolates could be roughly categorized according to their morphological characteristics. Among those, 20 belonged to the genus *Fusarium* representing 5 different morphotypes based on microscopical observations of micro- and macroconidia typical for this genus (TABLE I). Several *Fusarium* species have been reported as root endophytes (Rubini et al 2005; Götz et al 2006) and tomato was also shown as a potential host (Hallmann and Sikora 1996; Kim et al 2007). In spite of their interesting features (Hallmann and Sikora 1996; Athman et al 2007; Rodriguez et al 2008), the present study concentrated on the characterization of non-fusaria endophytic fungal species from tomato roots, due to the sparse information about their taxonomy. Hence, identification and plant colonization tests were carried out with the 14 remaining isolates. This could be further divided into six morphotypes producing conidia or

TABLE I. Grouping of tomato root endophytic fungi isolated of four different sites in Colombia according to their morphology. 30 fragments per root system were analyzed from each three plants of four sites in Colombia under commercial management in Villa de Leyva (VL), semi-commercial management in Cota (CT), traditional management in Tena (TN) and organic management in Madrid (MD). The number of isolates and morphotypes (in brackets), as well as the infection frequencies (isolates per root fragment) are shown.

| Crop site | Latitude/ Altitude | T[a] | Cultivar | Isolate number (Morphotype number) | | | | | IF |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *Fusarium sp.* | DSE/ SM[b] | OMT[c] | NG[d] | Total | |
| VL | 5°38'N 2045 m | 18 C | Chonto | 3 (2) | - | 1 (1) | 2 (1) | 6 (4) | 0.07 |
| CT | 4°49'N 2566 m | 13 C | Santa Clara | - | - | 3 (3) | 2 (2) | 5 (5) | 0.06 |
| TN | 4°39'N 1384 m | 21 C | Chonto | 7 (3) | 4 (2) | 1 (1) | 6 (3) | 18 (9) | 0.20 |
| MD | 4°50'N 2554 m | 14 C | Rocio | 10 (5) | 4 (3) | 1 (1) | 7 (6) | 22 (15) | 0.24 |
| Total of isolates | | | | 20 (5) | 8 (5) | 6 (6) | 17 (11) | 51 (27) | - |
| Isolation Frequency (IF) | | | | 0.06 | 0.02 | 0.02 | 0.05 | 0.14 | - |

a average of environmental temperature
b dark septate endophytes and sterile mycelia
c others morphotypes which produced conidia or spores
d no growth after recovering from oil

spores and five morphotypes represented by 8 isolates showing features of dark septate endophytes (DSEs) or other sterile mycelia (TABLE I).

*Characterization of fungal endophytes.*— In order to characterize the 14 endophytes three different approaches were followed. At first, the ITS region of the rRNA gene cluster was sequenced and compared with sequences of other fungi (TABLE II). Alignments of ITS regions from the endophytes and related genera were phylogentically analyzed by different programs giving highly similar results. Those obtained with the program PUZZLE were displayed as dendrograms (APPENDIX 1). Secondly, the isolates were cultivated on different media, where colony growth and the different morphological characteristics were monitored (TABLE II; APPENDIX 2). Finally, tomato roots were *in vitro* inoculated and colonization was monitored at three dates (physical contact, two and four weeks after inoculation) by confocal microscopy (TABLE III; APPENDIX 3).

Sequence comparisons revealed that the ITS regions of all 14 isolates were highly homologous to sequences from fungal species of the phylum Ascomycota (TABLE II). The six isolates, which produced conidia on PDA (OMT in TABLE I), were related to species among the Hypocreales

TABLE II. Identification of root fungal endophytes. Molecular characterization was based on amplification and sequencing the ITS region of the nrDNA and matching of the sequences by BlastN to those from other fungi in GenBank. Morphological characterization of nine isolates based on growth features and microscopic observation.

| Isolate | Crop site | GenBank accession number | Highest BLAST affinities | Identity | Percent coverage | Morphology on PDA | Microscopic features | Taxonomic identification |
|---|---|---|---|---|---|---|---|---|
| E20 | CT | AM944352 | *Plectosphaerella cucumerina* AJ492873.1 (Phyllochorales) | 99% | 99% | Moist yellowish white | Amero- and didimoconidia ovoid and hyaline (5.5 µm x 2.4 µm) | *Plectosphaerella cucumerina* (Palm *et al.* 1995) |
| E27 | CT | AM944351 | *Bionectria rossmaniae* AF210665 (Hypocreales) | 97% | 94% | Beige – pale pink powdery | Penicillate conidiophores; conidia one-celled ellipsoidal (5.7 µm x 3.4 µm) | *Clonostachys rossmaniae* (Schroers 2001) |
| E36<br>E41 | TN | AM944360<br>AM944361 | Fungal endophyte sp. DF4 DQ459005 (Pleosporales) | 99% | 99% | Grey – olive green | Dark septate hyphae; no production of conidia; dematiaceous chlamydospores like structures. | * |
| E48 | TN | AM944359 | Fungal endophyte sp. DF4 DQ459005 (Pleosporales) | 99% | 99% | Dark grey | Dark septate hyphae; no production of conidia; dematiaceous chlamydospores. | * |
| E49 | TN | AM944355 | Fungal endophyte FN394699 (Pleosporales) | 97% | 96% | Reddish brown; diffusible red pigment | Hyaline and dematiaceous hyphae; formation of small sclerotia. | * |
| E63 | MD | AM944362 | *Pyrenochaeta lycopersici* AY649593 (Pleosporales) | 99% | 98% | Grey silky colony | Dark septate hyphae; no production of conidia. | molecular identification not verified |
| E67 | MD | AM944350 | *Trichoderma* sp. AY514865 (Hypocreales) | 99% | 99% | Yellowish green fast growth colony | Subglobose amero- gloio-hyaloconidia with smooth cell wall; intercalar and terminal chlamydospores | *Trichoderma harzianum* (Rifai 1969) |

* No identification possible, morphological features are shown in APPENDIX 2

TABLE II (continued)

| Isolate | Crop site | GenBank accession number | Highest BLAST affinities | Identity | Percent coverage | Morphology on PDA | Microscopic features | Taxonomic identification |
|---|---|---|---|---|---|---|---|---|
| E131<br>E135 | MD | AM944357<br>AM944358 | *Leptodontidium orchidicola*<br>GQ302678<br>(Helotiales) | 98% | 99% | Greyish brown; colony with diffusible dark red pigment | Production of few conidia directly from dematiceous hyphae; formation of small sclerotia | *Leptodontidium orchidicola*<br>(Sigler & Currah 1987) |
| E133 | MD | AM944356 | Uncultured ascomycete<br>EU002996<br>(Chaetosphaeriales) | 85% | 97% | Whitish to beige; mycelium partly immersed in the agar | no production of conidia and typical conidiophores of *Codinaeopsis* sp. | * |

* No identification possible, morphological features are shown in APPENDIX 2

(E9, E27 and E67), the Phyllachorales (E20), the Sordariales (E22) and the Pleosporales (E52). Among those, E22 (ITS sequence: AM944353) and E52 (ITS sequence: AM944354) turned out to colonize plant roots neither *in vitro* nor in a pot culture (data not shown). They are therefore not further described. Eight isolates were classified as dark septate endophytes or sterile mycelia (DSE/SM in TABLE I), because they produced brown hyphae and/or no spores or other reproductive structures. Their ITS regions were highly homologous to sequences from species among the Pleosporales (E36, E41, E48, E49 and E 63), the Heliotales (E131 and E135) and the Chaetosphaeriales (E133).

*Hypocreales.*—Among the three isolates belonging to the order Hypocreales, E9 (ITS sequence: AM944349) clustered within *Fusarium* and presented 99% similarity to *F. equiseti* (Corda) Sacc. Because macroconidia were only observed after subsequent plating on PDA, the isolate was not identified as *Fusarium* in the first morphological screen. It was, however, omitted from the further characterization for the reasons mentioned above.

The endophytic isolate E27 (TABLE II) produced typical unicellular ellipsoidal conidia and diverticillate phialides of *Clonostachys rossmaniae* Schroers which is the anamorph of *Bionectria rossmaniae* (Schroers 2001). This corresponded to the phylogenetic analysis (APPENDIX 1a). At the first contact with root cells the endophyte E27 formed appressorium-like structures (TABLE III; APPENDIX 3, T1). Symplastic colonization increased over time (TABLE III, APPENDIX 3, T2 and T3), but symptoms in the roots were not observed. *B. rossmaniae* has been characterized as a destructive mycoparasite and is being used as biocontrol agent of fungal plant pathogens (Schroers 2001). Plant-colonizing abilities of this species have been to our knowledge described here for the first time.

BlastN analysis of the ITS region obtained from E67 indicated that it is closely related to species of the genus *Trichoderma* (TABLE II). Also the phylogenetic analysis of the sequence (APPENDIX 1b) clustered it among *Trichoderma* species with closest neighborhood to *T. citrinoviride, T. atroviride, T. tomentosum, T. harzianum* and *Hypocrea lixii*. However, the morphological characterization (TABLE II; APPENDIX 2) showed that E67 produced hyaline mycelium with divergent branched conidiophores, phialides and conidia corresponding to *T. harzianum* Rifai (Dodd et al 2003). Upon inoculation of the tomato roots, the endophyte formed singly appearing adhesion tips from which the

roots were colonized (TABLE III; APPENDIX 3). The genus *Trichoderma* is well known for harboring important biocontrol agents due to their antagonistic activities (Hagn et al 2003; Grosch et al 2007). Colonization of roots has been also reported (Yedidia et al. 1999). A response of the plant to the colonization as it was shown by Yedidia et al (2000) could not be observed for E67 under our conditions. Whether the present isolate is nevertheless able to influence plant-pathogen interactions as other *Trichoderma* species (Cordo et al 2007) remains to be shown.

*Phyllachorales.*—Sequence analysis of the endophyte E20 showed its close relation to *Plectosphaerella cucumerina* (Lindf.) W.Gams (Phyllachorales). Phylogenetic analysis including different species of this genus and of other fungi belonging to the Phyllachorales confirmed this assumption (APPENDIX 1c). Morphological characterization further substantiated the taxonomic classification. E20 produced ovoid hyaline conidia and phialides (TABLE II) that corresponded to the anamorph of this species named *Plectosporium tabacinum* (J.F.H.Beyma) M.E.Palm, W.Gams & Nierenberg. Colonization of the root started similar to E67 with singly formed adhesion tips (TABLE III; APPENDIX 3, T1). In the following, E20 did not colonize the root intensively, but was restricted to the cells of the rhizodermis (TABLE III; APPENDIX 3, T2). In contrast to the former described endophytes, the isolate E20 produced disease symptoms on roots after two weeks of co-cultivation. Tomato seedlings inoculated with E20 on WA plates showed small bright brown spots in the root tips far from the fungal plug (TABLE III, APPENDIX 4a) and the roots revealed local thickening of the cell wall and fluorescence (TABLE III, APPENDIX 4d). Such a fluorescence response was also detected in roots on PNM where no visual symptoms were, however, observed (data not shown). In this respect, E20 behaves like the *P. cucumerina* strain described as a pathogen on barley (Martinez et al 2003) and not like a different isolate which has been reported as a potential nematophagous biological control agent (Jacobs et al 2003).

*Pleosporales.*—Six isolates were grouped by the sequence of their ITS region among the order Pleosporales. One (E52) did not colonize roots under our conditions and were not further characterized. The other five presented melanized hyphae without production of conidia (E36, E41, E48, E49, E63) and were therefore classified as dark septate endophytes. Sequences of two isolates (E36, E41) were identical except for one base and differed only in three nucleotides from the sequence of isolate E48. They showed closest similarity (99%)

with the ITS region of a root fungal endophyte (DQ459005; TABLE II) isolated from a Chinese yam plant (Xu et al 2008), but also similar to sequences of dark sterile mycelia obtained from roots of a Mediterranean ecosystem (Girlanda et al 2002) and lower score but the same similarity with the fungus *Rhizopycnis vagum* (AF022786) isolated from roots of melons and sugarcane (Farr et al 1998). Phylogenetic analyses with species among the Pleosporales showed that E36, E41 and E48 clustered together with fungi of the genera *Rhizopycnis* and *Phoma* (APPENDIX 1d). The Megablast restricted to mitosporic ascomycota showed that the ITS sequences of E36, E41 and E48 are 99% similar to part of the according sequence of *Rhizopycnis vagum* (AF022786), but the phylogenetic support after bootstrap analysis was low (APPENDIX 1d). *R. vagum* was described by Farr et al (1998) as a coelomycetous fungus isolated from roots of melon and sugarcane that contributes to the disease complex known as vine decline in cucurbits (Armengol et al 2003), but has also been isolated from roots from several other hosts without causing symptoms (Ghignone et al 2003). Morphological characterization of the three isolates showed that the colony of E41 was identical to E36 and produced on PDA an olive green - grey colony without any conidia or pycnidia in the different media tested for sporulation (TABLE II; APPENDIX 2). A purple pigment was produced in old culture in presence of tomato roots (APPENDIX 2). The colony appearance of the isolate E48 was different in comparison to that produced by the endophyte E41. The endophyte E48 showed a dark grey colony with chlamydospore-like cells (TABLE II; APPENDIX 2). However, typical conidia of *R. vagum* were also not produced by these two isolates in any media evaluated. Colonization assays were carried out with E41 (identical to E36) and with E48. Both fungi developed adhesion tips on the surface of the tomato roots (TABLE III, APPENDIX 3, T1) which were singly (E41) or singly and branched (E48). Growing intracellular hyphae showed a constriction at sites where the fungus traverses the cell wall (TABLE III, APPENDIX 3, T2). Further research has to show if they have any impact on tomato development. None of the three isolates E36, E41 and E48 induced any visible responses in the tomato roots at least in the *in vitro* system used in the present analysis (TABLE III). In this respect they do not resemble the original pathogenic *R. vagum*. Based on the low bootstrap values in the phylogenetic analysis and on the absence of structures typical for *R. vagum* we suggest that the endophytes represent a new unknown species of DSEs among the Pleosporales.

The isolate E49 revealed highest similarity (97%) with the fungal endophyte FN394699 (TABLE II) isolated from the grass *Holcus lanatus* (Sánchez et al 2010), as well as with an unknown fungus colonizer (FJ613827) from roots of the medicinal plant *Artemisia annua* (Asteraceae). It clustered only with uncultured soil fungi (APPENDIX 1e) related to the order Pleosporales. The next known relatives seem to belong to the genera *Massarina* sp. and but the bootstrap-support was extremely low (APPENDIX 1e). The fungus produced on PDA a reddish brown flat colony with a diffusible red pigment in PDA. Hyaline conidia-like cells and dark sclerotia-like structures were observed (TABLE II; APPENDIX 2). None of these structures did match to any known fungi in the Pleosporales. It might be therefore a totally new DSE species or a new anamorphic from a teleomorph with unknown ITS sequence. In the colonization assays, E49 produced swollen cells attached to the intercellular spaces, as well as single or branched adhesion tips (TABLE III; APPENDIX 3, T1). As described for E41, constrictions were observed, when intracellular hyphae traverses cell walls (TABLE III, APPENDIX 3, T2). E49 was the only fungus in the present study producing fluorescent microsclerotia-like structures inside the roots (TABLE III, APPENDIX 3, T3).

The isolate E63 was classified as DSE based on colony appearance and showed 99% similarity with *Pyrenochaeta lycopersici* R.W.Schneid. & Gerlach (TABLE III), the causal agent of corky roots in tomato (Doganlar et al 1998). The phylogentic analysis also grouped it close to this species with a high bootstrap support (APPENDIX 1f). Its colony was grey and produced dark microsclerotia-like structures, but hyaline conidia like those described for *P. lycopersici* were not produced in any media tested (TABLE II, APPENDIX 2). Upon contact with tomato roots, E63 formed single or branched tips in addition to swollen cells and appressorium-like structures (TABLE III; APPENDIX 3, T1). Inside the root, heavy hyphal development and the formation of intracellular hyphal coils was observed (TABLE III; APPENDIX 3, T3). E63 produced disease symptoms on roots after two weeks of co-cultivation (TABLE III; APPENDIX 4). The tomato seedlings inoculated with this fungus showed necrotic areas on PNM and WA (APPENDIX 4). In summary, E63 resembles the pathogen *P. lycopersici* concerning the ITS sequence and the interaction with the tomato roots. The classification could, however, not be confirmed by morphological characters due to the

absence of any reproductive structures. It has to be therefore currently regarded as a new strain highly related to *P. lycopersici*.

*Helotiales.*—The isolates E131 and E135 showed an identical sequence that revealed 98% similarity with *Leptodontidium orchidicola* Sigler & Currah (GQ302678) (TABLE II). The molecular phylogeny (APPENDIX 1g) showed that it belongs to a polyphyletic group of DSEs among the Helotiales (Grünig et al 2002). This result was confirmed by the morphological characterization (TABLE II; APPENDIX 2). At grayish brown velvet colonies with diffusible dark red pigment conidia were produced directly from dematiaceous hyphae and corresponded to *L. orchidicola*. Production of microsclerotia-like structures inside of WA was also observed. E135 (as 131) attached the epidermal cells by appressorium-like structures and also by single tips (TABLE III, APPENDIX 3, T1). The hyphae connected root hairs (APPENDIX, T2) and root colonization was inter- and intracellular from epidermal to cortical cell layers (TABLE III, APPENDIX 3, T3). *L. orchidicola* has been isolated from roots of a wide range of plant species including orchids, herbaceous and woody plants (e.g. Fernando and Currah 1995). *In vitro* experiments revealed that *L. orchidicola* can form different type of interactions. Depending on the host and the strain, the effect could range from parasitic to beneficial (Fernando and Currah 1996). Here we show for the first time the presence of this fungus in a crop. No pathogenic features were detected indicating that the present isolates could behave as neutral endophytes.

*Chaetospaeriales.*—The endophyte E133 with hyaline hyphae showed only low homology to an uncultured ascomycete (TABLE II). This isolated is not related to any DSE group and clustered between *Codinaeopsis* and *Thozetella* species belonging to the order Chaetosphaeriales (APPENDIX 1h). The most similar known fungus (84% ITS sequence identity) was a strain of *Codinaeopsis sp.*, but it clustered with an unknown endophyte near to the *Thozetella* group (APPENDIX 1h). The endophyte produced a whitish colony with a dark zone close to the center (TABLE II; APPENDIX 2). This morphology resembles features of both genera, *Codinaeopsis* and *Thozetella* (APPENDIX 2) and is most similar to the colony described for *Codinaeopsis gonytrichoides* (Shearer & J.L.Crane) Morgan-Jones (Morgan-Jones 1976). Also the conidiogenous cells with conspicuous collarets reminded to this species. However, the typical conidiophores of *C. gonytrichoides* were not seen. Therefore, the taxonomy of this root colonizer remains open at the current stage. E133 contacted tomato roots in the *in vitro* system with single or branched

TABLE III. Colonization patterns of root fungal endophytes. Tomato roots were grown on water agar or PNM and inoculated with the endophytes. Roots were stained upon first contact (T1), 2 (T2) and 4 (T3) weeks after inoculation with WGA-AF 488 (chitin antibody) and microscopically analyzed.

| Isolate | T1 | T2, T3 | reactions of the plant |
|---|---|---|---|
| E20 | single adhesion tips | rhizodermis colonization | small bright brown spots in the root tips; local thickening of the cell wall and fluorescence |
| E27 | appressorium-like structures | symplastic colonization | no symptoms |
| E36<br>E41 | single adhesion tips | cortex colonization, constriction upon cell wall traversion | no symptoms |
| E48* | single and branched adhesion tips | cortex colonization, constriction upon cell wall traversion | no symptoms |
| E49* | swollen cells; single or branched adhesion tips | cortex colonization; constriction upon cell wall traversion | no symptoms |
| E63 | appressorium-like structures; swollen cells; single or branched adhesion tips | heavy hyphal development; intracellular hyphal coils | necrotic areas |
| E67 | single adhesion tips | low colonization on surface of root tip emerging; cortex colonization | no symptoms |
| E131<br>E135 | appressorium-like structures and single tips | inter- and intracellular colonization in epidermis and cortex | no symptoms |
| E133* | single or branched adhesion tips | heavy surface colonization; constriction upon cell wall traversion | brown spots in the root tips; fluorescence in single cells without necrosis |

adhesion tips (TABLE 3; APPENDIX 3, T1) and showed constrictions when traversing a cell wall (TABLE 3; APPENDIX 3, T2). As the fungal isolates E20 and E63, roots responded also to E133 after two weeks of co-cultivation and presented brown spots on tips (TABLE 3, APPENDIX 4). The microscopic observation showed heavy colonization of the endophyte on the root

surface associated with fluorescence in single cells of the root without tissue necrosis (TABLE 3; APPENDIX 4).

**DISCUSSION**

The presented screening for fungi being root endophytes of the important crop tomato resulted in the identification of eleven new isolates. Comparing the results from the four different sites interestingly showed that differences did not depend on the cultivar or the altitude of the site. However, four times less root endophytes were obtained from plants of conventional farming system (VL and CT) than from semi-commercial- and organic-grown crops (TN and MD). This could indicate an impact of conventional cultivation techniques and investigations for bacterial endophytes supported this finding (Smalla et al 2001). If this is a general pattern has to be confirmed in future work by a systematically screening with higher numbers of different crops. The study also showed that root endophytic fungi seem to be highly adapted to their ecological niche and very sensitive. Only 67% of the primary isolates could be recovered on plates. This indicates on the one hand that roots of crops should be also screened with methods identifying non-culturable endophytes and on the other hand that the methods for cultivation and conservation of this kind of fungi have to be improved.

Colonization of roots by Ascomycota fungi has been principally described for two DSE species. This shows different modes of penetration, depending on the host and the fungal species being involved. *Phialocepahala fortinii* shows penetration of the cell wall with narrow hyphae without distorting the cell wall (Currah et al 1993) and at least in the plant family *Salicaceae*, the most frequently site of hyphal penetration are the root hairs (Peterson et al 2008). Another endophyte related to *Mycocentrospora* (Pleosporales) colonizes only the epidermal layer without intracellular penetration by hyphae swollen at sites of root penetration (Wu et al 2010). None of these patterns resembled those observed in the current study. The endophytes belonging to the orders Phyllachorales, Chaetosphaeriales, Hypocreales, Pleosporales and Helotiales showed appressorium-like structures, swollen cells or single and branched adhesion tips. The penetration was in most cases through the intercellular space. Comparison of the observed patterns demonstrated a high variability among microfungal endophytes to enter the roots of their hosts.

After penetration, colonization further progressed inter- and intracellular in the epidermal cell layer and in most cases also through the cortex. None of the fungal strains invaded the vascular cylinder

or produced degradation of cortical and vascular parenchyma cells which has been reported for some DSEs (Peterson et al 2008). Intracellular growth of the hyphae presented in some cases a constriction at sites where the fungus traverses the cell wall (see E41, E48, E49, E133). Colonization for most of the isolates was limited and formation of hyphal coils or vesicles which can be characteristic for the fungus-host combination (Abdellatif et al 2009) was rarely observed. Only E63 produced coils which were localized in necrotic tissues of the root. One particular structure was the formation of microsclerotia-like structures by the unknown endophyte E49. Microsclerotia are frequently found associated with root hairs of dicotyledonous and monocotyledonous species (Peterson et al 2008). Under some conditions they were also detected inside the cells of the root cortex of members of the Pinaceae colonized by *P. fortinii*, where they were typically melanized (Jumpponen and Trappe 1998). In tomato roots, however, they fluoresce by the WGA-AF stain and did not show melanization. This could be interpreted as hyphae knots formed by growing mycelium in living root cells. Such a phenomenon has been observed in roots of durum wheat seven days after inoculation with two different mitosporic fungal strains (Abdellatif et al 2009). In such cells, the colonization pattern was irregular, while in parallel dead cells the hyphae grow regularly in a linear direction.

Comparing colonization patterns, the hyphae of most endophytes deviated from the direction along the root axes when they grew inside of cells. Only E63 and E133 still showed a regular linear growth even four weeks after co-cultivation. Abdellatif et al (2009) showed that differences in colonization pattern depend on the metabolic status of the root cells. While the mitosporic fungi presented a significant irregularity in hyphal direction, when they colonized living cells, growth of hyphae was linear along the root axes in dead plant cells. The regular colonization pattern of E63 and E133 in the current status correlated with the pathogenicity of the two fungi. They probably possess necrotrophic features which killed most of the root cells they colonized without any barriers, while all other endophytes were forced by the living cells to an irregular growth.

In summary, a number of root-endophytic fungi are presented in this study and taxonomically described, as well as characterized for their colonization ability of tomato roots. Some of them seem to be new species or new anamorphic stages of known teleomorphs, others are new isolates from already described plant-interacting fungi. The fact that fungal

isolates identified as closely related based on their ITS sequence possessed features different from the endophytes presented here, confirms that phylogenetic linkage does not necessarily indicate the same ecological function as has been shown many times before. In some cases, strains were identified as belonging to plant pathogenic species. These isolates could simply represent avirulent races or they might act as endophytes or pathogens depending on the host as it has been shown for example for *Colletotrichum acutatum* (Freeman et al 2001). Another possibility is a prolonged endophytic phase and that putative pathogenic features appear only at very late stages of the development as described for some Botryosphaeriaceae (Slippers and Wingfield 2007). This possibility has to be tested in future experiments along with the possibility to apply the new isolates in production systems to improve the growth of tomato plants, their resistance against pathogens or their tolerance for abiotic stressors. Up to now, only rarely indigenous fungal endophytes that inhabit tomato roots in commercial production systems were tested as biological agents (Kim et al 2007). Instead many endophytes have been studied which have been newly introduced in this ecological niche (Bargmann and Schönbeck 1992; Fakhro et al 2010). However, due to their colonization capacity and permanence in the plant, the newly characterized endophytes could posses an advantage over agents that mainly live in the surrounding or on the surface of roots (Hoitink and Boehm 1999; Narisawa et al 2002). These endophytes would be therefore much more effective to improve plant growth or to confer nonhabitat/habitat–adapted benefits to the plant host (Saikkonen et al 2004; Rodriguez et al 2009).

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# Chapter 3

## Screening of tomato endophytic fungi for potential biological agents

# Screening of tomato endophytic fungi for potential biological agents

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## Abstract

Tomato plants from four different sites in Colombia were used for the isolation of fungal endophytes. In total 51 isolates were obtained from roots and further characterised. Growth on different agar media and classification according to their morphology revealed that 20 isolates belong to *Fusarium* species, whereas 17 isolates were difficult to cultivate. The effect of the remaining 14 isolates on growth characteristics of tomato plants was analysed and root colonisation ability was controlled by reisolation and by microscopical observation. Those isolates not showing obvious endophytic growth or which had negative effects on at least one of the growth parameters were omitted. This procedure resulted in the selection of three fungal isolates which could be used as potential biological agents in tomato crops.

**Key words**: fungal root endophytes, tomato

- All experiments were carried out by me under supervision of the co-authors.

Supplemental results (Appendix 6)

- The corresponding manuscript is in press in the IOBC Bulletin.

## Introduction

Endophytes are microorganisms able to colonize internally plant tissues without causing visible disease symptoms for at least part of their life cycle (Kogel et al. 2006). Among the endophytes, mycorrhizal fungi are known since a long time (Smith & Read 2008), but numerous non-mycorrhizal endophytes can be found inside plant tissues in a wide diversity of habitats (Saikkkonen et al. 1998). Fungal endophytes are mainly divided into two groups (Petrini 1996). The "clavicipitaceous system" (Ascomycota, Clavicipitaceae) contains fungi, which colonise the leaves of grasses and often confer resistance to herbivores. Among the second group, dark septate endophytes (DSE) are commonly found in roots from alpine and arctic habitats in the northern temperate region (Mandyam & Jumpponen 2005), but little is known about fungi which symptomless colonise roots of agri- and horticultural crops.

Tomato (*Solanum lycopersicum* L.) is the most commonly grown fresh market vegetable world wide (http://faostat.fao.org), but the producers are confronted with great losses in the production caused by plant pathogens. High fungicide input can control these pathogens, but leads to the development of resistant strains (e.g. Chapin et al. 2006). Another possibility is by means of using tomato resistant cultivars, but the pathogens overcame these resistances (Parlevliet 2002) and resistances against soil-borne pathogens do only rarely exist. An alternative is the use of microbial biocontrol agents that have been shown to be able to suppress diseases caused by such soil-borne pathogens (Kloepper et al. 1999). The success of their application however requires that these agents are able to prevail against the other microorganisms in the hostile environment of the soil. Due to their permanence in the plant, endophytes could posses an advantage over agents that mainly live in the surrounding soil or direct on root surfaces and would be therefore much more effective (Hoitink and Boehm 1999). Based on these considerations, the present study was aimed to increase the spectrum of tomato root endophytes, which could be used in future as biocontrol agents against tomato root pathogens.

## Materials and methods

### *Isolation of fungal endophytes*

Roots from tomato plants without disease symptoms of three commercial managed and one experimental organic crop production site in Colombia were intensively washed with tap

water and disinfected with 70% ethanol (1 min) and 5% sodium hypochlorite (7-10 min). One cm fragments were cut and shortly pressed on potato dextrose agar (PDA; Oxoid, Lenexa, USA) as control for the presence of epiphytic microorganisms. Subsequently, the same fragments were incubated on PDA, Sabouraud dextrose agar (Oxoid) and 2% malt extract agar (Oxoid) containing 500 mg/l chloramphenicole. Single isolates growing from the root fragment were transferred to new plates and incubated at 21°C in the dark. After ensuring their purity, the isolates were macro- and microscopically characterized and kept for conservation in sterile oil at 4°C.

***Inoculation of tomato roots***

Fungal isolates were grown in liquid complete medium (Pontecorvo et al. 1953) for three to four weeks at 28°C under moderate speed agitation (100 rpm). The cultures were mixed by a blender (Model D72, Moulinex, Leipzig, Germany) for 15 sec at minimal speed. The number of propagules was estimated by counting in a Thoma chamber and their viability was checked by plating on PDA. The suspensions were adjusted to a concentration of 5 x $10^5$ cfu/ml with sterile water containing 0.025% Tween 20 (Merck, Darmstadt, Germany).

Disinfected tomato seeds were germinated on 0.8% water agar for five days at 24°C and seedlings were subsequently transplanted into pots containing humid sterilised sand (1:1 of EN12620:EN1339; Euroquarz, Dorsten, Germany). These pot cultures were placed in a green house (26±3°C, 60-70% relative humidity) for eight days and watered twice during this period with 40 ml of nutrient solution (De Kreij et al. 1997). Eight tomato seedlings per treatment were inoculated by root dipping in the fungal suspension for three hours and transplanted into 580 ml pots with a sterilised 1:1 mixture of substrate (Fruhstorfer Erde Typ P; Archut, Vechta, Germany) and the sand mixture mentioned above. The substrate was mixed with an endophytic suspension of $10^5$ cfu/gr before transplanting. After five weeks under conditions mentioned above, two plants per treatment were harvested, thirty root fragments from each root were stained with trypan blue and acid fuchsine and microscopically evaluated. In addition, root fragments were disinfected for two to three minutes with 0.5% sodium hypochlorite and placed on PDA and water agar (1.2% w/v). Outgrowth and morphology of fungal endophytes was controlled under the microscope. After 10 weeks of greenhouse cultivation, the remaining six plants per treatment were harvested to evaluate plant growth parameters (shoot and root fresh and dry weight, leaf and bloom number). Leaf areas were scanned with a LI-3100 Area Meter (LI-COR, Bad Homburg, Germany).

## Results and discussion

### *Isolation and selection of fungal endophytes*

A collection of 51 isolates from roots of tomato plants from Colombia were screened for putative biological agents. Seventeen out of 51 isolates showed no or only limited *in vitro* growth suggesting that they might be at least partially dependent on the plant root. They were omitted, because they would not be very useful for a survey aimed at inoculum production. The morphological screen of the remaining root isolates revealed that 20 belonged to the genus *Fusarium*. This could either indicate that this species dominates the inside tissues of tomato roots or that these fungi overgrow others during the isolation procedure. Different species of this genus have been reported as effective biocontrol agents against various fungal pathogens like the intensively studied strain FO47 (Paulitz & Belanger 2001). Nevertheless isolates of the *Fusarium* species were omitted due to their potential pathogenicity against different types of organisms. Seven isolates were classified as dark septate endophytes (DSE) or sterile mycelia, because they produced on the agar plates brown hyphae but no spores or other reproductive structures under the conditions used. These together with seven other morphotypes producing spores or conidia were further analyzed.

### *Colonisation and impact on plant development*

Tomato seedlings were inoculated with the 14 different endophytes and grown in pot cultures in the greenhouse. Endophytic growth was controlled after five weeks, while different plant growth parameters were evaluated after ten weeks (Tab. 1). Among the 14 isolates, E22, E52 and E133 could not be recovered from roots on agar plates and endophytic growth was observed by microscopy only for seven isolates (E9, E36, E48, E49, E63, E131, E135). Significant negative effects on plant development were detected in the treatment with the isolates E9, E22, E27, E63 and E131. These results could be principally reproduced in a second experiment under slightly different conditions (data not shown). Three of the fungal isolates (E48, E49 and E135) were selected for further analysis because they clearly colonised the roots of tomato and because they never showed a negative effect on any of the plant growth parameters. These three fungi were morphological grouped to the DSE which could be confirmed by sequence analysis (data not shown). DSE fungi produce melanised septated hyphae and microsclerotia in the roots (Mandyam & Jumpponen 2005). This morphological feature could be observed in the roots inoculated with the isolates E48, but was difficult for the isolates E49 and E135. This was probably a consequence of the poor chitinisation of their

fungal wall (Mandyam & Jumpponen, 2005), which would result in an underestimation of the true presence of this type of endophytes in plant tissues. Most of the described DSE isolates are from artic, alpine, antartic and temperate habitats (Mandyam & Jumpponen, 2005), while nothing is known about them in tropical ecosystems and none of those was obtained from tomato roots. Studies about their function revealed that they can influence plant growth both positively and negatively (Mandyam & Jumpponen 2005). Future analysis will show, if the three new DSE could be used as biological agents by analysing their capacity to protect the plants against pathogens and/or increasing the yield and the quality of tomato fruits.

Table 1. Interaction of fungal isolates and tomato plants in two pot culture experiments.

| isolate | endophytic growth | | effect on growth parameters[b] | | | | |
|---|---|---|---|---|---|---|---|
| | isolation from roots | microscopy | shoot FW/DW | root FW | leaf N° | leaf area | florescence N° |
| E9 | yes | yes | ns/- | - | - | ns | - |
| E20 | yes | ?[a] | ns/ns | ns | ns | ns | ns |
| E22 | no | ? | ns/- | ns | ns | ns | - |
| E27 | yes | ? | -/- | - | ns | ns | - |
| E36 | yes | yes | ns/ns | ns | ns | ns | ns |
| E41 | yes | ? | ns/ns | ns | ns | ns | ns |
| E48 | yes | yes | ns/ns | ns | ns | ns | ns |
| E49 | yes | yes | ns/ns | ns | ns | ns | ns |
| E52 | no | ? | ns/ns | ns | ns | ns | ns |
| E63 | yes | yes | -/- | ns | ns | - | ns |
| E67 | yes | ? | ns/ns | ns | ns | ns | ns |
| E131 | yes | yes | -/- | ns | ns | ns | ns |
| E133 | no | no | ns/ns | ns | ns | ns | ns |
| E135 | yes | yes | ns/ns | ns | ns | ns | ns |

a If hyphae were thin and spores not formed, endophytes were difficult to observe (?).
b Significant positive (+) or negative (-) effects on growth parameters were monitored (ns = no significant difference; one-way ANOVA; $P < 0.05$; $n = 6$)

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# Chapter 4

# Effects of dark septate endophytes on tomato plant performance

## Effects of dark septate endophytes on tomato plant performance

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**Abstract** Non-mycorrhizal fungal root endophytes can be found in all natural and cultivated ecosystems, but little is known about their impact on plant performance. The impact of three mitosporic dark septate endophytes (DSE48, DSE49) and *Leptodontidium orchidicola* previously isolated from tomato roots on plant characteristics were studied in more detail. Their effect on tomato root and shoot growth, their influence on fruit yield and fruit quality parameters and their ability to diminish the impact of the pathogen *Verticillium dahliae* were investigated. While shoot biomass of young plants was enhanced by the endophyte DSE49 and *L. orchidicola*, vegetative growth parameters of old plants were not affected except a reproducible increase of root diameter by the isolate DSE49. Concerning fruit yield and quality, *L. orchidicola* increased biomass and glucose content of tomatoes, but this was dependent on date of harvest and on root colonization density. Additionally, the endophytes DSE49 and *L. orchidicola* slightly decreased the negative effect of *V. dahliae* on tomato, but only at a low dosage of the pathogen. The mitosporic DSE48 showed no reproducible effect on any of the parameters studied. This indicates that the three dark septate endophytes have a significant impact on tomato characters under horticultural practice conditions, but that this is negligible concerning the production of marketable fruits.

**Keywords** Dark septate endophytes (DSE); Fruit quality; *Leptodontidium orchidicola*; *Solanum lycopersicum* L.; *Verticillium dahliae*

- All experiments were carried out by me under supervision of the co-authors. This work was submitted to the Journal Mycorrhiza. See supplemental material (Appendices 7 – 9).

## Introduction

Tomato (*Solanum lycopersicum* L.) is the most commonly grown fresh market vegetable world wide with about 130 million tons produced in 2008 (htpp://faostat.fao.org). Cultivation of tomato has been increasing through the five continents because of the variety of cultivars with optimum growth to different conditions and for the properties of its edible fruit which is an important source of carotenoids, flavonoids, vitamins and minerals (Minoggio et al. 2003; Guil-Guerrero and Rebolloso-Fuentes 2009). However, pathogens like *Fusarium oxysporum*, *Verticillium dahliae* (Kleb.) or *t*he Oomycete *Phytophthora infestans* (Brayford 1996; Shattock 2002; Fradin and Thomma 2006) are responsible for economically important crop losses especially in field grown tomato as for example in Colombia (Salgado et al. 2008). The pathogens are mainly controlled by application of fungicides, but the high input of fungicides has negative effects on the environment (Soares and Porto 2009) and leads to resistance of the pathogens (Stammler et al. 2006). Also the increasing demand of consumers for food with less pesticide residue contamination stimulates the use of alternative control methods. The use of tomato resistant cultivars is the best method in controlling such diseases but the occurrence of new races of pathogens has overcome the resistance (e.g. Parlevliet 2002). This circumstance has stimulated the research in alternatives to combat fungal pathogens by biocontrol agents (Whipps 2001). A number of studies have already demonstrated that the use of biocontrol agents suppressed diseases caused by soil-borne pathogens (e.g. Alabouvette et al. 2009). However, less biocontrol candidates are available for using in the field due to lack in effectiveness compared to previously shown suppression effect under controlled conditions (Ojiambo and Scherm 2006). The complex interaction in the rhizosphere could be one reason affecting the success of biological agents, because they are not able to compete efficiently with other microorganisms in this hostile environment (Conn and Franco 2004). Due to their colonization capacity and permanence in the plant, indigenous endophytes, which live within host-plant tissues without causing any visible symptoms of disease (Wilson 1995) could posses an advantage over introduced agents and would be therefore much more effective (Hoitink and Boehm 1999; Conn and Franco 2004). Concerning tomato, non-pathogenic strains of *Fusarium oxysporum* were isolated from sterilized tomato roots (Hallmann and Sikora 1994). One of these strains produced secondary

metabolites that were highly toxic to the nematode *Meloidogyne incognita*, a second one reduced significantly *in vitro* the growth of *Phytophthora cactorum, Pythium ultimum* and *Rhizoctonia solani* (Hallmann and Sikora 1996).

Root fungal endophytes form symbiotic associations with a very wide range of plant species showing a high phylogenetic diversity and possess multiple functions (Rodriguez et al. 2009). The plant - fungus genotype and physiology combination determine the outcome of this symbioses varying from parasitism to mutualism (Redman et al. 2001; Schulz and Boyle 2005; Kogel et al. 2006). Despite variable and complex interactions, fungal endophytes have been related to plant growth, fitness and stress responses. This has been shown many times and in a broad variation of plant-fungal combinations for the mycorrhizal fungi belonging to different taxonomic phyla (Smith and Read 2008). Another interesting group is the order *Sebacinales* (phylum Basidiomycota) which interact with the roots of a broad spectrum of plants forming mycorrhizal and non-mycorrhizal interactions (Weiss et al. 2004; Deshmukh et al. 2006; Selosse et al. 2009). *Piriformospora indica*, which serves as model for the non-mycorrhizal interactions of the Sebacinales, is able to colonize roots of many plants (Varma et al. 1999) and also interacts with tomato (Fakhro et al. 2010). Among the Ascomycota, *Trichoderma* species have been extensively studied. They colonise root surfaces, penetrate the first cell layers and are used as opportunistic plant symbionts for disease control and yield enhancement (Harman et al. 2004). Another group among the ascomycetous fungi interacting with plant roots are the dark septate endophytes (DSE). Mostly, they showed no impact (Jumpponen 2001), but some investigations revealed positive effects on plant growth and yield (Shivanna et al. 1994; Wu and Guo 2008) and on nutrient uptake (Jumpponen et al. 1998; Upson et al. 2009). Recently, it was shown for one DSE that it enhanced not only root development and plant biomass of a Chinese medicinal plant, but also increased the level of the flavonoid rutin (Wu et al. 2010).

In order to enlarge the number of indigenous putative biological agents, 51 isolates from tomato roots were screened and 14 were analysed in more detail concerning their taxonomic position and their *in vitro* colonization ability (Andrade-Linares et al. 2010a; unpublished). Based on pot culture experiments under green house conditions (Andrade-Linares et al. in press) three dark septate endophytes (DSEs) were selected for further studies. In the present study, the effects of the

unknown DSE48 and DSE49 and of DSE135 identified as *Leptodontidium orchidicola* were further evaluated. Six experiments were carried out to analyse (1) their impact on vegetative tomato growth characters in young and older plants, (2) their capacity to protect tomatoes against the pathogen *Verticillium dahliae* in order to proof their suitability to be used as biological agents and, (3) their influence on the yield of tomato fruits and their quality concerning taste related compounds such as sugars and titratable acids as well as health promoting carotenoids.

## Materials and methods

*Experimental design*

To investigate the disease suppression effect of the three endophytes four experiments (A-D; Table 1) with tomato plants (cv. Hildares F1, Hild Samen GmbH, Marbach, Germany) artificial infested with *V. dahliae* (see below) were carried in the greenhouse in Großbeeren (52°N, 13°E). Experiment A and B were also used to show the impact of the endophytes on vegetative growth of young plants. Tomato seeds were disinfected with 70% ethanol for 5 min and subsequently two times with 3.5% NaOCl for 15 min. After each disinfection step the seeds were washed with sterile water. Seeds were pre-germinated on 0.8% water agar at 24°C to select homogenous and not contaminated plants for the experiments. The tomato seedlings were transplanted into pots (1 L) at 1-2 leave stage and inoculated with the endophytes (see below). The pots were filled with sterilised substrate (Fruhstorfer Erde Typ P; Archut, Vechta, Germany; chemical analysis (mg per 100 g): N=75, P= 75, K= 125; pH 5.9) in experiments A and B or with a sterilised mixture of this substrate and quartz sand (EN12620:EN1339; Euroquarz, Dorsten, Germany) at a 1:1 ratio (v/v) in experiment C and D. The plants were further cultivated under greenhouse conditions (Table 1). Each treatment consisted of three replicates with six plants each (experiment A and B) or three replicates with four plants each (experiment C and D) arranged in a randomized block design. The pots were watered daily to maintain the substrate moisture and twice a week with 40 to 200 ml nutrient solution (De Kreij et al. 1997; EC = 2 dS $m^{-1}$; pH 5.5) dependent on the growth stage. Shoot fresh and dry weights of tomato plants were measured and dry matter contents were calculated after a cultivation time of six (experiment A and B) or seven weeks (experiment C and D). Disease severity caused by *V. dahlia* was assessed (experiment A-D) on the following scale according to Morgan et al. (1992): 0 = no symptoms, 1 = slight

yellowing of leaf, stunting or wilting, 2 = moderate yellowing of leaf, stunting or wilting, 3 = severe yellowing of leaf, stunting or wilting and 4 = leaf death at harvest.

Two experiments (E and F) were carried out under controlled conditions in a climate chamber (York, Mannheim, Germany; 23/20°C, 60/70 % relative humidity day /night, 16 h light h 33.7 $Mol*m^{-2}*d^{-1}$) to assess the impact of the endophytes on vegetative growth of older plants and on fruit yield and quality. Seeds were prepared as mentioned above for experiment A-D, but pre-germination were carried out first in liquid MS medium (Murashige and Skoog 1962) and after seven days in plant nutrition medium (PNM; Shahollari et al. 2007) at 23°C with a photoperiod 16/8 h (day/night) to improve the root growth and finally the endophyte root colonization. Seedlings were transplanted into pots (1.2 L) at 2-3 leaf stage. The pots were filled with a substrate sand mixture inoculated with the endophytes (see below). Each treatment in each experiment included 6 plants. Watering of plants and the nutrient application were done as described above. The plants were cultivated in the climate chamber until flowering and then transferred to the greenhouse (Table 1). Fresh and dry weights of shoots and roots were measured and dry matter content were calculated 22 weeks after endophyte inoculation. Additionally, root length and diameter were estimated with a root scanner (Epson Perfection *V700* Photo).

*Preparing of the endophyte inoculum and plant inoculation*

Each dark septate endophyte isolate was grown in a 300-ml Erlenmeyer flask with 150 ml liquid medium (Pontecorvo et al. 1953) for three to four weeks at 25°C and 100 rpm agitation. Fresh mycelium was filtered and washed with sterile distilled water until the liquid became clear. The fresh mycelium was weighted and part of it was mixed with sterile tap water by a blender (Model D72, Moulinex, Leipzig, Germany) for 1 minute at minimal speed.

Number of propagules of the endophytes was estimated by Thoma chamber and their viability was checked by plating on PDA (VWR, Berlin, Germany). For inoculation, the suspensions were adjusted with sterile tap water to a concentration corresponding to each experiment. For Experiments A-D, tomato plants were treated with the endophytes by root dipping ($5 \times 10^5$ cfu/ml) at 1-2 leaf stage before planting. The substrate was also drenched with fresh mycelium suspension of the endophytes (1% w/v).

For Experiment E and F, the pots were filled with a substrate sand mixture

inoculated with fresh mycelium suspension of the endophytes (1% w/v). Control plants were mock-inoculated without fungal mycelium.

*Inoculation of the pathogen V. dahliae*

The isolate *Verticillium dahliae* (accession number GU060637, kindly provided by Valerie Grimault, GEVES, Angers, France) was grown in 150 ml sucrose sodium nitrate (SSN) liquid medium (Sinha and Wood 1968) at 28°C and 100 rpm for one week, transferred to 200 ml fresh medium and further incubated for two weeks. The culture was thereafter mixed by a blender (Model D72, Moulinex, Leipzig, Germany) for 40 seconds at minimal speed. Number of conidia was estimated by Thoma chamber and their viability was checked by plating on PDA (VWR, Berlin, Germany). Tomato plants were inoculated at 3-4 leaf stage two weeks after endophyte treatment with the pathogen by drenching with 30 ml conidia suspension ($2x10^5$ conidia mL-1 in experiment A and B or $7x10^6$ conidia $mL^{-1}$ in experiment C and D).

*Effect on fruit yield and quality*

In experiment E and F, fruit harvest was started 15 weeks after inoculation and was carried out twice a week for 7 weeks. Numbers of red-ripe fruits, fresh and dry weights were monitored and results were summarised in three groups (week 15-16; week 17-18; week 19-21). For quality analysis, red-ripe tomatoes harvested 19 and 21 weeks after inoculation from plants of experiments E and F were manually sorted, mixed and marketable fruits were separated from blossom-end rot- fruits according to the CBT-scale (Anonymous, 1992). Fruits of the colour stage 10-11 were selected for quality analysis carrying out a double estimation with a mixture of 12 fruits from approximately 10 different plants per treatment (four treatments with each six plants x 2 experiments). Carotenoids were analysed according to Krumbein et al. (2006). Briefly, 1 g calcium carbonate, 30 g sodium sulphate and 30 ml acetone were added to 15 g homogenised tomatoes and mixed for 2 minutes. The extract was filtered under suction and the solid materials were repeatedly extracted with acetone until the resulting filtrate was colourless. Carotenoid composition and content were determined by HPLC using a C-18 reversed-phase column (Lichosphere 100; 5 µm, 250 x 4 mm; Merck, Darmstadt, Germany) with an isocratic eluent of 75% acetonitrile, 15% methanol and 10% methylene chloride. In parallel 50 g of homogenised samples were frozen for acid and reduced sugar (glucose and fructose)

analyses. Titratable acid content was determined by potentiometric titration with 0.1 M NaOH, while sugars were enzymatically determined (Krumbein et al. 2004). All results were referred to 100 g fresh weight.

*Microscopy*

For confirmation of endophyte colonization, tomato root fragments were sampled 2 and 3 weeks after inoculation, stained with WGA-AF 488 (Molecular Probes, Karlsruhe, Germany) according to Andrade-Linares et al. (submitted). Three plants per treatment and experiment were randomly selected and samples from different parts of the root system were stained. 15 root fragments per sample were mounted on glass slides and analyzed with an Axioscop 2 Plus Microscope (Zeiss, Germany). WGA-AF 488 was excited with a 488-nm laser line and detected at 505–540. Quantification of the endophytic colonization was based on a method for arbuscular mycorrhizal fungi (Trouvelot et al. 1986) modified for the root endophyte *Piriformospora indica* (Bütehorn et al. 2000). Colonization was classified from 0 to 5 representing < 1%, between 1% and 10%, between 10% and 50%, between 50% and 90% and > 90% percent of fragment colonized by fungal hyphae. Infection frequency (F) and total colonization intensity (C) were calculated with F = Ne/Nt*100 (Ne: number of fragments colonized by the endophyte; Nt: total number of analysed root fragments), and $C = (95n_5 + 70n_4 + 30n_3 + 5n_2 + n_1)/Nt$ ($n_x$: fragment amount in infection class x).

*Statistical analyses*

The STATISTICA program version 6.0 (StatSoft Inc., Tulsa, Oklahoma, USA) was used for all analyses. Disease severity was non-parametrically analysed by Kruskal-Wallis test. Tomato growth parameters and fruit yield were analysed by one-way ANOVA and the Fisher's protected LSD ($P$ = 0.05). Data given as percentages (shoot weight losses after pathogen infection) were transformed to arcsin before one-way ANOVA with LSD-test ($P$ = 0.05).

**Results**

*The endophyte impact on vegetative plant growth*

Tomato plants of two parallel experiments (experiment A and B) were harvested six weeks after planting in order to determine the impact of the endophytes DSE48, DSE49 and *Leptodontidium orchidicola* on shoot growth of young tomato plants (Table 2). DSE48 increased shoot fresh

**Table 1** Conditions for tomato cultivation. Plants were grown and inoculated with endophytes (En) for analysing their impact on growth, on the interaction with the pathogen *Verticillium dahliae* (Vd) (A, B, C, D) or on vegetative growth of older plants and on fruit yield and quality (E, F).

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Temperature [day/night; $^{o}$C] | 23.9/18.0 | 24.8/18.5 | 23.8/19.2 | 23.8/19.2 | 22.5/18.4 | 22.5/18.4 |
| Humidity [day/night; %] | 63.3/82.4 | 54.4/69.3 | 64.6/79.2 | 64.6/79.2 | 70.8/73.1 | 70.8/73.1 |
| Mean daily radiation [Mol*m$^{-2}$*d$^{-1}$] | 40.3 | 28.9 | 32.2 | 32.2 | 34.2 | 34.2 |
| Treatments | En + Vd | En + Vd | En + Vd | En + Vd | En | En |

**Table 2** Impact of endophytes on young tomato plants. Plants from two experiments (A and B) were harvested six weeks after inoculation with the fungal endophytes DSE48, DSE49 and *Leptodontidium orchidicola* (*L.o.*). Average values of shoot fresh weights (FW) and dry matter contents (dmc%) ± standard deviations are shown. Values in bold denote significant differences between colonized and non-colonized control plants (one-way ANOVA according to LSD-test; $P = 0.05$).

| | Experiment A | | Experiment B | |
|---|---|---|---|---|
| Treatment | FW | dmc% | FW | dmc% |
| control | 63.4 ± 0.5 | 13.4±0.1 | 76.5 ± 1.4 | 11.4±0.1 |
| DSE48 | **73.7 ± 2.8** | 14.0±1.0 | 75.8 ± 1.3 | 11.6±0.6 |
| DSE49 | **76.7 ± 2.1** | **14.1±0.1** | **84.8 ± 0.1** | **12.2±0.2** |
| *L. o.* | 66.7 ± 1.7 | 13.5±0.4 | 78.8 ± 1.3 | **13.0±0.4** |

weight in one experiment, while *L. orchidicola*-colonised plants showed a higher dry matter content compared to control in the second experiment. The endophyte DSE49, however, significantly enhanced shoot fresh weight and dry matter content of young tomato plants in both experiments. In contrast, experiments E and F showed that the three endophytes affected neither shoot and root biomasses nor leaf numbers of older plants after 22 weeks of cultivation (Table 3). Only inoculation with DSE48 resulted in lower root lengths in experiment E, but this effect on the root was not evident in experiment F. Differences in both experiments were detected for DSE49. Colonized plants had roots with larger diameter than those from the controls.

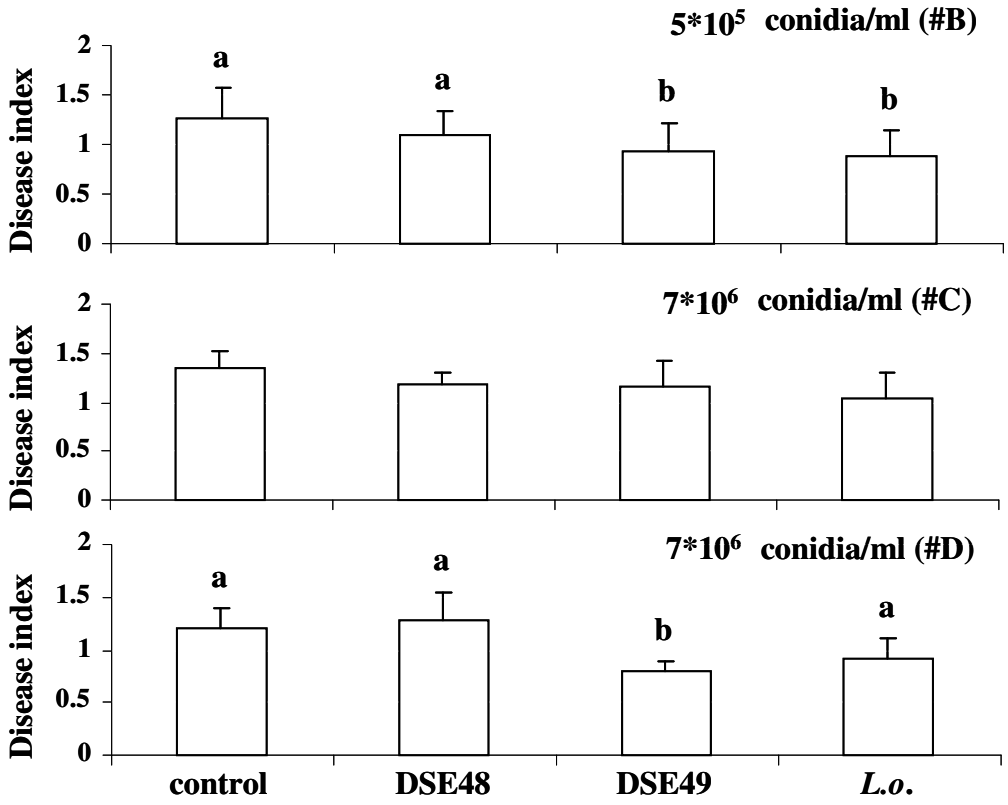


**Fig. 1** Bioprotective effects of fungal endophytes. Disease indices of leaf symptoms caused by *Verticillium dahliae* infection in tomato control plants and plants colonized by the endophytes DSE48, DSE49 or *Leptodontidium orchidicola* were estimated based on a 0 - 4 scale 4 weeks after inoculation with 30 ml of a $2x10^5$ conidia $mL^{-1}$-pathogen suspension (experiment B) or 5 weeks after inoculation with 30 ml of a $7x10^6$ conidia $mL^{-1}$-pathogen suspension (experiment C and D). Significant differences of the endophyte-colonized plants to the respective controls are indicated by different letters above the columns. Statistical comparisons between treatments were performed by Kruskal-Wallis test ($P$ = 0.05). No disease symptoms were observed in experiment A.

*The endophyte impact on plant-pathogen interaction*

In order to analyse if DSE48, DSE49 and *L. orchidicola* could potentially be used as biocontrol agents, plants colonized or not by the three endophytes for two weeks were infected in four experiments with two concentrations ($2x10^5$ and $7x10^6$ conidia $mL^{-1}$) of the fungal pathogen *Verticillium dahliae*. Four or five weeks after pathogen inoculation, fresh and dry weights of shoots were evaluated, weight losses calculated (Table 4) and disease severity was assessed (Fig. 1). In experiment A, no disease symptoms could be observed and the pathogen showed no effect on plant growth within cultivation time. In experiment B, however, symptoms were obvious and weight losses were above 20% in control plants. Plants colonized by

**Table 3** Impact of endophytes after 22 weeks of cultivation. Averages values ± standard deviations of vegetative growth parameters from tomato plants colonised with the fungal endophytes DSE48, DSE49 and *Leptodontidium orchidicola* (*L.o.*) are shown. Statistical comparisons between colonised and non-colonised plants were carried out by one-way ANOVA according to LSD-test ($P$ = 0.05). Values in bold denote significant differences between colonized and non-colonized control plants.

| | | Shoot | | Root | | | Root length | Root diameter | |
|---|---|---|---|---|---|---|---|---|---|
| | | FW[a] [g/plant] | dmc[b] [%] | FW [g/p] | dmc [%] | Shoot/Root FW ratio | [cm/g FW] | [mm] | Leaf N° |
| E | control | 1733.3 ±101.6 | 12.69 ±1.23 | 96.3 ±19.7 | 8.47 ±0.84 | 18.7 ±3.54 | 340.7 ±28.4 | 0.26 ±0.01 | 43 ±0.9 |
| | DSE48 | 1654.8 ±202.4 | 12.30 ±0.72 | 108.2 ±28.5 | 8.10 ±1.11 | 16.3 ±4.12 | **270.5 ±55.8** | 0.27 ±0.02 | 44 ±2.9 |
| | DSE49 | 1634.7 ±53.7 | 12.62 ±1.05 | 82.4 ±14.9 | 9.82 ±1.64 | 20.6 ±4.39 | 370.9 ±43.7 | **0.30 ±0.01** | 44 ±2.3 |
| | *L.o.* | 1564.1 ±137.3 | 12.81 ±0.94 | 86.1 ±9.7 | 8.32 ±0.73 | 18.4 ±2.39 | 403.0 ±69.9 | 0.26 ±0.02 | 44 ±1.4 |
| F | control | 1669.7 ±69.8 | 14.18 ±2.20 | 61.8 ±8.5 | 13.80 ±1.43 | 27.6 ±4.43 | 316.7 ±39.0 | 0.29 ±0.03 | 45 ±3.0 |
| | DSE48 | 1576.1 ±159.6 | 13.17 ±1.79 | 63.2 ±9.7 | 12.44 ±0.90 | 25.4 ±3.71 | 344.9 ±59.2 | 0.30 ±0.05 | 41 ±4.1 |
| | DSE49 | 1695.3 ±169.2 | 13.73 ±1.70 | 63.8 ±9.8 | 15.28 ±2.45 | 27.5 ±6.34 | 305.9 ±14.9 | **0.35 ±0.02** | 43 ±4.6 |
| | *L.o.* | 1635.1 ±180.9 | 13.42 ±1.19 | 56.6 ±5.7 | 13.77 ±1.95 | 29.0 ±3.1 | 369.3 ±81.7 | 0.30 ±0.03 | 45 ±1.7 |

[a] fresh weight, [b] dry matter content.

DSE48 behave similar to controls. In contrast, leaves of plants colonized by DSE49 or by *L. orchidicola* clearly showed less disease symptoms (Fig. 1) and weight losses were significantly reduced (Table 4). In the experiments C and D with the higher pathogen dosage, disease index in controls was similar as before, but weight losses were even higher (between 25 and 30%). Reduction of the symptoms and the weight losses were again observable in plants pre-inoculated with DSE49 and *L. orchidicola*, but differences were only significant for the decrease in the disease index with DSE49 in one experiment (Fig. 1, experiment D).

*The endophyte impact on fruit yield and quality*

Fruits from plants inoculated with DSE48, DSE49 and *L. orchidicola* were harvested between 15 and 21 weeks after inoculation

(6 x 10 plants per treatment), grouped and three groups were analysed concerning number, total FW and DW of tomatoes (Fig. 2). In one experiment, fruit number from plants colonized by *L. orchidicola* was significantly higher than those from controls during the first harvest period and biomasses (FW and DW) consequently were also greater (harvest I; Fig. 2E). Any significant differences were not evident for the other endophytes and also not for the later two harvest periods. This increase could, however, not be observed in the second experiment, where numbers and biomasses were similar in all treatments at the three harvest periods (Fig. 2F). If marketable fruits were analysed separated from fruits with blossom-end rot, results were similar (data not shown) indicating that none of the endophytes had any influence on this type of disease. In order to find a reason for the difference between the two experiments, root colonization by the endophyte *L. orchidicola* was quantified. This showed no significant difference between the experiments concerning the infection frequency (68% and 44%) but total colonization intensity was significant higher in root of experiment E (16.7% and 3.4%).

Fruit quality analysis was carried out with mixed samples of red-ripe tomatoes from two dates of the harvest (12 fruits from 10 plants per treatment). Amount of titratable acids, reducing sugars and carotenoids (lycopene and ß-carotene) were measured (Table 5). Significant differences, however, could not be detected among the treatments respect to the control plants, except at the first harvest, where glucose levels were higher in fruits from

**Table 4 Weight loss percentages of tomato plants after *Verticillium dahliae* infection.** Two weeks after inoculation with the endophytic fungi DSE 48, DSE 49 and *Leptodontidium orchidicola* (*L.o.*), plants were infected with 30 ml of a $2x10^5$ or $7x10^6$ conidia $mL^{-1}$ pathogen suspension. Fresh weights were measured and fresh weight losses were calculated as the ratio of the values from pathogen-infected to non-infected plants. Statistical comparisons between treatments were performed by one-way ANOVA according to LSD-test ($P = 0.05$). Values in bold denote statistically significant differences between endophyte-colonized and non-colonized plants (control).

| | $2x10^5$ conidia $mL^{-1}$ ($n = 18$) | | $7x10^6$ conidia $mL^{-1}$ ($n = 12$) | |
|---|---|---|---|---|
| | A | B | C | D |
| | Fresh weight loss percentages | | | |
| control | 9.6 | 22.2 | 30.2 | 25.1 |
| DSE48 | 11.0 | 23.4 | 29.0 | 22.5 |
| DSE49 | 12.7 | **14.4** | 25.1 | 19.1 |
| *L.o.* | 7.9 | **4.5** | 28.9 | 17.1 |

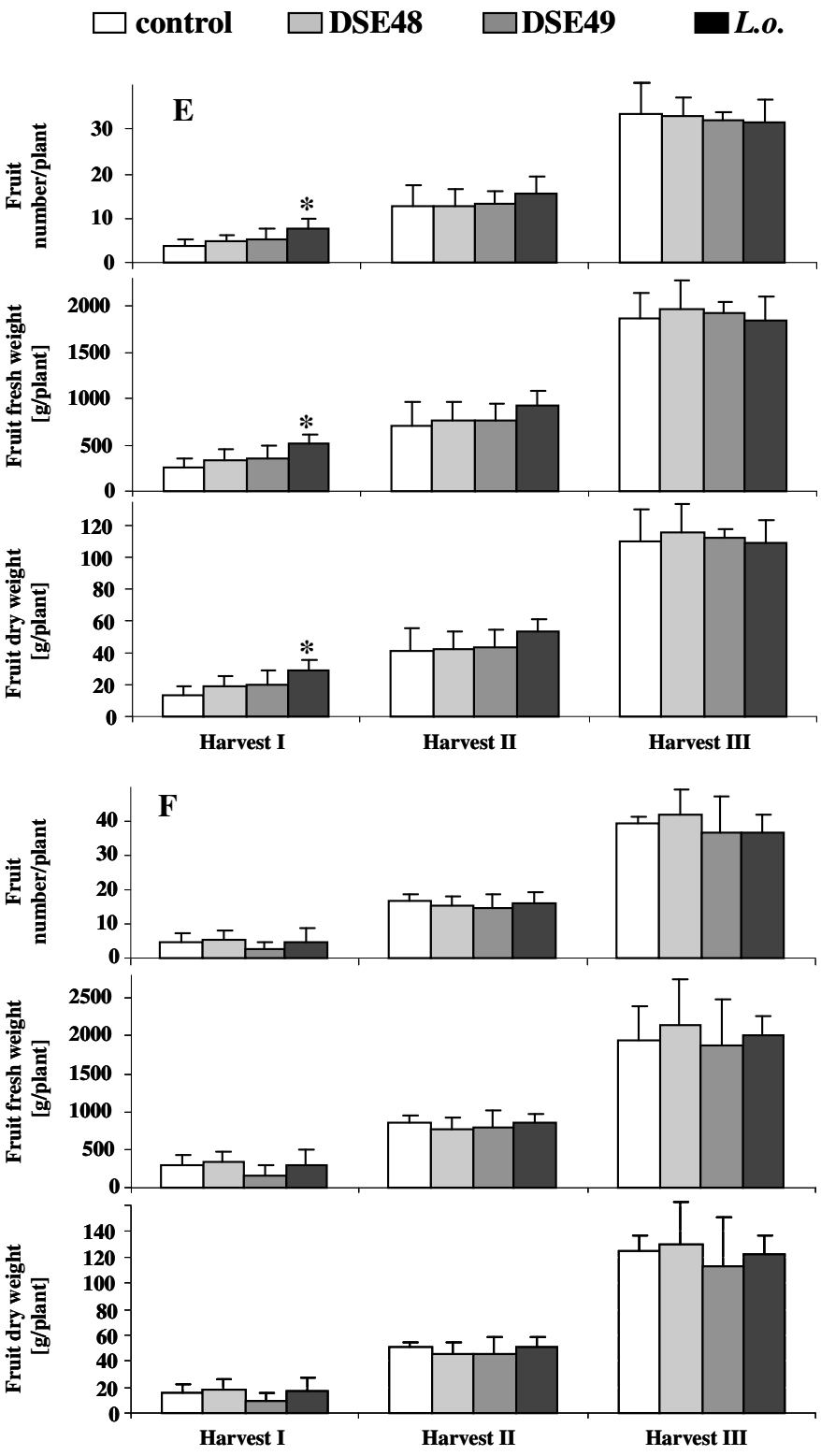


**Fig. 2** Influence of the endophytes on yield. Fruit number, fresh and dry weights and marketable fruit dry weight per plant were estimated in two parallel experiments (E and F). Harvests were carried out between week 15 and week 22 after endophyte inoculation and fruits were grouped into I (week 1-2), II (week 3-4) and III (5-7). Significant differences of endophyte-colonized plants to the respective controls are indicated by asterisks above the columns. Statistical comparisons between treatments were performed by one-way ANOVA according to LSD-test ($P = 0.05$).

**Table 5 Metabolite contents in tomato fruits.** Marketable fruits with the same red intensity were sampled from plants 19 and 21 weeks after inoculation with the fungal endophytes DSE48, DSE49 and *Leptodontidium orchidicola* (*L.o.*) and analysed. Averages values are shown with their ± standard errors. Statistical comparisons between treatments were performed by one-way ANOVA according to LSD-test ($P$ = 0.05). Value in bold denotes significant differences between the colonized and non-colonized control plants (C).

| | 19 weeks after inoculation | | | | 21 weeks after inoculation | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolites in 100 g FW | C | DSE48 | DSE49 | *L.o.* | C | DSE48 | DSE49 | *L.o.* |
| Acids [mg] | 386 ±22.6 | 395.3 ±7.41 | 415.1 ±11.84 | 398.7 ±8.62 | 368.2 ±0.70 | 360.7 ±10.35 | 372.8 ±2.06 | 349.8 ±13.97 |
| Glucose [g] | 1.07 ±0.01 | 1.13 ±0.038 | 1.15 ±0.004 | **1.26 ±0.044** | 1.57 ±0.035 | 1.52 ±0.053 | 1.55 ±0.043 | 1.6 ±0.081 |
| Fructose [g] | 1.22 ±0.041 | 1.34 ±0.043 | 1.33 ±0.012 | 1.39 ±0.077 | 1.75 ±0.007 | 1.71 ±0.018 | 1.73 ±0.038 | 1.78 ±0.068 |
| Lycopene [mg] | 5.5 ±0.295 | 5.34 ±0.016 | 4.79 ±0.025 | 4.66 ±0.196 | 7.62 ±0.054 | 6.57 ±0.519 | 7.34 ±0.406 | 6.60 ±0.152 |
| ß-Carotene [mg] | 0.32 ±0.04 | 0,31 ±0.042 | 0.36 ±0.017 | 0.32 ±0.000 | 0.30 ±0.025 | 0.27 ±0.023 | 0.29 ±0.035 | 0.27 ±0.003 |

plants colonized by *L. orchidicola* than in those from control plants.

**Discussion**

Dark septate endophytes (DSEs) are in some cases able to form mutualistic interactions with plants (Jumpponen 2001). We therefore analysed the impact of three newly isolated DSEs on their natural host tomato concerning (1) vegetative growth, (2) interaction with a pathogen and (3) fruit yield and quality.

When plants were harvested six weeks after inoculation, two of the dark septate endophyte showed a reproducible impact on shoot development of tomato plants. This effect, however, disappeared 22 week

weeks of cultivation. These differences in vegetative plant growth promotion were probably not a result of inoculation and growth conditions, but depend very likely more on plant growth stage of harvest. This indicates that the beneficial effect of the endophytes seems to be only transient up to the stage around inflorescence emergence (about 7 weeks) and can be compensated in controls at later times of development. Most studies on DSE – plant interactions could not show positive effects (Jumpponen 2001). Plant growth promotion could be, however, observed in some investigations, but either plants were

relatively young (Wu et al. 2010) and/or effects were dependent on the host (Fernando and Currah 1996) or on environmental conditions like elevated $CO_2$ concentrations (Alberton et al. 2010) or N fertilisation (Jumpponen et al. 1998; Upson et al. 2009). Another factor influencing the outcome of the interaction was the experimental design. Results were different under sterile conditions compared to those obtained in open pot cultures (reviewed in Jumpponen and Trappe 1998). No studies on the influence of DSE on plant performance at different stages have been conducted, but a similar phenomenon was observed for the endophyte *Piriformospora indica*, colonized barley plants showed significantly higher shoot lengths at 9 and 12 weeks after inoculation, but shoots were not taller at the end of the vegetation period (Achatz et al. 2010). Also experiments in tomato with *P. indica* showed that endophyte-inoculated plants increased shoot fresh weights 5 to 8 weeks after inoculation (Fakhro et al. 2010), but such differences disappeared at later harvests (Andrade-Linares et al. 2010b; in press).

Acceleration of early processes of plant development can be caused by several characteristics of root endophytic fungi. It has been shown that such organisms directly produce auxin-like substances (Sirrenberg et al. 2007; Vinale et al. 2008) or indirectly influence hormone signal transduction and hormone-regulated gene expression (Barazani et al. 2007; Vadassery et al. 2008; Schäfer et al. 2009). The growth of younger plants might be more sensitive for such changes and also the observed modifications in root architecture in the older plants could be caused by phytohormones. Root length was reduced in one experiment by DSE48 and root diameter enhanced in both experiments by DSE49 (Table 3). As auxin inhibitors increase root length and reduce root diameter (Zhao and Hasenstein 2009) it will be interesting to analyse if these DSEs also produce auxin-like substances like other fungal root endophytes do (see above).

Isolates of DSEs have been shown to reduce disease symptoms in Chinese cabbage and eggplant after challenge with *Verticillium* pathogens (Narisawa et al. 2002; 2004). It was therefore analysed in four experiments (A-D), if the three DSEs are able to reduce the symptoms in tomato caused by *Verticillium dahliae*. At low concentrations ($10^5$ conidia $mL^{-1}$) of the pathogen weight loss percentage and yellowing of the leaves were only observed in experiment B and two of the endophytes (DSE49 and *L. orchidicola*) were able to reduce these symptoms (Table 4 and Fig. 1). At higher pathogen concentrations ($10^7$

conidia $mL^{-1}$) symptoms were in the same range, but only DSE49 could reduce the disease index in one out of two experiments. These differences could be based on the variations in the conditions in the four experiments. Disease symptoms were developed when inoculum density was high (experiment C and D) or at reduced density only, if humidity was low enough (experiment B in Table 1). Stem resistance of water flow is increased in infected plants because of the vessels occluded by the pathogen and it is reflected in lower relative water content in wilted leaves (Pegg and Brady 2002). This phenomenon could be enhanced by the decreased humidity in experiment B, although inoculum density was also low (Table 1). Another factor could be the high radiation in experiment A compared to the other three experiments (Table 1). Leaf photosynthesis is greatly impaired by *V. dahliae* infection, with lower net assimilation rates (Pegg and Brady 2002) and at the high radiation in experiment A might had compensated this effect. If disease symptoms were detectable, reduction of these symptoms by the two DSEs was only clear in one out of three experiments. One might argue that this is due to the higher inoculum density in experiment C and D. The concentration of the pathogen in the plant must not be correlated with the severity of the symptoms (Veronese et al. 2003) but it could have affected the plant protection ability of DSE49 and *L. orchidicola*. Another variation between experiment B on the one site and C or D on the other is the use of pure substrate versus substrate mixed with sand. In spite of the same mineral nutrient solution in all experiments, the amount of organic compounds is higher in the pure substrate. This could be important for the plant-DSE interaction and DSE ability to induce resistance. DSEs can facilitate the uptake of organic nitrogen, phosphate, sulphur compounds and of carbon (reviewed in Mandyam and Jumpponen 2005) and positive growth promotion by different DSEs was only observed when plants were grown in substrate amended with organic nitrogen (Upson et al. 2009). It is interesting to note that plant growth promotion at early stages was much lower in experiment C and D than in A and B (data not shown) also indicating the role of the substrate for the functioning of the DSEs. The mechanism by which DSE49 and *L. orchidicola* protect the tomato plants and whether this involves plant tolerance or resistance remains to be studied. Antagonistic activity against *V. dahliae* was evaluated for these isolates, but no growth inhibition or mycoparasitism were observed (data not shown). This might indicate that the induction of plant

defence called priming (Conrath et al. 2006) could be the mechanism behind the observed phenomenon. Priming is based on the activity of particular plant hormones like jasmonate, ethylene or salicylic acid (Gutjahr and Paszkowski 2009; Shoresh et al. 2010). If the level of phytohormones is affected by the DSEs remains to be shown.

Two weeks after first flowering, fruit numbers were increased in experiment E by a factor of 3.5 in plants colonized by *L. orchidicola* and 1.75 and 2.2 for DSE48 and DSE49 inoculated plants. All these differences could not be observed at later dates of harvest (Fig. 2). As already discussed for the vegetative growth, it seems that inoculated plants reach earlier the generative phase of development and therefore show higher numbers of flowers and fruits in the beginning. At later phases control plants catch up and develop at the end similar numbers of generative organs. This phenomenon seemed to be related to the intensity of endophytic colonization which was lower in experiment F where no effect on fruit biomasses was observable. Low temperature of (18°C/15°C day/night) at the beginning of the experiment was monitored in the first five weeks after inoculation in the climate chamber for experiment F. This might had directly affected fungal growth rates or indirectly the colonization process due to influences on plant physiology at low temperatures mirrored by the increased dry matter of the plants compared to those in experiment E (Table 3). Other differences of plants growing at lower temperature are higher starch accumulation and lower rate of net photosynthesis (Venema et al. 1999) or increased abcisic acid contents (Daie and Campbell 1981) which all might influence the colonization capacity of the fungal endophytes.

Quality analyses were carried out with fruits mixed from both experiments. This mode of sampling was possible, because deviations among the values obtained were low (Table 5). Measuring the contents of titratable acids, reducing sugars and carotenoids (lycopene and ß-carotene) showed only one significant effect by endophytic colonization: glucose concentrations were increased in tomatoes from plants where the roots were colonized by *L. orchidicola* (Table 5). As influence on vegetative growth and on the development of generative organs, this could be however only observed at the early date. Glucose amounts depend among others on enzymatic activity of the acid invertase (e.g. Johnson et al. 1998) and this could be also regulated by phytohormones. High levels of gibberellic acid, auxins and abscisic acid induce the expression of the corresponding genes (Roitsch et al. 2003). Hence, differences

between inoculated and control plants could be controlled by plant hormones which play an integral role not only in controlling growth and development but also in regulating the sink strength (Roitsch et al. 2003). One could speculate that glucose levels in fruits are enhanced during certain stages in plants colonized by *L. orchidicola* due to systemic induction of biosynthesis of these phytohormones.

## Conclusion

Tomato plants grown under horticultural conditions and colonized by the dark septate endophytes DSE48, DSE49 and *L. orchidicola* showed some positive responses. The ability of DSEs to confer benefits to the plants seems, however, to be restricted to the early stages of vegetative or generative development. This does not lead to an overall increased yield or fruit quality. Also a plant protective effect could be only observed under conditions when inoculum pressure of the pathogen was low. Therefore the effects of the fungal endophytes are negligible under the cultivation management employed in the present experiments. Under natural conditions these slight differences in rate of development and disease resistance might, however, give DSE-colonized plants an advantage which finally leads to a better ecological performance. If this could be used in horticulture under detrimental conditions like drought, low plant-available nutrients or natural occurring pathogens has to be further investigated.

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# Chapter 5

## The root endophyte *Piriformospora indica*

## 5.1 Impact of *Piriformospora indica* on tomato

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**Abstract**

*Piriformospora indica* is a basidiomyceteous fungus and able to colonize roots of many monocotyledonous and dicotyledonous plants. This root endophyte belongs to the Sebacinales order and can be cultivated on different media for inoculum production. *P. indica* shows a potential to be used in agricultural and horticultural systems due to its benefits conferred to the plant after root colonization. Different plant hosts show increased growth of root and shoot, resistance against root and foliar pathogens and higher yield, but tomato as potential host has been not studied. The influence of *P.indica* on tomato growth was evaluated under different conditions of inoculation, nutrition and growth. The fungus influences positive or negatively the tomato plants according to its inoculum density and mineral nutrient conditions. *P. indica*-colonized plants can show accelerated growth during the vegetative phase that is not obvious any more during generative development. Its potential for application in crop production systems is discussed.

- Three out five experiments were done by me:
    - Impact on vegetative growth : Table 2 and Figure 4
    - Impact on generative growth: Figure 5, Figure 7 and Figure 8.
- The Materials and Methods are described in Appendix 10.
- This works is in press in: Oelmüller R., Kost G., Tripati S., Varma A. (eds.), Sebacinales. Springer-Verlag, Heidelberg.

## Introduction

Among the heterobasidiomycetous fungi, a new order was proposed six years ago based on molecular phylogeny studies, the Sebacinales (Weiss et al. 2004). This order contains species forming all kind of mycorrhizal associations except arbuscular mycorrhiza which is restricted to the phylum Glomeromycota (Schüssler et al. 2001). Some Sebacinales are even able to form different types of mycorrhiza thereby connecting plants from very diverse plant families (Kottke et al. 2008). In a recent study with 39 plant species among monocot and dicot families, Sebacinales have been detected whenever sequences were obtained (Selosse et al. 2009). This suggest that in addition of being ecto-, ericoid and orchid mycorrhizal fungi, this order seems to contain species which belong to the group of the non-mycorrhizal root endophytes. It was in retrospect therefore not surprising that *Piriformospora indica* firstly isolated in the Thar Desert of India (Verma et al. 1998) later turned out to form orchid mycorrhizal structures (Blechert et al. 1998), and was able to colonize a number of other plant species (Varma et al. 1999). This fungus (together with some isolates of the closely related species *Sebacina vermifera*) is able to establish associations with roots of many different plants. Moreover it influences whole plant physiology often improving vegetative growth, inducing resistance against pathogens and increasing yield (Tab. 1). The fact that the fungus can be cultivated on different media without a host (Verma et al. 1998) and that large biomass quantities can be easily obtained, let researchers from the beginning propose its application in future plant production systems (Varma et al. 1999). Important crop plants among the monocots (maize, barley, wheat) have been already investigated, but analysis of dicotyledonous plants was up to now restricted to models like Arabidopsis (discussed in other chapters of the book) or to plants with less economical importance (Tab. 1).

**Tab. 1** Plant hosts of *Piriformospora indica*. The results of experiments with different plant species and various methods of inoculation are shown, where statistical analyses of the effect on plant performance were carried out.

| species | inoculation | increases … | reference |
|---|---|---|---|
| *Zea mays* | in pots | vegetative growth[a] | Varma et al. 1999 |
| *Petroselinum crispum* | in pots | vegetative growth[a] | Varma et al. 1999 |
| *Artemisia annua* | in pots | vegetative growth[a] | Varma et al. 1999 |
| *Bacopa monnieri* | in pots | vegetative growth[a] | Varma et al. 1999 |
| *Nicotiana tabaccum* | in pots | vegetative growth[a] | Varma et al. 1999 |
| *Populus tremula* | in pots | vegetative growth[a] | Varma et al. 1999 |
| *Nicotiana tabaccum* | of calli | vegetative growth[a] | Varma et al. 1999 |
| *Withania somnifera* | of seeds for field | vegetative growth and flowering | Rai et al. 2001 |
| *Spilanthes calva* | of seeds for field | vegetative growth and flowering | Rai et al. 2001 |
| *Arabidopsis thaliana* | in pots | vegetative growth | Peskan-Berghofer et al. 2004 |
| *Nicotiana attenuata* | of seeds[b] | vegetative growth | Barazani et al. 2005 |
| *Nicotiana attenuata* | of seeds[b] | seed production | Barazani et al. 2005 |
| *Nicotiana attenuata* | of seeds[b] | susceptibility for herbivores | Barazani et al. 2005 |
| *Hordeum vulgare* | of seedlings | vegetative growth | Waller et al. 2005 |
| *Hordeum vulgare* | of seedlings | disease resistance | Waller et al. 2005 |
| *Hordeum vulgare* | of seedlings | salt tolerance | Waller et al. 2005 |
| *Hordeum vulgare* | pots in the field | yield | Waller et al. 2005 |
| *Pelargonium x hortorum* | of cuttings | enhanced rooting | Drüge et al. 2007 |
| *Euphorbia pulcherrima* | of cuttings | enhanced rooting | Drüge et al. 2007 |
| *Petunia hybrida* | of cuttings | no effect | Drüge et al. 2007 |
| *Triticum aestivum* | in pots | vegetative growth | Serfling et al. 2007 |
| *Triticum aestivum* | in pots | disease resistance | Serfling et al. 2007 |
| *Triticum aestivum* | in the field | only straw, not yield | Serfling et al. 2007 |
| *Triticum aestivum* | in the field | partial disease resistance | Serfling et al. 2007 |
| *Arabidopsis thaliana* | in pots | seed production | Shahollari et al. 2007 |
| *Arabidopsis thaliana* | in Petri plates | drought tolerance | Sherameti et al. 2008 |
| *Arabidopsis thaliana* | of seedlings | disease resistance | Stein et al. 2008 |
| *Linum album* | CF[c] to cells | lignan production | Baldi et al. 2008 |
| *Cicer arietinum* | of seeds | yield | Meena et al. 2010 |

[a] no statistical analysis, but large differences between controls and inoculated plants and low standard deviations

[b] effects also achieved with *Sebacina vermifera*; [c] culture filtrate

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

**Conditions for analysing *Piriformospora indica* – tomato interactions**

Tomatoes are commercially cultivated in the soil under field or under protected conditions. In greenhouses or under plastic cover soilless cultivation using different substrates became more and more important during the last thirty years (Savvas 2003). It was therefore necessary to find out the optimal inoculation conditions (mode of inoculum production, inoculum amounts, time point of inoculation) in a soilless system. Such a system had not been used for plant-inoculation with *P. indica*, but it turned out that the fungus is able to successfully colonize tomato roots in nutrient solution (Fig. 1) and also influences growth characteristics of tomato similar as in horticultural substrate or sand (Fakhro et al. 2010).

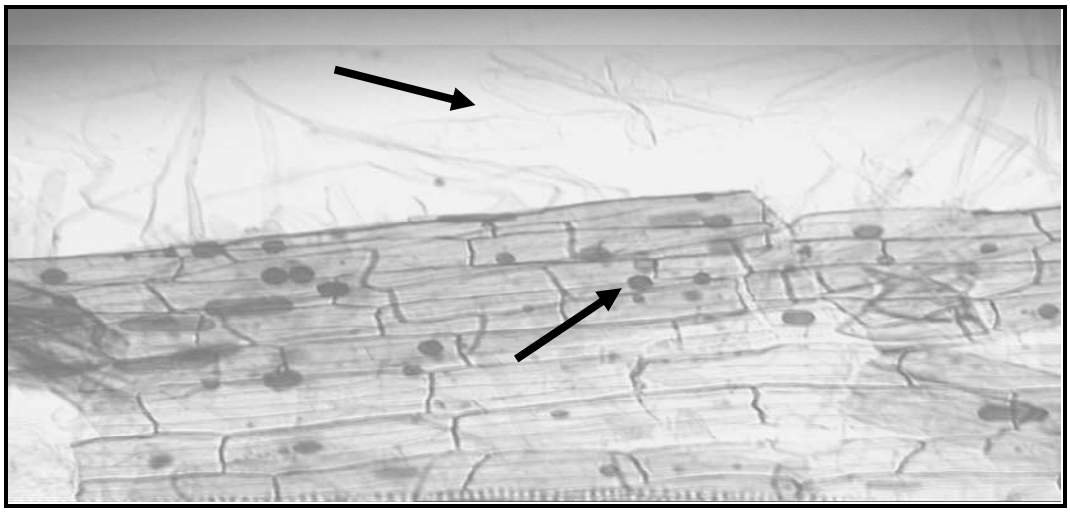

**Fig. 1** *Piriformospora indica* colonization of tomato roots. Tomato plants (cv. Hildares) were grown in a hydroponic system and inoculated with spores and hyphae from *P. indica*. Eight weeks after inoculation, roots were harvested and stained with trypan blue. Arrows indicate fungal hyphae outside and chlamydospores inside of the root.

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

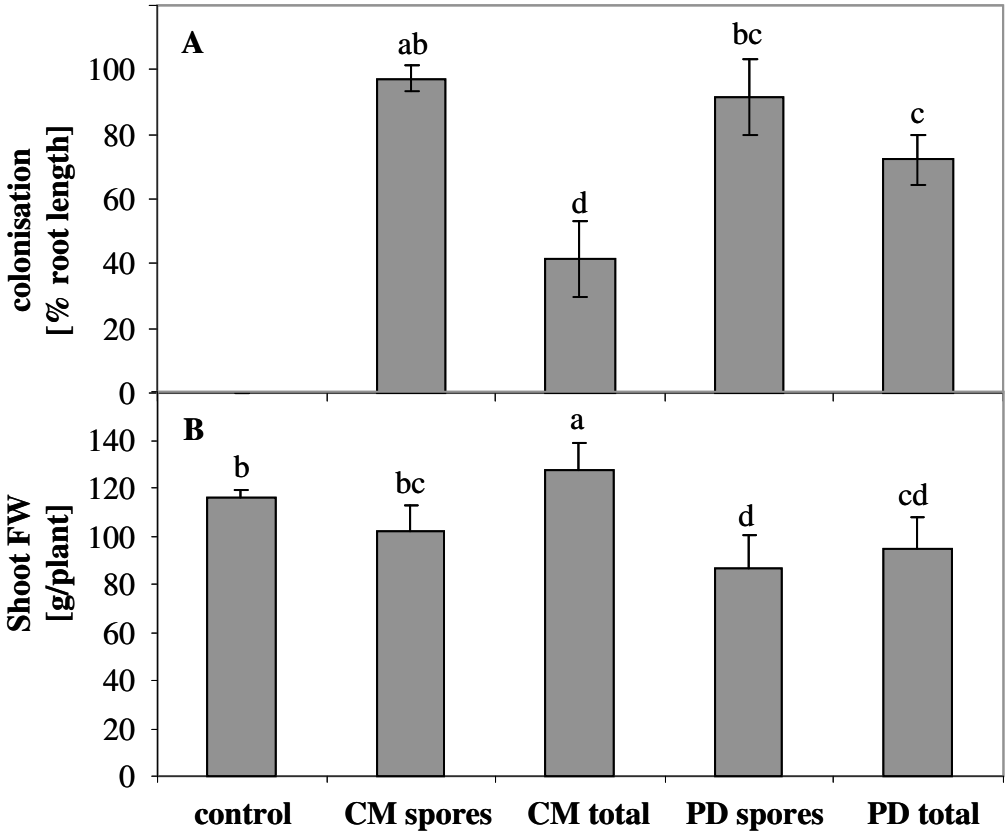


**Fig. 2** *Piriformospora indica* colonization intensity and plant growth promotion. *P. indica* was cultivated on complex medium (CM) or on potato dextrose (PD) in liquid medium or on agar plates. Spores were harvested from agar plates (spores) or hyphae and spores from liquid medium (total) and used for inoculating of tomato plants (cv. Hildares) grown in a hydroponic system. Six weeks after inoculation, roots were harvested, stained with trypan blue and colonization intensity was estimated by calculating the percentage of root length occupied by fungal chlamydospores (A). In parallel fresh weights of shoots were determined (B). Significant differences between treatments are indicated by different letters above the columns (one-way ANOVA; $P = 0.05$; $n = 3x10$).

These results indicate that the mode of inoculum production has an influence on the level of colonization. If spores were used, fungal spread in the root was high, while inoculation with total mycelium resulted in lower colonization intensities. Interestingly, impact on plant growth was negative, if *P. indica* inocula were produced on PD and colonization levels were inverse correlated with shoot fresh weights. The latter indicates that at least in tomato, *P. indica* can perform negative effects, if it occupies a too large area of the root. This is supported by experiments with different amounts of inocula in nutrient solution, sand or substrate, where positive effects were achieved with 3 x $10^5$ cfu/ml, but *P. indica* impact was negative at 9 x $10^5$ (Fakhro et al. 2010). Such effects were even more drastic using soil fertilised with low amounts of nitrogen or phosphorous (Fig. 3).

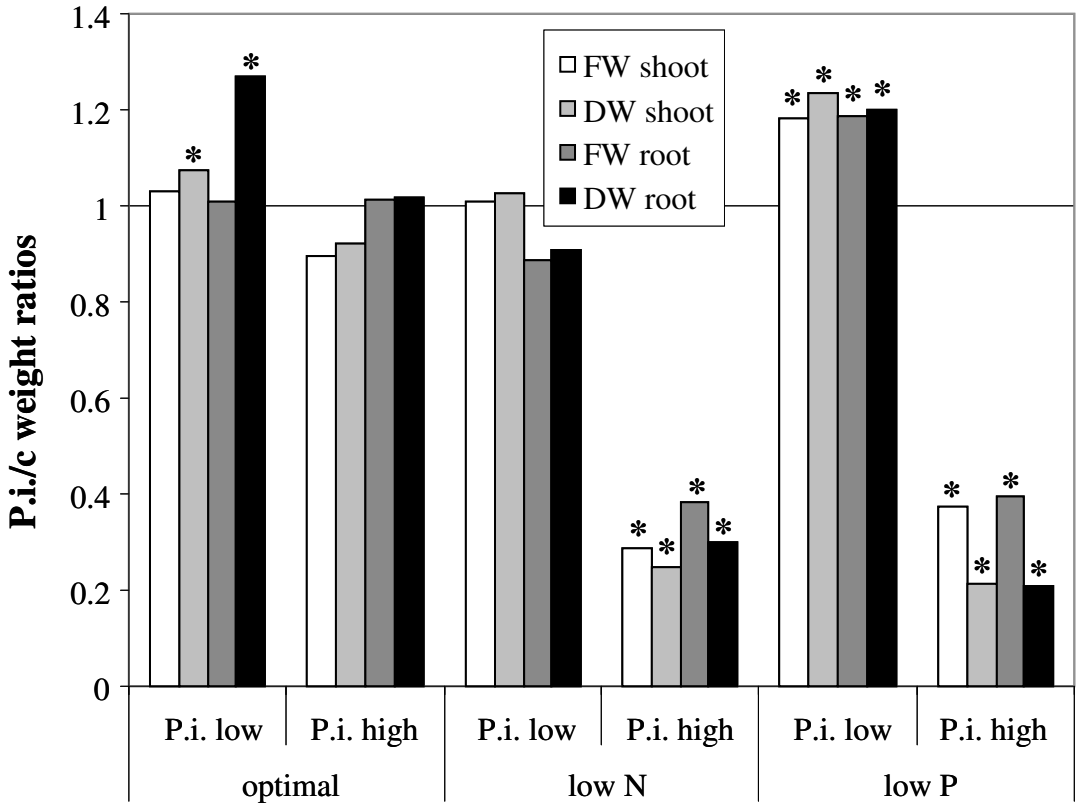


**Fig. 3** *Piriformospora indica* inoculum amounts and fertilisation. Roots of tomato plantlets (cv. Liberto) were dipped in a suspension of 5x$10^5$ spores $ml^{-1}$ (P.i. low and high) and transferred to pots with a phosphorous-poor soil where half of the plants were additionally inoculated with 10 g mycelium per 250 ml substrate (P.i. high) or not (P.i. low). The soil was optimal fertilised for tomato growth (De Kreij et al. 1997) or with only 10% nitrogen (low N) or 0% phosphate (low P). Plants were harvested four weeks after inoculation and fresh and dry weights of shoots and roots were measured. Ratios of values obtained for inoculated plants to the values of the controls are shown. Significant differences between inoculated and non-inoculated plants are indicated by asterisks (two-way ANOVA; $P = 0.05$; $n = 5$). Interaction between the factors inoculation and fertilisation were significant.

At optimal fertilisation, positive effects of *P. indica* disappeared at high inoculum concentrations. No growth promotion was observed at low nitrogen, but was significant for all four growth characteristics at low phosphate fertilisation. This might indicate that the fungus supports phosphate supply of the plant. Such a role in plant nutrition could not be observed in other plants, such as tobacco and barley (Barazani et al. 2005; Achatz et al. 2010), but a phosphate transporter was recently isolated from *P. indica* and suppressing its expression resulted in a disappearance of plant-growth promotion abilities in maize (Yadav et al. 2010). Interestingly, when plants grown under such nutrient-limiting conditions were inoculated with high amounts of *P. indica*, severe negative effects could be observed. Endophyte inoculation reduced biomasses by 60 to 80 percent.

It became clear from these studies that *P. indica* interacts with tomato in soil, in different substrates and also in hydroponic cultures. The outcome of this interaction, however, depends on type and the amount of inoculum being used, the time point of inoculation and the nutrient conditions in the environment. The basis for negative effects could be the unique mode of root-colonization: the fungus induces programmed cell death as it has been shown at least in barley (Deshmukh et al. 2006) which leads to higher percentage of dead root cells as in the corresponding controls (Franken et al. 2000). Why the negative effects are so severe under low nutrient conditions, is not clear. It is, however, interesting to note that such strong negative effects were also observed, when the fungus was cultivated on ammonium instead of nitrate as sole N source (Kaldorf et al. 2005). The authors suggested the production of toxins by the fungus under certain growth conditions. Further analysis will be necessary to prevent such phenomena.

**Impact on vegetative growth**

In contrast to the results obtained in other plants (Tab. 1), the effects of *P. indica* on vegetative development of tomato are marginal. There are some enhancements of biomasses between 4 and 10 weeks after inoculation (Fig. 2 and 3; Fakhro et al. 2010), but they never exceeds a value of 30 % and rarely concerns the whole plant. Moreover, this enhancement could not be observed in older plants. Already 10 weeks after inoculation plants inoculated with *P. indica* showed no difference to the control plants (Tab. 2) and also in a long term experiment carried out to monitor yield (see next paragraph), biomasses of leaves in plants with colonized or non-colonized roots were nearly identical (Fig. 4). This indicates that *P. indica* accelerates development in the beginning as it has been already discussed for barley (Achatz et al. 2010), but that this advantage is later being caught up by non-colonized plants.

This can only be explained by a negative effect of *P. indica*, because tomato shoot growth is under the applied conditions not limited by other factors. If root growth is slowed down while colonization is further progressing, the increasing percentage of dead cells as discussed above might explain such a negative effect at later stages of the interaction.

**Tab. 2** *Piriformospora indica* impact on vegetative growth 10 weeks after inoculation. Tomato plants (cv. Hildares) in pot cultures with commercial garden substrate were inoculated (P.i.) or not (C) in three consecutive experiments. After 10 weeks plants were harvested and fresh weights (FW) of shoots and roots, dry weights (DW) of roots, leaf numbers and leave areas were measured.

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

* significant differences are shown in bold (one-way ANOVA; $P = 0.05$; $n = 6$)

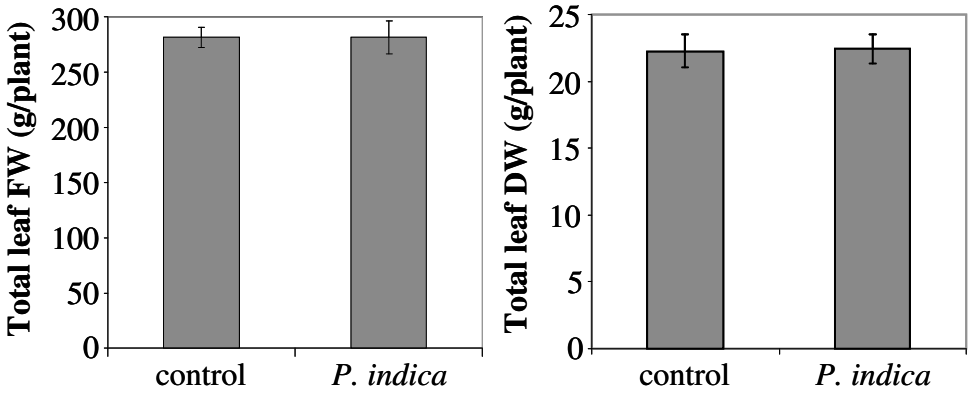


**Fig. 4** *Piriformospora indica* impact on vegetative biomass after four months. Tomato plants (cv. Hildares) inoculated or not with *P. indica* were cultivated in a hydroponic system (3 gullies with each 10 plants). At the end of the experiment (4 months after inoculation) total leaf fresh weight (FW) and dry weight (DW) were measured. Significant differences between inoculated plants and controls were not detected (one-way ANOVA; $P = 0.05$; $n = 3x10$).

**Impact on generative development and yield**

Comparing plants colonized with *P. indica* and the corresponding controls, differences in flowering time could be observed in many experiments. Colonized plants developed inflorescences already 12 days after inoculation where flowers could not be observed at control plants (Fig. 5). After three weeks all plants bore flowers, but the number still was different. Plants inoculated with the fungus had significant higher numbers of flowers than controls. Similar to the vegetative growth, however, these differences became non-significant with increasing plant age (Fig. 6).

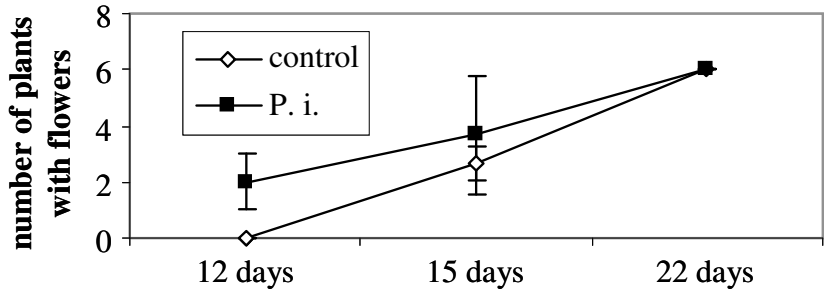


**Fig. 5** *Piriformospora indica* impact on flowering time. Tomato plants (cv. Hildares) inoculated or not with *P. indica* were cultivated in substrate. Plants with flowers were counted 12, 15 and 22 days after inoculation. Significant differences between inoculated plants and controls were only detected at the first date (one-way ANOVA; $P = 0.05$; $n = 6$).

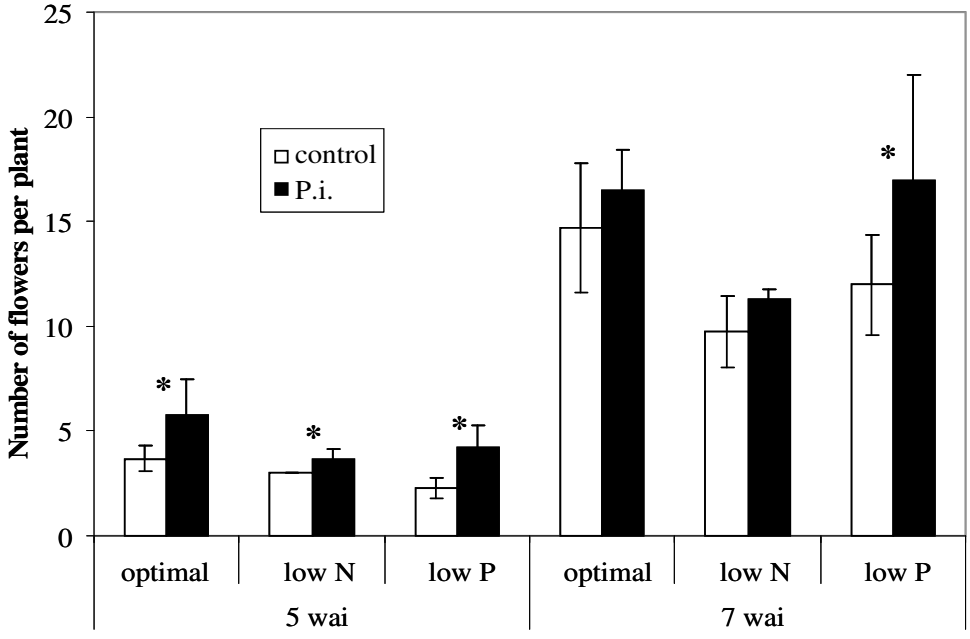


**Fig. 6** *Piriformospora indica* impact on flower number. Tomato plants (cv. Liberto) inoculated or not with *P. indica* were cultivated in soil. Number of flowers was counted five or seven weeks after inoculation. Significant differences between inoculated plants and controls are indicated by asterisks above the columns (one-way ANOVA; $P = 0.05$; $n = 5$).

Because flower numbers were different at the early dates, it was not surprising that biomasses of fruits at the early harvests also showed significant differences. Hence, at an early harvest, fruit fresh weights were double in plants colonized by the fungus compared to the corresponding controls (Fakhro et al. 2010). Repeating the experiments showed at the first two harvests 30% and 70% increase in fruit fresh weights, but thereafter colonized plants did not bear more tomatoes than the corresponding controls (Fig. 7).

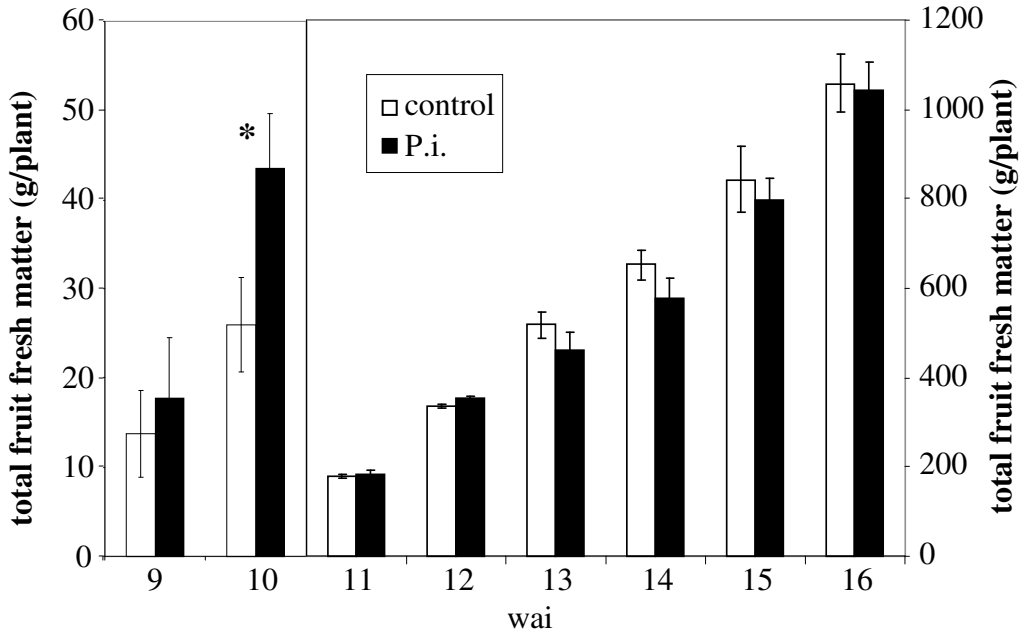


**Fig. 7** *Piriformospora indica* impact on fruit biomasses. Tomato plants (cv. Hildares) inoculated or not with *P. indica* were cultivated in hydroponic cultures. Fruits were harvested between 9 and 16 weeks after inoculation (wai) and fresh weights were measured. Significant differences between inoculated plants and controls are indicated by asterisks above the columns (one-way ANOVA; $P = 0.05$; $n = 4x10$).

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

amounts of marketable fruits harvested from plants being colonized by the endophyte and from control plants (Fig. 8).

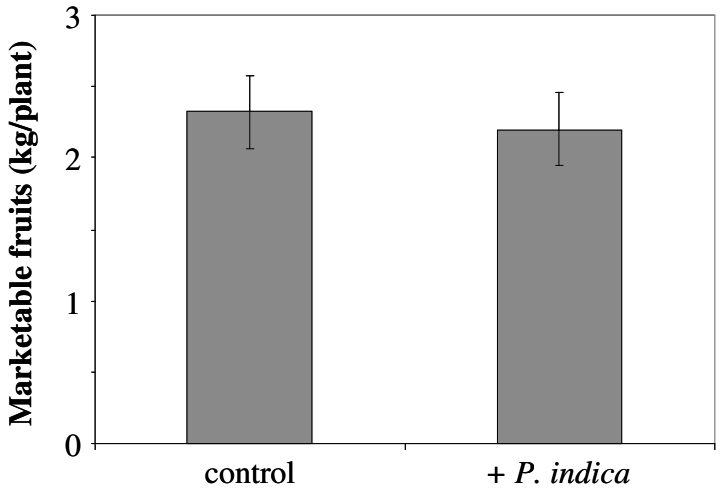


**Fig. 8** *Piriformospora indica* impact on yield. Tomato plants (cv. Hildares) inoculated or not with *P. indica* were cultivated in hydroponic cultures. Fruits were harvested between 9 and 16 weeks after inoculation and those possessing blossom end rot disorder were removed. Fresh weight sums of marketable fruits from all harvests are shown. No significant differences between inoculated plants and controls were detected (one-way ANOVA; $P = 0.05$; $n = 4x10$).

## Conclusion

*Piriformospora indica* seems to accelerate vegetative and generative development in tomato plants. This has been already observed in barley (Achatz et al. 2010). While this accelerated development results in increased yields in certain cultivars of this cereal crop (Waller et al. 2005) without any losses in quality (Achatz et al. 2010), this was not the case with tomato. It has, however, to be mentioned that *P. indica* does reduce disease symptoms in case of the fungal pathogen *Verticillium dahliae* and confines the spread of *Pepino mosaic virus* (Fakhro et al. 2010). The application of this endophyte can therefore be recommended although yield seems not to be increased. Moreover it will be interesting to investigate, if the fungus also helps tomato plants to overcome abiotic stresses as it has been shown for higher salt concentrations during cultivation of barley and Arabidopsis (Baltruschat et al. 2008; Sherameti et al. 2008). Finally, it might be possible that yield increases can be achieved under particular conditions e.g. at low nutrient availability or with other cultivars which have not been tested yet.

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## 5.2 Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens.

- The final publication is available at www.springerlink.com (Mycorrhiza, Volume 20, Number 3, 191-200, DOI: 10.1007/s00572-009-0279-5).

- In this paper, the experiment concerning the interaction of *P. indica* with the pathogen *Verticillium dahliae* was carried out by me (Figure 1 and Supplemental results appendix 11).

# Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens

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**Abstract** *Piriformospora indica* is a root endophytic fungus with plant-promoting properties in numerous plant species and induces resistance against root and shoot pathogens in barley, wheat and Arabidopsis. A study over several years showed, that the endophyte *P. indica* colonised the roots of the most-consumed vegetable crop tomato. *P. indica* improved the growth of tomato resulting in increased biomass of leaves by up to 20%. Limitation of disease severity caused by *Verticillium dahliae* by more than 30% was observed on tomato plants colonised by the endophyte. Further experiments were carried out in hydroponic cultures which are commonly used for the indoor production of tomatoes in central Europe. After adaptation of inoculation techniques (inoculum density, plant stage), it was shown that *P. indica* influences the concentration of *Pepino mosaic virus* in tomato shoots. The outcome of the interaction seems to be affected by light intensity. Most importantly the endophyte increases tomato fruit biomass in hydroponic culture concerning fresh weight (up to 100%) and dry matter content (up to 20%). Hence *P. indica* represents a suitable growth promoting endophyte for tomato which can be applied in production systems of this important vegetable plant not only in soil, but also in hydroponic cultures.

**Keywords** Hydroponic cultures; *Lycopersicon esculentum*; *Pepino mosaic virus*; *Verticillium dahliae*

## Introduction

*Piriformospora indica* was originally isolated from the spore of an arbuscular mycorrhizal (AM) fungus, but inoculation experiments showed its ability to colonise plant roots (Verma et al. 1998). It is an anamorphic strain of the Sebacinales (Basidiomycota), a group with many plant-interacting organisms including ecto-, ericoid and orchid mycorrhiza (Weiss et al. 2004; Selosse et al. 2007). The endophyte *P. indica* possesses positive influence on growth and development of many different plant species like AM fungi. Inoculation leads to increased fresh weights (Varma et al. 1999), supports the establishment of micro propagated plantlets (Sahay and Varma 1999) enhances flower and seed production (Rai et al. 2001; Barazani et al. 2005; Shahollari et al. 2007), promotes the rooting from cuttings (Drüge et al. 2007) and results in higher yield (Waller et al. 2005). It could be also shown that *P. indica* induces tolerance against salt stress and resistance against root and shoot pathogens (Waller et al. 2005; Serfling et al. 2007; Deshmukh and Kogel 2007; Sherameti et al. 2008; Baltruschat et al. 2008; Stein et al. 2008). In contrast to AM fungi however, the endophytic fungus colonises the roots and promotes the development of the model plant *Arabidopsis thaliana* (Peskan-Berghöfer et al. 2004). In this experimental system, a number of plant proteins and genes were identified which are important for the interaction between the endophyte and the plant root (Shahollari et al. 2005, 2007; Sherameti et al. 2005, 2008) and the basis for induced resistance was analysed (Stein et al. 2008). However, no data are available for the interaction between *P. indica* and plants of the *Solanaceae* which contain important crop species and models like potato, tomato, tobacco, eggplant or petunia.

Tomato is the most commonly grown fresh market vegetable world wide, but various pathogens can lead to dramatic losses in yield. One widespread disease of field grown tomato plants is verticillium wilt caused by the soilborne fungus *Verticillium dahliae* (Pegg and Brady 2002). The disease symptoms are characterized by V-shaped yellowing of the leaves, browning of vascular bundles and wilting. The fungus infects the root system directly or through wounds, invades the xylem and moves upward. Once in plant tissues, it produces toxins (Mansoori et al. 1995) and in course of the interaction, the disease symptoms progress up the stem and the plant becomes stunted (Gold et al. 1996). Control strategies are soil fumigation, crop rotation and growing cultivars with the *Ve* resistance gene (Talboys 1984; Huisman and Ashworth 1976; Ligoxigakis and Vakalounakis 1994). However, these strategies are limited by the longevity of the *V. dahliae* microsclerotia in soil (Pegg 1974; Talboys 1984), by the economically unpractical long rotation cycles (Harrington and

Dobinson 2000) and by the appearance of new race 2 strains which cause typical disease symptoms also on resistant *Ve* cultivars (Dobinson et al. 1996)

Besides production in the field, the importance of soil-less cultivation of tomato using open or closed hydroponic systems has been increasing worldwide during the last three decades (Savvas 2003). In these production systems, plants are usually supplied with a nutrient solution circulating to allow a more accurate control of the root environment. This can result in an optimal use of water and nutrients, and thus in higher yields and better fruit quality. However, recirculation of the nutrient solution and high plant density facilitates the rapid and efficient spread of pathogens and may thus increase the risk for epidemics if not managed well (Stanghellini and Rasmussen 1994). Since 1999, *Pepino mosaic virus* (PepMV) has attracted much attention because it is found widely in tomato greenhouses in many European countries, in Morocco, South and North America and, in China (see references in Spence et al. 2006). The origin of its sudden occurrence is not clear. However, rapid transmission and spread of PepMV within and between greenhouses can be mechanically caused by tools, clothes, and the hands of workers during crop handling (Jones et al. 1980). Typical symptoms of infected leaves are rolling, light-yellow mosaics, dark-green discolouration, and leaf distortion (Jordá et al. 2001; Van der Vlugt et al. 2002). Fruits may show yellow blotches, necrotic or yellow spots, and irregular ripening (French et al. 2008). Symptom expression can be affected by the tomato cultivar, the genotype of the virus, and the environmental conditions (French et al. 2008; Hanssen et al. 2009). There are conflicting reports on yield losses due to PepMV infection varying from low up to the collapse of the crop (Soler-Aleixandre et al. 2005). Apart from total yield losses significant decreases in fruit quality and thus marketable yield reductions up to 40% are reported (Spence et al. 2006; Schwarz et al. 2009).

The present investigation was carried out first to analyse, if the endophyte *P. indica* is able to reduce the symptoms of verticillium wilt in substrate-grown tomato. Secondly it was aimed to establish the interaction between *P. indica* and tomato in hydroponic culture systems for analysing, if the accumulation of PepMV within the apical shoot is influenced by fungal colonisation of the roots. At third, the impact of *P. indica* on tomato fruit biomass in the hydroponic system was determined.

## Materials and methods

*Cultivation of fungi and Pepino mosaic virus*

The endophyte *P. indica* was propagated on potato dextrose agar (PDA, VWR, Berlin, Germany) at 28°C. Chlamydospores were collected after two weeks from the agar plate and used to inoculate 100 ml potato dextrose broth (PDB, VWR) in 300 ml Erlenmeyer flasks. These flasks were further incubated at 28°C and 90 rpm for 4 weeks. Mycelium and spores were filtered through gauze and mixed with a blender (Model D72, Moulinex, Leipzig, Germany) for 2 min at lowest speed in 100 ml of sterile tap water. Propagule (chlamydospores, hyphal fragments) concentration was examined in a Thoma chamber and the number of viable propagules by plating on PDA.

*Verticillium dahliae* (kindly provided by Valerie Grimault, GEVES, Angers, France) was grown in 150 ml sucrose sodium nitrate liquid medium (Sinha and Wood 1968) at 28°C and 100 rpm. After one week, the culture was transferred to 200 ml fresh medium and further incubated for 2 weeks. The culture was mixed by a blender (Model D72, Moulinex) for 40 seconds at minimal speed. The mixed solution was filtrated and washed once by centrifugation. The number of conidia was estimated by counting in a Thoma chamber and their viability was checked by plating on PDA. For inoculation, the suspensions were adjusted with sterile tap water to a concentration of $10^5$ conidia/ml based on counted colonies.

The virus isolate *Pepino mosaic virus*-Sav E397 used in all experiments was obtained from tomatoes purchased in a German supermarket that were labelled to be imported from France. The virus isolate was recovered from these tomato fruits by maceration of crude fruit in ELISA sample buffer (10% PBS buffer, 2% polyvinylpyrrolidone) followed by mechanical inoculation using 0.05% Celite as an abrasive to tobacco plants (*Nicotiana benthamiana*) on which it was further propagated as described (Schwarz et al. submitted).

*Cultivation and inoculation of tomato plants*

For the experiment with the pathogen *V. dahliae*, surface disinfected tomato seeds (*Lycopersicon esculentum* Mill. cv. Hildares) were germinated on 0.8% water agar for 1 week and subsequently transplanted into pots containing 1 litre of the substrate 'Fruhstorfer Erde Typ P' (Archut, Vechta, Germany). These pot cultures were placed for the whole experiment in a greenhouse at day/night temperature of 25°C/19°C, relative humidity 54%/69% and a mean daily radiation of 31.3 Mol $m^{-2}$ $day^{-1}$. The plants were watered twice per week with 40 ml of nutrient solution (De Kreij et al. 1997; pH = 5.5; EC = 2 dS $m^{-1}$). Half of the plants (36) were inoculated with *P. indica* before planting into 'Fruhstorfer Erde Type P' by dipping the roots over night in a suspension of 105 cfu/ml of tap water. In addition, fresh *P. indica* mycelium was mixed 1/100 (w/w) with this substrate to achieve heavily colonised plants.

Two weeks after inoculation with *P. indica*, half of the plants (18 colonised by *P. indica* and 18 controls) were drenched with 30 ml of the conidia suspension of *V. dahlia* ($10^5$ conidia/ml).

For all other experiments (Table 1), seeds were germinated for two weeks in sterilized sand and further grown in pots filled with 0.5 litre sterilized sand (v/v: 1 (0.2 – 1 mm)/1 (2 – 3 mm); Euroquarz, Laußnitz, Germany) fertilised with the nutrient solution mentioned above. 1-4 weeks later, plants were inoculated by dipping the roots for two hours in a *P. indica* suspension or a control solution (Table 1). Thereafter inoculated plants and controls were transferred to buckets or gullies (10 plants per biological replicate), containing the nutrient solution, sand (as above) or substrate (Fruhstorfer Erde Type P). Twice a week, nutrient solution was renewed or added to sand or substrate until it drained off the buckets. In gullies, nutrient solution was applied as described (De Kreij at al., 1997). Daily climate data averages are shown in Table 1. For ensuring root colonisation by *P. indica*, five to six fragments (3 – 4 cm) of plant roots were sampled 10 days after inoculation, incubated on PDA and analyzed for the appearance of chlamydospores with characteristic morphology (Verma et al. 1998) by means of light microscopy. Two weeks after inoculation with *P. indica*, when tomato plants had developed 9 to 10 leaves, half of the *P. indica*-inoculated or the control plants were mechanically inoculated on leaf number 8 or 9 by abrading a homogenate containing PepMV which was obtained from leaves of host plants (*N. benthamiana*) using 0.05 % Celite as an abrasive. These leaves were thereafter washed with sterile water. Successful infection was determined one week later by DAS-ELISA (see below).

*Analysis of plants*

Disease severity in *V. dahliae*-inoculated plants was assessed at harvest (8 weeks after sowing, 6 weeks after *P. indica*- and 4 weeks after pathogen-inoculation) based on an arbitrary scale of disease classes: 0 = no symptoms, 1 = slight yellowing of leaf, stunting or wilting, 2 = moderate yellowing of leaf, stunting or wilting, 3 = severe yellowing of leaf, stunting or wilting and 4 = leaf death. Values were estimated for the different set of plants using the formula ($\Sigma$ n° of $\text{leaves}_{\text{disease class}}$ x disease class)/total number of leaves. The value obtained for the control plants infected with *V. dahliae* was set at 100%. In parallel, pieces of the stem base were placed on PDA and the outgrowth was microscopically confirmed. Fresh and dry weights of shoots were estimated at harvest time.

PepMV infection of tomato plants was determined by testing the upper most leaves in DAS-ELISA (modified after Clark and Adams 1977) using commercially available polyclonal

antibodies (immunglobulin IgG) according to the instructions provided (AS-0632; DSMZ, Braunschweig, Germany). Each ELISA test included a negative and a positive control. Samples were rated positive if the absorbance measured at 405 nm was greater than twice the level obtained from healthy controls (Cordoba-Selles et al. 2007).

Fresh and dry weights of shoots were measured at the end of all experiments, while fresh and dry weights of fruits at the end of experiment 4 and 5 (Tab. 1). Dry matter content was calculated.

**Table 1** Conditions of tomato growth in soilless cultures

| Experiment number | 1 | 2 | 3 | 4 | 5a | 5b |
|---|---|---|---|---|---|---|
| date of *P. indica* inoculation (weeks after sowing; developmental stage*) | 3-6; 102-110 | 4; 104-105 | 4; 104-105 | 4; 104-105 | 4; 104-105 | 4; 104-105 |
| Inoculum density ($10^5$ cfu/ml) | 3 | 3 or 9 | 3 | 3 | 3 | 3 |
| Substrate | sand | sand, substrate, nutrient solution | nutrient solution | nutrient solution | nutrient solution | nutrient solution |
| Containment | 1 l buckets | 2 l buckets | gullies | gullies | 10 l buckets | 10 l buckets |
| pathogen | no | no | PepMV | PepMV | PepMV | PepMV |
| plants per treatment | 6 | 6 | 2 x 10 | 4 x 10 | 4 | 4 |
| Temperature (day/night) (°C) | 21.0/ 17.6 | 22.2/ 19.2 | 21.7/ 20.0 | 23.4/ 18.9 | 25.8/ 20.8 | 25.8/ 20.8 |
| Humidity (day/night) (%) | 63.2/ 63.8 | 59.1/ 57.4 | 78.2/ 75.3 | 76.3 79.9 | 67.4/ 78.8 | 67.4/ 78.8 |
| Mean daily radiation (Mol $m^{-2}$ $d^{-1}$) | 18.5 | 7.8 | 9.0 | 12.8 | 11.0 | 6.0 |
| $CO_2$ concentration (ppm) | 479.6 | 454.5 | 365.7 | 396.3 | 452.0 | 452.0 |
| end of the experiment (weeks after sowing) | 9 | 11 | 10 | 13 | 12 | 12 |

* according to Feller et al. (1995)

*Statistical analysis*

Disease severity after *V. dahliae* infection was analysed by the non-parametric Kruskal-Wallis test, while all other experimental data were processed by analysis of variance procedures. Means at the different measurement dates were separated by Tukey's test procedure at P = 0.05. Significant differences are presented by different letters, standard deviation bars are added in the figures and significant interactions between factors are mentioned in the legends and. STATISTICA 6.0 software (2003) was used for all statistical analyses indicated in the figure legends.

## Results

*Impact of Piriformospora indica on verticillium wilt*

Tomato plants grown in substrate and infected with the pathogen *V. dahliae* showed typical symptoms as leaf yellowing, stunted growth, wilting and death of the plant. Fresh weights were significantly reduced in the pathogen-infected control plants, while the dry matter content was only slightly different (Fig. 1a). Tomato plants inoculated with the endophyte *P. indica* showed a significant increase in fresh weights. Moreover, the negative effect of the pathogen on plant growth was alleviated when the plants were colonised by the root endophytic fungus. Differences in fresh weight and dry matter content between *P. indica*-inoculated plants and the corresponding controls were higher, when the plants were infected with *V. dahliae*. This was mirrored by the result of a two-way ANOVA showing a significant interaction between the two factors 'pathogen' and 'endophyte'. Estimation of the disease symptoms also showed the protecting effect of *P. indica* (Fig. 1b). The disease severity was reduced by 32% in plants colonised by the endophytic fungus.

*Establishment of Piriformospora indica inoculation in a soilless system*

In order to reveal the best conditions for *P. indica* infection in soilless systems, at first the optimal plant stage of inoculating the endophyte was determined (experiment 1 in Table 1). For this purpose, tomato plants were inoculated at different stages (Fig. 2). Significant positive effects were only detected when the fungal inoculum was added four weeks after sowing (stage 104-105 according to Feller et al. 1995). This experiment showed in addition that the medium used for growing *P. indica* had no influence on tomato biomass as it has been seen before in soil cultures (Varma et al. 1999).

The second experiment (Table 1) was carried out for analysing the effects of growth medium and inoculum density on tomato growth (Fig. 3). Comparison of substrate, sand and nutrient solution showed a significant positive effect of *P. indica* on shoot fresh weight with a low density of inoculum only in sand. Significant negative effects were obvious with a high inoculum dosage in nutrient solution. No differences were detected for the dry matter content and interaction between the two factors 'inoculum density' and 'growth medium' could not be observed.

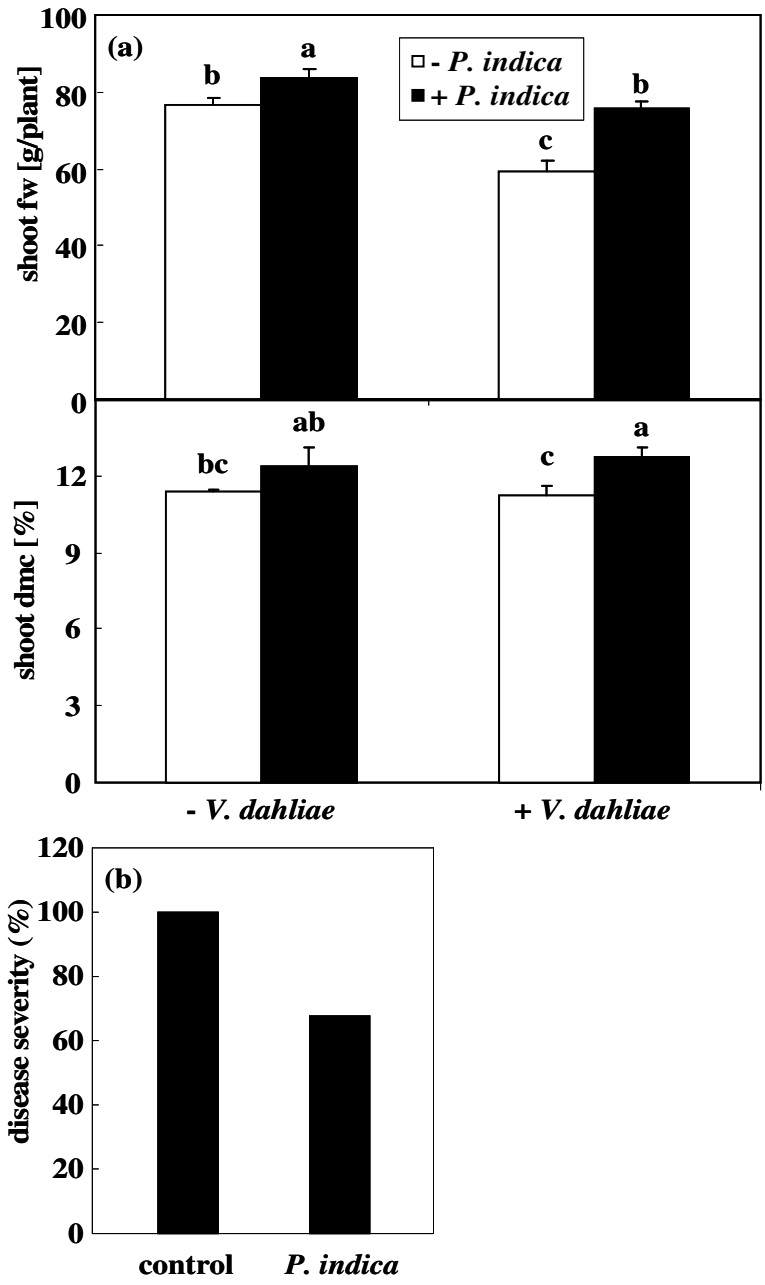


**Fig. 1** Influence of *Piriformospora indica* on tomato-*Verticillium dahliae* interaction. Tomato plantlets were transferred to pots containing substrate supplemented or not with *P. indica.* After two weeks half of the controls and the inoculated plants were infected or not with the pathogen *V. dahliae*. Four weeks later, shoot fresh weights (fw) and dry weights were measured and dry matter content (dmc) was calculated (a). Significant different values are indicated by different letters above the columns. The factors *P. indica* and *V. dahliae* showed a significant interaction for both parameters (two way ANOVA; $P = 0.05$; $n = 18$). In addition, disease severity was estimated and set for the control plants as 100% (b). Statistical analysis showed that disease severity was significantly different (Kruskal-Wallis test; $P = 0.05$; $n = 3$ x 6 plants).

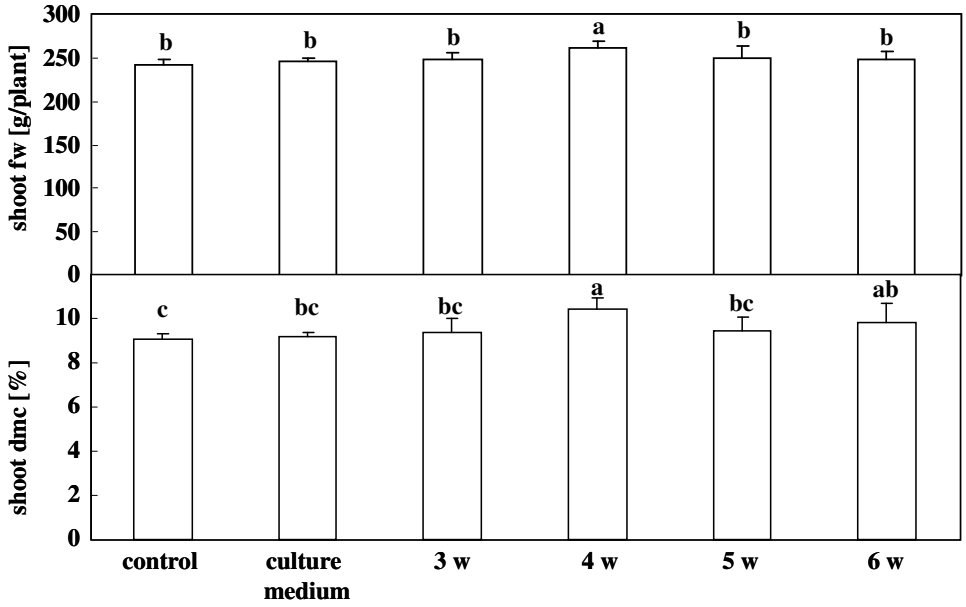


**Fig. 2** Influence of inoculation date. Tomato plantlets (3 weeks after sowing) were transferred to pots containing a nutrient solution and supplemented with the *Piriformospora indica* inoculum immediately (3 weeks after sowing) or after 7, 14 or 21 days (4-6 weeks after sowing). Control plants obtained no supplement (control) or culture medium without the fungus. Nine weeks after sowing, shoot fresh weights (fw) and dry weights were measured and dry matter content (dmc) was calculated. Significant differences between different types of inoculum are indicated by different letters above the columns (one way ANOVA; $P = 0.05$; $n = 6$).

*Impact of Piriformospora indica on Pepino mosaic virus spread*

Experiments 3-5 (Table 1) were carried out to analyse the influence of *P. indica* on the concentration of *Pepino mosaic virus* (PepMV). Typical symptoms of PepMV infection were observed in all experiments, as it has been described by Jordá et al. (2001) and Van der Vlugt et al. (2002), but not quantified. The concentration of PepMV decreased over time (14, 41, 57 dai) in the upper most leaves in experiment 3, but was always between 10% and 20% higher in tomato plants colonised by *P. indica* than in non-colonised controls (Fig. 4a). The difference was significant at the latest date (57 dai). The virus responded opposed in experiment 4 (Fig. 4b). First, virus concentration increased during the course of the experiment (10, 38, 64, 81 dai). Secondly, the virus was detected at all dates except the second (38 dai) with lower concentrations in plants, which were inoculated with the root endophyte, than in the controls. This reduction of virus spread was significant at the first date of sampling (10 dai). In order to find out the differences between the two experiments, climate conditions during the cultivation were compared and, light intensity was revealed as the major variation between the two experiments (Table 1).

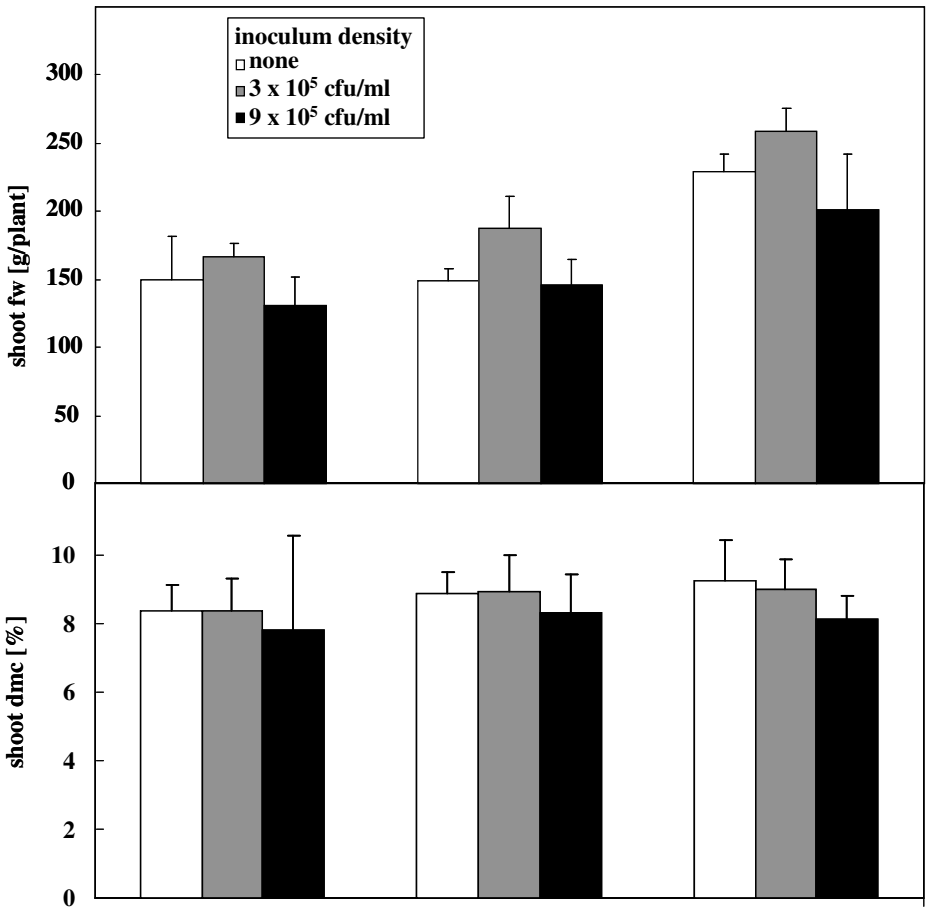


**Fig. 3** Influence of cultivation system and *Piriformospora indica* inoculum density. Tomato plantlets were transferred into buckets containing a nutrient solution, sand or a commercial garden substrate. Each cultivation system was supplemented with no, with 3 or with 9 x $10^5$ cfu/ml of *P. indica* inoculum. Shoot fresh weights (fw) and dry weights were measured and dry matter content (dmc) was calculated eight weeks after inoculation. Two way ANOVA ($P$ = 0.05; $n$ = 6) revealed significant differences in shoot fw for the influence of inoculum density and of the cultivation system, but not for the interaction of the two factors. No significant differences were revealed for shoot dmc.

Consequently, half of the plants were shaded in experiment 5 (Fig. 4c). In these shaded plants, *P. indica*-inoculation led to a significantly increased content of PepMV in the apical leaves at the first two dates (17, 31 dai). In plants however, which obtained higher light intensities, a decreased virus concentration was detected in the leaves at the last two dates (31, 59 dai) when the roots were colonised by the endophytic fungus (significant at 59 days after inoculation).

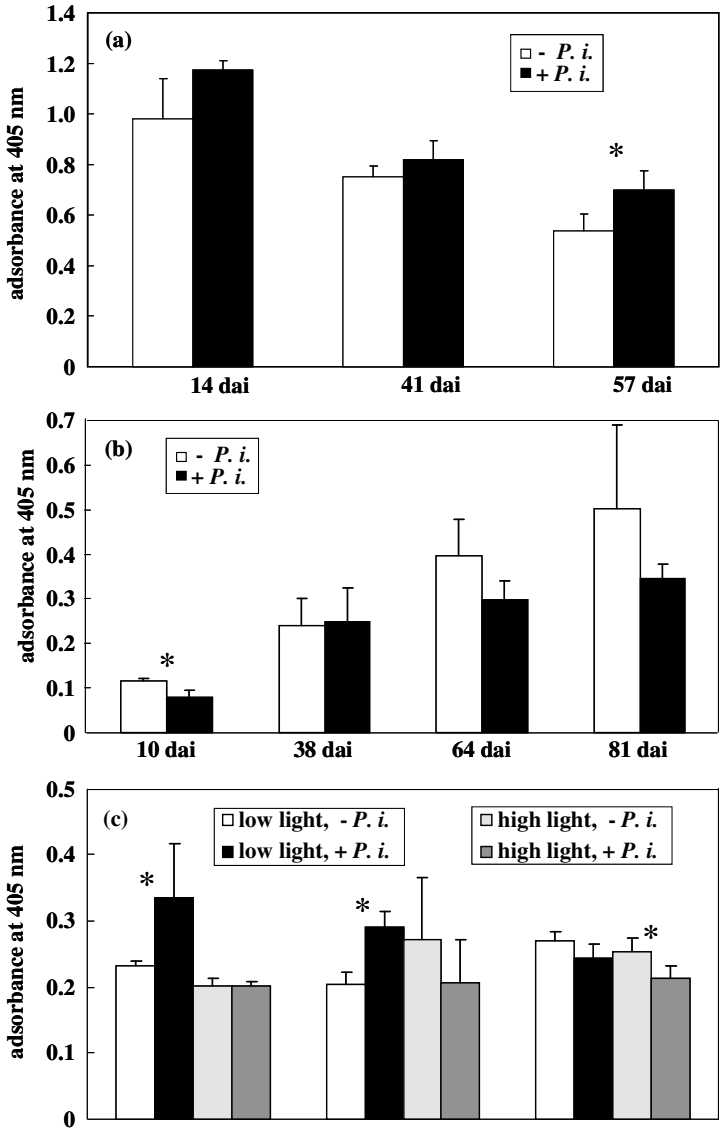


**Fig. 4** Influence of *Piriformospora indica* on *Pepino mosaic virus* spread. Tomato plants were grown in nutrient solution in three consecutive years (a: winter 2006; b: summer 2007, c: late summer 2008 under two light regimes) and inoculated or not with *P. indica*. When roots were colonised, youngest leaves of half of the plants were inoculated with PepMV. At different days after inoculation (dai), youngest leaves were harvested and PepMV colonisation was measured by ELISA. Significant differences between plants inoculated or not by *P. indica* are indicated by an asterisk. (One way (a+b) and two way (c) ANOVA; $P = 0.05$; $n = 2$ (a) or 4 (b and c)). An interaction between light and *P. indica* was detected at 31 dai (c).

*Impact of Piriformospora indica on fruit biomass*

Higher numbers of flowers or setting of fruits were observed in the plants inoculated with *P. indica* in all experiments. Plants of experiment 4 and 5 were therefore used to harvest and to analyse the fruits (Fig. 5 shows results of experiment 5). This revealed a significant influence of *P. indica* on total fruit biomass (Yield of marketable fruits was not determined). At the date of harvest, tomato fruit fresh weights per plant were increased between 50% and 100% and dry matter content between 10% and 20%. The increase in fresh weights was not due to

differences in the single fruit, but due to higher numbers of fruits. Significant differences were also obtained in experiment 4 with fresh weight increases between 40% and 50% and a 7% higher dry matter content (data not shown).

**Discussion**

*Piriformospora indica* is a root-endophytic fungus with plant growth promoting abilities. The increase of fresh weights was in some studies between 20 and 40% (Peskan-Berghöfer et al. 2004; Barazani et al. 2005) while it could reach in others up to 100% (Varma et al. 1999; Waller et al. 2005; Serfling et al. 2007). This characteristic could be confirmed in the present experiments using tomato as a plant host. Fresh weight of *P. indica*-colonised plants were, however, in the best case not more than 20% higher than in controls and reached although significant in most experiments increases of only 10%. This is probably on the one hand dependent on the plant species. For instance rooting of cuttings was strongly enhanced in *Euphorbia pulcherrima* and *Pelargonium* x *hortorum*, while no effect could be observed in *Petunia hybrida* although cultivated under the same conditions (Drüge et al. 2007). On the other hand, conditions of inoculation (plant stage, substrate, inoculum density) and growth clearly played an important role. Best results for tomato were obtained in sand compared to substrate and nutrient solution. A similar result comparing sand and soil has been obtained with wheat (Serfling et al. 2007). Nutrient poor conditions in the sand compared to the substrate cannot be the reason, since nutrient supply was optimal and *P. indica* does not improve at least P and N content in tobacco (Barazani et al. 2005) or in barley (Achatz et al. 2009). Hence an explanation for the influence of the substrate has for the moment to be left open. The other conditions tested for tomato were the inoculum density and the plant stage of inoculation. This showed that the amount of fungus should not exceed a certain extent, because negative effects on plant growth were obtained. Such negative effects with high amounts of inoculum of *P. indica* have been also seen in an experiment with tomato using field soil as substrate (data not shown). Another study using poplar as host in a Petri dish system also revealed negative effects of the endophyte (Kaldorf et al. 2005). This was not dependent on inoculum amount, but on the mode of cultivation of the fungus. If *P. indica* was grown on media containing ammonium as N source, the fungus started to colonise not only the cortex, but also the vascular cylinder of the roots and necrotic lesions occurred.

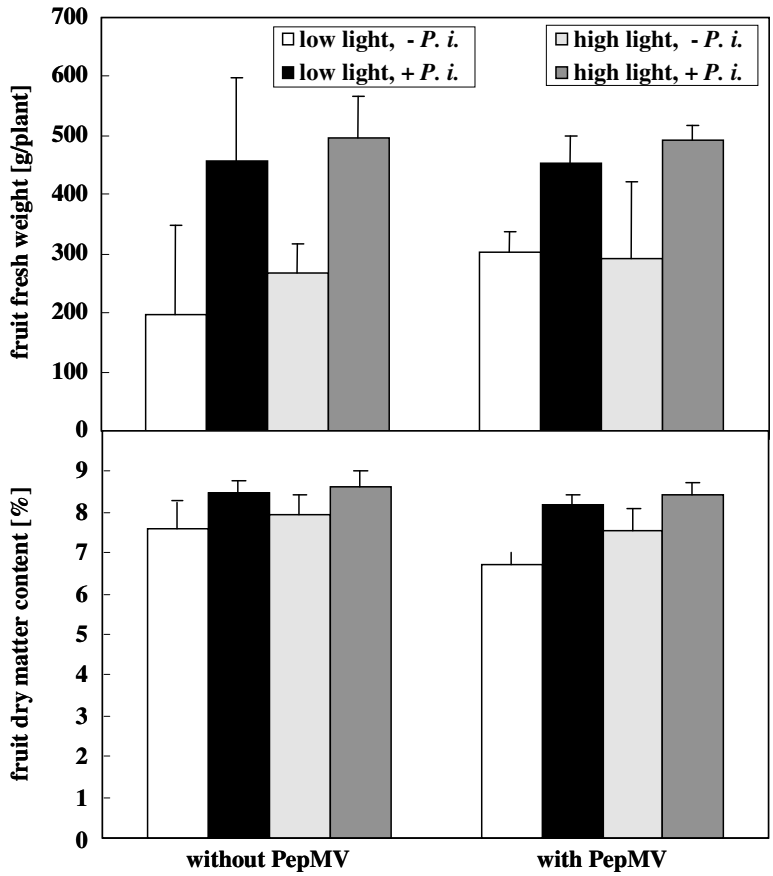


**Fig. 5** Influence of *Piriformospora indica* on fruit fresh weight and dry matter content. Tomato plants were grown in nutrient solution under two light regimes and inoculated or not with *P. indica* and *Pepino mosaic virus*. 12 weeks after germination fruits were harvested, fresh and dry weights measured and dry matter content calculated. A three way ANOVA ($P$ = 0.05; $n$ = 4) showed significant influence on fresh weight for *P. indica* and on dry weight for all three factors (light, PepMV, *P. indica*). Interactions between any of the factors were not detected.

Positive effects were observed, if the endophyte derived from cultures with nitrate. Negative effects could be based on the mode of colonisation. The fungus is not a biotroph as e.g. the arbuscular mycorrhizal fungi, but increases the number of dead cells in the root (Franken et al. 2000). Interestingly, *P. indica* seems to induce the programmed cell death of plants as numerous pathogens do, but in contrast to these pathogens the colonisation of the root by the endophyte depends on the cell death program of the plant (Deshmukh et al. 2006). If the number of dead cells in the root exceeds a particular threshold, *P. indica* could exert a negative influence on plant growth and development. This might be the case, if the amount of inoculum is too high and the colonisation is from the beginning too intense. An optimal balance between positive and negative effects of *P. indica* could also explain the observation that best effects were obtained, if the fungus was applied at the 4-5 leaf stage. At the earlier

date the percentage of dying cells could reach the point, where positive and negative effects are in equilibrium, while at the later dates, the root is not susceptible anymore for the positive activity of the fungus. Variable effects of *P. indica* on vegetative growth are probably not simply due to the extent of colonisation, because plants showing between 10% and 50% colonisation intensities were not different in their shoot fresh weights (data not shown). All this indicates that the outcome of the interaction between tomato and *P. indica* depends on experimental conditions and, future experiments will be directed to proof more variables in this respect. Preliminary experiments indicate for instance that the culture medium for growing the fungus also seems to influence the effect of the endophyte on plant performance. In addition, different isolates of *Sebacina vermifera*, a close relative of *P. indica*, have to be tested, if they promote tomato growth even more as it has been shown for tobacco and barley (Barazani et al. 2005; Deshmukh et al. 2007). The mechanisms behind the growth promoting effects of *P. indica* are a matter of debate. Phytohormones as ethylene, auxin and cytokinin seem to play a role as different analyses of the fungal culture filtrate and particular plant mutants indicate (Barazani et al. 2007; Sirrenberg et al. 2007; Vadassery et al. 2008). To make things even more complicated a recent analysis has shown that *P. indica* contains bacteria inside its cytoplasm which show similar effects on plant growth and defence reactions as the fungus (Sharma et al. 2008).

In addition to promoting vegetative growth, *P. indica* also exerts a positive influence on the generative organs of plants. In contrast to the relatively low enhancement of shoot fresh weights, the increase in tomato fruit biomass was surprisingly high. This was not due to increased fresh weight of single fruits, but the fruit number was larger than in control plants. Higher number of inflorescences and fruit settings was already observed in the experiments 1-4 (data not shown). In addition to the fresh weight, *P. indica* also enhanced the dry matter content of the fruits. This indicates that more biomass was transported into the generative organs during the growth period of the plants. Because the vegetative organs were not reduced in size, there must have been a higher production of these biomasses during growth of the tomatoes in interaction with the endophyte. In barley, were yield increases up to 11% were observed in open door experiments (Waller et al. 2005), different parameters have been tested (Achatz et al. submitted). While improved mineral nutrition or protection against pathogens did not play a role, enhanced $CO_2$ assimilation under low light conditions was observed. Indeed in the last experiment without the virus, fresh weights were increased by a factor of 2.3 under low light and by a factor of 1.9 under high light. This difference was not significant as no interaction could be observed between the two factors endophyte and light.

Nevertheless, further experiments will be carried out with higher differences in the light intensities and measurements of C assimilation and total C in the different organs of the tomato plant in order to better elucidate the basis for the increased biomasses. In addition, the total yield until the last fruit setting has to be monitored for excluding the possibility that *P. indica*-colonised plants are just developmentally progressed compared to controls. Such an accelerated development was indicated by particular gene expression patterns in barley roots (Waller et al. 2008). However, yield of seeds in tobacco, barley and Arabidopsis was not only increased at a particular date, but also overall at the end of the whole growth period (Barazani et al. 2005; Waller et al. 2005; Shahollari et al. 2007).

In barley, wheat and in Arabidopsis it has been shown that *P. indica* is able to alleviate the symptoms after attack of the plants by fungal pathogens (Waller et al. 2005; Serfling et al. 2007; Stein et al. 2008). It was therefore not surprising to find that the endophyte is also reducing the symptoms of verticillium wilt in tomato. The disease severity was lowered by more than 30% and *P. indica* balanced the fresh weight decrease caused by the pathogen. This was in the range what has been observed in the other systems mentioned in the introduction. A similar effect on verticillium wilt has been also observed by using the non-virulent isolate Dvd-E6 of *V. dahliae* as a competitor (Chen et al. 2004). In this case the colonisation of Dvd-E6 nearly totally excluded the infection by the virulent race (Shittu et al. 2009). If this is also the case for *P. indica* remains to be analysed by assessing the biomass of the pathogen in the plant. Such resistance reactions induced by *P. indica* have been observed in case of powdery mildew in barley, wheat and Arabidopsis (Waller et al. 2005; Serfling et al. 2007; Stein et al. 2008). The increased production of antioxidants in roots and shoots of *P. indica*-colonised plants were discussed as one reason for the induced resistance (Waller et al. 2005; Serfling et al. 2007) and also particular genes known to be involved in plant defence reactions were shown to be systemically induced after *P. indica* inoculation (Waller et al. 2008). Such type of studies could be also carried out for tomato, where many defence and pathogenesis-related genes are known.

Although not significant at all dates of investigation, the overall picture of the last three experiments suggested that *P. indica* interferes with *Pepino mosaic virus* accumulation in the apical shoot and, that the outcome of this interference is dependent on light intensities during tomato cultivation. Such interactions between fungal root endophytes and viral pathogens were up to now only reported for arbuscular mycorrhizal fungi and the tobacco mosaic virus (Dehne 1982; Shaul et al. 1999). In both cases, viral occurrence and resulting symptoms were increased in leaves. In contrast, when tomatoes were co-inoculated with PepMV and a fungal

pathogen, such as *Verticillium* spp. (Spence et al. 2006) or *Pythium aphanidermatum* (Schwarz et al. 2009), the virus colonisation of the plant was inhibited. The root necrosis caused by the fungal pathogens could perhaps induce resistance mechanisms affecting virus multiplication and spread (Van Loon, 1997) and/or biochemical and structural changes in root architecture reduced the efficiency of PepMV uptake of roots through the nutrient solution. Similarly to the AM fungi, *P. indica* might increase under low light conditions the carbohydrate content of cells due to a higher photosynthetic rate and in this way stimulates the number of virus particles in the tissues as detected by ELISA. Increases in C assimilation of *P. indica*-colonised plants have been observed in barley (Achatz et al. submitted) and in *Pelargonium* x *hortorum* (unpublished). The difference in carbohydrate contents between control and endophyte-colonised plants would be lower under high light conditions and another mechanism would become evident. Such a mechanism could be similar to the systemic induced resistance (SIR) against viruses which was obtained with particular plant growth promoting rhizobacteria (Raupach et al. 1996; Jetiyanon and Kloepper 2002) and results in a decrease of the accumulation of PepMV in tomato. It has to be mentioned that usually systemic acquired resistance and not SIR is acting against viruses (Ton et al. 2002). However, a SIR-similar mechanism dependent on jasmonate signalling was revealed as being responsible for the reduction of powdery mildew in *P. indica*-colonised Arabidopsis plants (Stein et al. 2008).

**Conclusion**

*Piriformospora indica* reduces the disease symptoms caused by the fungal pathogen *Verticillium dahliae* and is able to repress the amount of *Pepino mosaic virus* provided that light intensities are high. Tomato plants colonised by the endophyte show only slightly enhanced vegetative development, but fruit biomass is strongly increased. More research is necessary to further optimize the application of *P. indica* and to ensure that quality of fruits concerning taste- and health-related compounds are not negatively affected. The presented results however let us already suppose that the plant-protecting and development-promoting abilities of *P. indica* could be used to improve the production of tomatoes in hydroponic cultures.

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## 5.3 Root colonization by *Piriformospora indica* enhances grain yield in barley under diverse nutrient regimes by accelerating plant development.

- The final publication is available at www.springerlink.com (Plant and Soil Volume 333, Numbers 1-2, 59-70, DOI: 10.1007/s11104-010-0319-0).

- In this paper, the experiments concerning *Glomus mosseae* and phosphate and nitrogen up take in shoots of barley plants was carried out by me (Figure 2 and appendix 12 and 13).

# Root colonization by *Piriformospora indica* enhances grain yield in barley under diverse nutrient regimes by accelerating plant development

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## Abstract

The *basidiomycete* fungus *Piriformospora indica* colonizes roots of a broad range of mono- and dicotyledonous plants. It confers enhanced growth, improves resistance against biotic and tolerance to abiotic stress, and enhances grain yield in barley. To analyze mechanisms underlying *P. indica*-induced improved grain yield in a crop plant, the influence of different soil nutrient levels and enhanced biotic stress were tested under outdoor conditions. Higher grain yield was induced by the fungus independent of different phosphate and nitrogen fertilization levels. In plants challenged with the root rot-causing fungus *Fusarium graminearum, P. indica* was able to induce a similar magnitude of yield increase as in unchallenged plants. In contrast to the arbuscular mycorrhiza fungus *Glomus mosseae*, total phosphate contents of host plant roots and shoots were not significantly affected by *P. indica*. On the other hand, barley plants colonised with the endophyte developed faster, and were characterized by a higher photosynthetic activity at low light intensities. Together with the increased root formation early in development these factors contribute to faster development of ears as well as the production of more tillers per plant. The results indicate that the positive effect of *P. indica* on grain yield is due to accelerated growth of barley plants early in development, while improved phosphate supply –a central mechanism of host plant fortification by arbuscular mycorrhizal fungi- was not observed in the *P. indica*-barley symbiosis.

**Keywords**

Mycorrhiza, barley, *Piriformospora indica*, phosphate uptake, grain yield

## Introduction

Associations of plant roots with beneficial fungi are common in the majority of terrestrial plants, and colonization of the land by the ancestors of today's vascular plants are thought to have been aided by beneficial fungi about 400 million years ago (Smith and Read 1997; Selosse and Tacon 1998; Reinhardt 2007). The symbiosis of root-colonizing fungi with plants plays an important role for the plants' fitness, specifically with respect to nutrient acquisition, resistance against biotic and tolerance to abiotic stress (Smith and Read 1997; Borowicz 2001). Several different morphological and functional types of fungus-root interactions have been described. The arbuscular mycorrhizal (AM) association formed by fungi of the phylum *Glomeromycota,* for example, is present in over 80 % of land plants (Newman and Redell 1987; Harrison 2005). AM fungi are obligate biotrophs and form specialised structures, called arbuscules, for symbiotic interaction. AM fungi provide mineral nutrients, specifically phosphate, to their host plant in exchange for carbohydrates (Marschner and Dell 1994; Smith and Read 1997; Smith et al. 2004; Bucher 2007).

*Piriformospora indica* is the best-studied member of the recently established basidiomycete fungal order *Sebacinales* (Verma et al. 1998; Weiss et al. 2004). It colonizes roots of a broad range of mono- and dicotyledonous plants, including important crop plants (Varma et al. 1999). Growth of this root endophyte is restricted to the cortical tissue of the root, predominantly in the differentiation and the root hair zone (Deshmukh et al. 2006). *P. indica* colonization of developmentally older root tissue is associated with host cell death, without visible negative effects on root viability or growth (Deshmukh et al. 2006). When seedlings are inoculated, the fungus can still be detected in the roots of barley and wheat plants at the time of harvest several months later (Waller et al. 2005; Serfling et al. 2007).

*P. indica* confers enhanced growth to a large number of plant species (Varma et al. 1999; Rai et al. 2001; Peskan-Berghöfer et al. 2004) and enhances grain yield in barley (Waller et al. 2005). Furthermore, the root endophyte induces enhanced tolerance to salinity and resistance against biotic stresses (Waller et al. 2005), suggesting a further exploration of its agricultural use. To identify possible mechanisms underlying *P. indica*-induced improved grain yield in crop plants, we extended our initial study (Waller et al. 2005) to test the roles of different fertilization levels and biotic stress on yield. As AM fungi improve the uptake of P into roots of host plants (e.g. Bucher 2007) and *P. indica* was shown to induce an enhanced uptake of P into the cotyledons of *Arabidopsis* grown on agar medium (Shahollari et al. 2005), we tested whether *P. indica*-mediated yield increase depends on P and N levels of the soil, and whether *P. indica,* in direct comparison to *Glomus mosseae*, increases total P content of barley plants.

In addition, two hypotheses which could explain enhanced growth of *P. indica* colonized plants were tested: (1) *P. indica*-induced enhanced defence-readiness of the host plant (Waller et al. 2005) could result in a lower metabolic 'cost' of pathogen defence thereby increasing yield , or (2) the root endophyte could increase the photosynthetic capacity of the host plant for faster development and biomass production. We therefore analyzed yield parameters in barley grown under outdoor conditions with additional biotic stress using a fungal pathogen, and tested effects of the fungus on host plant development and on photosynthesis.

## Material and Methods

For an overview of experiments and conditions tested please refer to table 1.

**Table 1 Overview of experiments and experimental conditions**

| Experiment | Tested conditions | Cultivar | *P.. indica* | Other conditions |
|---|---|---|---|---|
| **1** Outdoor | Yield. Fertilization: Control, NK, NPK, NPK+ | Ingrid | + / - | - |
| **1** Outdoor | Yield. Fertilization: Control | Annabell | + / - | - |
| **2** Outdoor | Yield. Fertilization: Control, NK, NPK, NPK+ | Ingrid | + / - | + / - *Fusarium* (control fertilisation only) |
| **2** Outdoor | Yield. Fertilization: Control | Annabell | + / - | + / - *Fusarium* |
| **3** Greenhouse | Photosynthetic rate | Ingrid | + / - | - |
| **4** Greenhouse | N and P uptake | Ingrid | + / - | + / - *Glomus mosseae* |

**Plant and fungal material, growth conditions for yield evaluations (experiments 1 & 2)**

Spring barley cultivars *Hordeum vulgare* cv. Ingrid and cv. Annabell were used for yield evaluations. *Piriformospora indica* was provided by Dr. R. Oelmüller, Jena (Peskan-Berghöfer et al. 2004) and was propagated on a rotary shaker at 18-22°C in liquid '*Aspergillus a'* complex medium (Pham et al. 2004), with the modification that vitamin concentrations were 0.1 ppm (w/v). Mycelium from liquid culture was washed with water three times to completely remove medium, and crushed with 5-10 short pulses (5 s) of a blender (Waring Blendor, VWR International, Darmstadt, Germany). For inoculation of barley plants, 2 g of crushed mycelium was added to 300 g of substrate before sowing. Barley was grown in a 2:1 mixture of expanded clay (Seramis, Masterfoods, Verden, Germany) and Oil-Dri (Damolin, Mettmann, Germany) for four weeks. In early April 2004 (experiment 1) and 2005 (experiment 2), respectively, plants were transferred into 6 l Mitscherlich pots (six plantlets per pot), and filled with a mixture of a loam (Loess) soil and sand (1:2 v/v), as previously described (Waller et al. 2005). Experiments comprised four to six non-inoculated (control) and *P. indica*-inoculated pots, respectively, for both barley cultivars. The soil

contained 13.35 mg/100 g $K_2O$ and 10.52 mg/100 g $P_2O_5$, and had a pH($CaCl_2$) of 6.3. Plants were watered with fully de-ionized water throughout the experiments.In all experiments 1.6 g K (as $K_2SO_4$) and 0.2 g Mg (as $MgSO_4$*7 $H_2O$) were added to the soil per pot. In addition, N and P were supplied to the soil (as $NH_4NO_3$ and $Ca(H_2PO_4)_2$*$H_2O$) according to four different fertilization regimes. The 'control' fertilization (which was used throughout experiments except experiment 4 which examined the effect of *G. mosseae* and *P. indica* on N and P uptake) consisted of the addition of 0.5 g N and 0.4 g P per pot; nitrogen was applied a second time at a rate of 0.25 g N per pot two weeks after transfer of the plants to Mitscherlich pots.

For testing the influence of P and N supply, parallel experiments with three different fertilization regimes (in addition to the 'control' fertilization regime described above) were performed in cv. Ingrid. For each fertilization regime, four to six pots with six plants per pot were used. Compared to the control, P fertilization was omitted in the fertilization regime 'NK', the double amount of P was added in 'NPK', and 'NPK+' received the double amount of P and the double amount of N. Amount of added nitrogen (first application and second application) and phosphate is indicated as '(N1, N2, P [g/pot])': 'Control' (0.5, 0.25, 0.4); 'NK' (0.5, 0.25, 0.0); 'NPK' (0.5, 0.25, 0.8) and 'NPK+' (1.0, 0.5, 0.8).

In addition, increased pathogen pressure was assessed in experiment 2: For infection, the soil was inoculated with spores of the pathogen *Fusarium graminearum* by adding a suspension of macroconidia to the roots of each plant ($10^6$ conidia/plant) immediately after transfer of *P. indica*-inoculated and non-inoculated control plants into Mitscherlich pots. For each of the combinations, four to six pots with six plants per pot were used.

For plant protection, the fungicide Opus® Top (250 g/L Fenpropimorph and 84 g/L Epoxiconazole; BASF, Ludwigshafen, Germany) at a concentration of 1.5 L/ha and the insecticide Karate® (100 g/L Lambda-Cyhalothrin; Syngenta, Basel, Switzerland) at a concentration of 150 ml/ha were used in the outdoor experiments.

Presence of *P. indica* was monitored throughout the vegetation period by fluorescence microscopy of lactic acid/fuchsin stained root samples (Kormanik and McGraw 1982).

**Analysis of plant development, yield parameters and grain quality**

For detecting differences in plant development, the emergence of the inflorescence was recorded in experiments 1 & 2 10, 11 and 12 weeks after sowing. Three classes representing different developmental stages based on emergence of the ears were defined and numbers of tillers corresponding to these classes were determined. The three classes represent growth stages (GS) GS49, GS50-GS57 and GS58, respectively, according to Zadoks et al. (1974). In

addition, plant height was measured at regular intervals: Shoot length was measured from the stem base to the base of the flag leaf (up to 9 weeks after inoculation) or from stem base to the tip of the ear at later time points.

Plants were harvested when the development of ears was completed, as it is practice in field experiments. Due to climatic differences, it took 19 weeks and 16 weeks in experiment 1 (2004) and 2 (2005), respectively, from sowing until harvest. After harvesting, grains were dried and yield was calculated corresponding to a relative moisture of 86 % dry weight. In addition, the number of ears per pot, grains per ear, thousand grain weight (TGW), straw yield (weight of the straw after drying at 65°C) and the resulting harvest-index (ratio of grain yield per straw yield) were determined. For grain quality assessment, N content of the grains was determined by the method of Kjeldahl using a Büchi distillation apparatus, and P content of grains was determined by a colorimetric method according to Gericke and Kurmies (1952). Starch content measurements were performed in duplicate at the LUFA-NRW (Münster, Germany) via a polarimetric method according to EG L209/23-27 1999.

**Statistical analysis of experiments**

For statistical analysis of results, the statistics software package SPSS Ver. 17 (SPSS Inc, Chicago, Ill, USA) was used.

Analysis of variance of yield parameters was done by general linear model (GLM). All possible interactions of the respective data set were calculated. If more than two factor levels were present (e.g. comparison of four different fertilization levels [Table 5]), the respective least significant differences (LSD) were calculated in order to allow multiple comparisons.

For plant development evaluations (Fig. 1 b), the numbers of tillers within respective classes were analyzed. General Loglinear Analysis (GENLOG) was used in order to analyze the frequency counts of observations falling into each cross-classification category in a contingency table. The interactions of class, treatment and cultivar with time were included into the model allowing varying cell frequencies between different time steps.

**Assessment of photosynthetic rate (experiment 3)**

Barley plants (cv. Ingrid) were grown and inoculated with *P. indica* as described above for experiments 1 & 2. Gas exchange of the third leaf was measured using the porometer LI-6200 (LI-COR, Lincoln, NE, USA) three weeks after inoculation with *P. indica.* Measurements were carried out at the place of cultivation (greenhouse) and different light intensities were adjusted using an externally regulated halogen lamp starting with the lowest intensity. First,

saturation curves were recorded for the plants and subsequently the exchange of $CO_2$ was measured at three different light intensities (PAR 200, 800 and 1200 µmol photons $m^{-2}*s^{-1}$) to calculate the photosynthetic rate for *P. indica*-colonized and non-colonized barley plants. Each value is the mean of measurements of three or four plants.

**Assessment of root and shoot P and N content (experiment 4)**

For comparison of AM fungi and *P. indica* effects on P and N uptake of barley, a specific nutrient-poor substrate was used: Loamy sand (45 kg) was air dried, sieved (5 mm), sterilised for two days at 80°C and supplemented with 100 mg $(NH_4)_2SO_4$, 100 mg $K_2SO_4$, 5.2 mg Fe-EDTA, 10 mg $ZnSO_4$ * 7 $H_2O$ and 10 mg $CuSO_4$ * 5 $H_2O$ per kg. *Glomus mosseae* BEG-12 inoculum (Biorize R&D, Pluvault, France) was filtered and half of the spores and hyphae, which remained on the filter, were autoclaved. Autoclaved and non-autoclaved filtered inoculum was mixed with the filtrate and served as *G. mosseae* control and *G. mosseae* treatment. For *P. indica* inoculation, a suspension of $5x10^4$ spores per ml was used. Half of the spore suspension was autoclaved for the *P. indica* control, the other half served as *P. indica* treatment. Previous experiments had shown that autoclaved material had no effect on plant growth (unpublished). Fertilized soil (65 g) was mixed with 7 ml of *G. mosseae* control (10 % w/w) or *G. mosseae* treatment inoculum, and filled in 50 ml-pots. Barley seeds were surface-disinfected for 10 min in 4 % sodium hypochlorite and germinated on filter paper soaked with a saturated solution of $CaSO_4$. Three-day-old seedling roots were dipped into the autoclaved or living spore suspension of *P. indica* for 5 min. Each seedling was transplanted into a pot with the respective substrate. Plants were grown in a greenhouse until harvest (26±3° C, 60-70 % relative humidity) and colonization with *G. mosseae* and *P. indica* was microscopically confirmed by trypan blue staining (Phillips and Hayman 1970) three weeks and twelve weeks after inoculation. Colonization intensities at the latter time point were estimated according to Trouvelot et al. (1986) for *G. mosseae* (M = 12 %) and according to Bütehorn et al. (2000) for *P. indica* (absolute spread of spores = 18 %). Both values were typically observed in our experiments. Barley plants were transferred into 250 ml pots two weeks and harvested twelve weeks after germination. Dried and ground shoot and root samples were dry-ashed and dissolved in 18.5 % HCl. Phosphorus was photometrically analyzed with an EPOS-analyzer 5060 (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. Nitrogen was determined after dry oxidation according to the manufacturer's protocol (Elementar Vario EL, Hanau, Germany).

## Results

### Growth and development of *Piriformospora indica* colonized barley plants

Barley plants with roots colonized by *P. indica* showed significantly higher shoot lengths in cv. Annabell up to 9 weeks after inoculation in both experiments (Table 2). In cv. Ingrid, higher shoot lengths were observed 9 and 12 weeks (experiment 1), and 12 weeks after inoculation (experiment 2). At the end of the vegetation period (two weeks before harvest), *P. indica*-colonized plants of both cultivars were not significantly taller than non-inoculated control plants (Table 2).

**Table 2 Development of shoot length of *Piriformospora indica* colonized and non-colonized barley plants**

| Experiment | Weeks after inoculation | Shoot length [cm] / difference cv. Ingrid | | | Shoot length [cm] / difference cv. Annabell | | |
|---|---|---|---|---|---|---|---|
| | | Control | *P. indica* | difference | Control | *P. indica* | difference |
| 1 (2004) | 8 | 30.9 | 32.8 | + 6 % | 25.9 | 32.6* | + 26 % |
| | 9 | 36.6 | 43.6* | + 19 % | 31.3 | 38.2* | + 22 % |
| | 12 | 94.0 | 99.5* | + 5.8 % | 79.8 | 85.9* | + 7.7 % |
| | 18[1] | 93.5 | 93.5 | 0 % | 75.4 | 76.2 | + 1.1 % |
| 2 (2005) | 7 | 25.4 | 24.3 | - 4 % | 14.1 | 19.4* | + 37 % |
| | 9 | 41.4 | 44.0 | + 6 % | 29.0 | 40.6* | + 40 % |
| | 12 | 95.0 | 100.4* | + 6 % | 88.3 | 89.2 | + 1 % |
| | 14[1] | 106.6 | 106.5 | - 0.3 % | 93.2 | 90.4* | - 3.0 % |

Shoot lengths of plants were determined in two experiments at the time points indicated. Asterisks indicate statistically significant differences of *P. indica* plants compared to respective control plants (Students t-test $p < 0.01$).

[1] Shoot length did not increase after this time point until harvest.

For detecting differences in plant development, the emergence of the inflorescence was recorded (Fig. 1). For *P. indica* colonized plants, higher numbers of further developed tillers were recorded as in respective control plants (Fig. 1). The higher frequency of further developed tillers within respective classes was significant ($p < 0.05$) for *P. indica* colonized plants in both cultivars at all three time points (Fig. 1). *P. indica* root colonization significantly elevated grain yield between 5.3 and 11 % in cv. Ingrid and cv. Annabel in experiments performed in two subsequent growing seasons (experiment 1 [Waller et al. 2005] and experiment 2 [data not shown]).

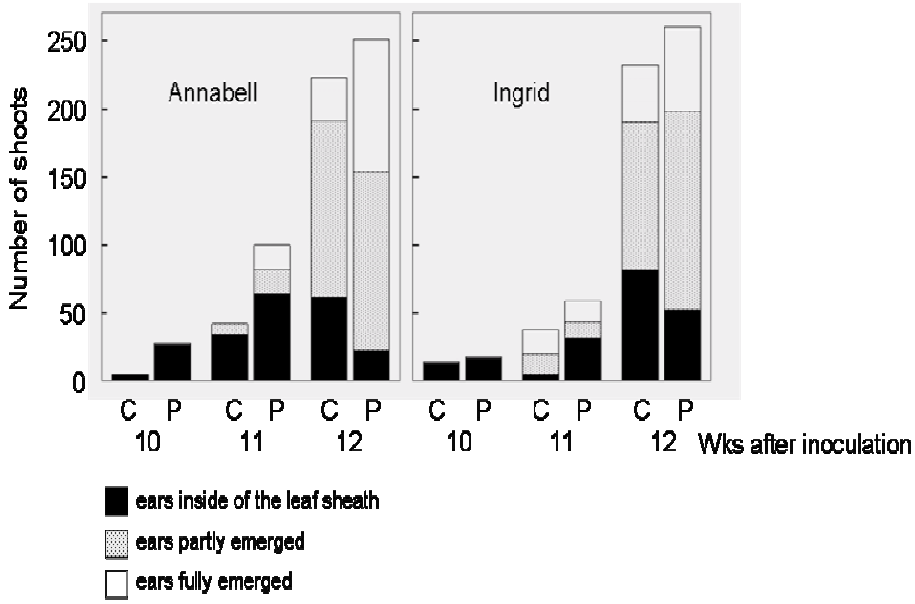


**Figure 1 Development of the inflorescence of *Piriformospora indica* inoculated and non-inoculated barley plants under outdoor conditions.** Emergence of ears from the flag leaf was assessed 10, 11, and 12 weeks after inoculation of roots with *P. indica* (P) and in non-inoculated control plants (C). Numbers of tillers with awns or ears emerging from the flag leaf sheath were determined for three classes: (1) emerged awns (ears still inside of the flag leaf sheath), (2) partly emerged ears and (3) ears fully emerged from the flag leaf sheath. Shown are mean values for spring barley cultivars Annabell and Ingrid from the experiment performed in 2005, with 36 plants per treatment. Similar results were obtained in 2004.

**Influence of *P. indica* on yield parameters under different nutrient regimes**

To determine if *P. indica* induced higher grain yield depends on soil P and N content, different fertilization regimes were tested in cv. Ingrid. Plants were fertilized without P (NK), with 200 % P (NPK), and with 200 % P and 200 % N (NPK+) as compared to control fertilization. Grain yield for NK (lacking P fertilization) was about 11 % lower and for NPK+ about 40 % higher than that of the control fertilization (Table 3a). *P. indica* significantly induced higher grain yield in a similar range in all four different fertilization regimes (Table 3a). The observed *P. indica* effects on yield parameters were similar in the two experiments performed in subsequent growing seasons (Supplementary Table 1).

Analysis of variance revealed that both *P. indica* and fertilization alone significantly influenced grain yield, straw yield, harvest index, ears per pot and thousand-grain-weight (Table 3b). In addition, 'fertilization' significantly influenced grain weight per ear (Table 3b). The factor 'experiment' influenced most yield parameters reflecting differences in climatic conditions of two subsequent growing seasons. A significant interaction of the factors '*P. indica*' and 'fertilization' was detected only for the harvest index, and all three factors

significantly interact with respect to the number of ears per pot and grain yield. This was also the case for the interaction of '*P. indica'* and 'experiment', which, in addition, was significant with respect to the harvest index.

**Table 3 Effect of *Piriformospora indica* on yield parameters under different fertilisation regimes**

Table 3a. Means of yield parameters of *P. indica* inoculated and non-inoculated barley plants (cv. Ingrid) grown under four different fertilisation regimes. LSD: Least significant difference between means at P = 0.05.

| Fertilisation regime ($N_1$, $N_2$, P [g / pot]) | *P. indica* inoculation | Grain yield per pot [g] | Straw yield per pot [g] | Harvest index | Ears per pot | Grains per ear | Grain weight per ear [g] | TGW** [g] |
|---|---|---|---|---|---|---|---|---|
| '**Control**'* (0.5, 0.25, 0.4) | - | 51.08 | 50.55 | 1.01 | 46.13 | 22.01 | 1.08 | 48.01 |
| | + | 53.90 | 51.79 | 1.04 | 49.58 | 23.14 | 1.11 | 47.79 |
| '**NK**' (0.5, 0.25, 0.0) | - | 45.30 | 46.18 | 0.98 | 43.96 | 22.48 | 1.04 | 45.07 |
| | + | 47.91 | 50.69 | 0.96 | 43.53 | 22.65 | 1.05 | 46.90 |
| '**NPK**' (0.5, 0.25, 0.8) | - | 47.68 | 47.67 | 1.00 | 45.75 | 22.81 | 1.13 | 47.80 |
| | + | 53.63 | 52.56 | 1.03 | 48.71 | 21.91 | 1.05 | 47.89 |
| '**NPK+**' (1.0, 0.5, 0.8) | - | 70.67 | 68.05 | 1.04 | 67.50 | 23.13 | 1.13 | 46.57 |
| | + | 77.83 | 69.27 | 1.13 | 69.63 | 22.91 | 1.18 | 47.61 |
| **LSD** | | 2.15 | 2.36 | 0.04 | 2.23 | 1.23 | 0.08 | 2.15 |

* Part of the 'Control' values (experiment 1) were published in Waller et al. 2005.
** TGW Thousand grain weight, P values lower than 0.05 are depicted in bold letters.

Table 3b. Analysis of variance. P values for the factors '*P. indica*', 'experiment' and 'fertilisation regime' and all possible interactions were calculated according to the general linear model (GLM).

| Factors | Grain yield per pot | Straw yield per pot | Harvest index | Ears per pot | Grains per ear | Grain weight per ear | TGW** |
|---|---|---|---|---|---|---|---|
| *P. indica (P.i.)* | **<0.001** | **0.001** | **0.029** | **0.012** | 0.913 | 0.944 | **0.031** |
| Experiment (Exp.) | **0.032** | **<0.001** | **<0.001** | **<0.001** | 0.141 | 0.086 | 0.753 |
| Fertilisation (Fert.) | **<0.001** | **<0.001** | **<0.001** | **<0.001** | 0.742 | **0.035** | **<0.001** |
| *Exp. x* Fert. | **<0.001** | **0.002** | **0.004** | **<0.001** | 0.091 | 0.472 | **0.002** |
| *P.i.* x Fert. | 0.093 | 0.235 | **0.039** | 0.322 | 0.420 | 0.341 | 0.089 |
| *P.i.* x Exp. | **0.001** | 0.875 | **0.001** | **0.003** | 0.661 | 0.595 | 0.611 |
| Exp. x *P.i.* x Fert. | **0.026** | 0.072 | 0.152 | **0.006** | 0.328 | 0.233 | 0.749 |

** TGW Thousand grain weight, P values lower than 0.05 are depicted in bold letters.

**Analysis of nutrient uptake in *Piriformospora indica* and *Glomus mosseae* colonized barley plants**

P and N contents were determined in barley plants inoculated with *P. indica* as well as with the AM fungus *Glomus mosseae*. Average root dry weights were 0.52, 0.53 and 0.71 g per plant for non-inoculated, *P. indica*- and *G. mosseae*-inoculated plants, with only the value of *G. mosseae* colonized plants being significantly higher than control plants (Students t-test $p < 0.05$). Average shoot dry weights were 2.86, 2.85 and 3.40 g per plant for non-inoculated, *P. indica*- and *G. mosseae*-inoculated plants, with both treatments not significantly different from control plants (Students t-test $p > 0.05$). N contents were only slightly elevated by *G. mosseae* colonization, while the P content was significantly increased in the shoots (+ 59 %) and the roots (+ 51 %) of *G. mosseae*-colonized plants (Fig. 2). *P. indica*-colonized plants, however, showed no significant increase of P or N contents (Fig. 2).

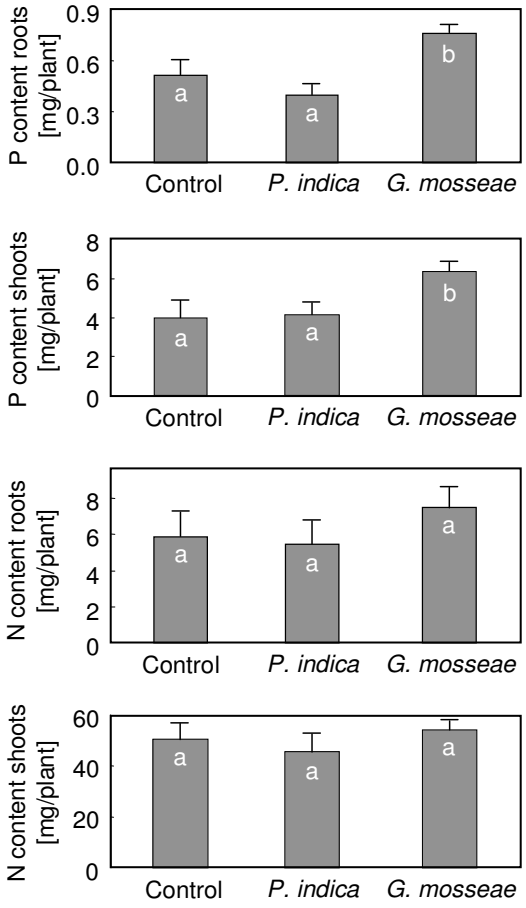


**Figure 2 P and N content of roots and shoots of barley inoculated and non-inoculated with *Piriformospora indica*.** Plants inoculated with *P. indica* (and autoclaved *G. mosseae*) and / or *Glomus mosseae* (and autoclaved *P. indica*) and plants inoculated with both autoclaved fungi (control plants) were grown on a nutrient-poor soil. At harvest, dry weights of roots and shoots and respective amounts of P and N were determined. Values are means of four plants per treatment. Error bars indicate standard deviation. Bars labelled with different letters are significantly different ($p < 0.05$) according to the Mann-Whitney U-test.

**Influence of *P. indica* on grain yield under elevated pathogen stress**

In order to determine whether the effect of *P. indica* on yield can be explained by an improved pathogen resistance of the host plant, we inoculated a set of plants within experiment 2 with the root-rot causing pathogen *F. graminearum.* This elevated biotic stress caused significant effects on the yield parameters harvest index, grains per ear, grain weight per ear and thousand-grain-weight (Table 4b). *P. indica* increased grain yield to a similar degree in *F. graminearum* challenged and unchallenged plants: *P. indica* increased grain yield in *F. graminearum* inoculated plants by 5.6 % (cv Annabell) and 6.8 % (cv Ingrid), while *P. indica*-dependent grain yield increase without pathogen challenge was in the range of 5 % and 5.3 %, respectively (Table 4a). In line with these results, analysis of variance revealed that the effect of the factor '*P. indica'* on all yield parameters was independent of the factor '*F. graminearum'* (Table 4b).

**Table 4 Influence of *Piriformospora indica* on yield parameters of *Fusarium graminearum* inoculated and non-inoculated plants**

Table 4a. Mean values of yield parameters of *F. graminearum* inoculated *P. indica*-colonized and non-colonized plants.

| Cultivar | *F. graminearum* inoculation | *P. indica* inoculation | Grain yield per pot [g] | Straw yield per pot [g] | Harvest index | Ears per pot | Grains per ear | Grain weight per ear [g] | TGW* [g] |
|---|---|---|---|---|---|---|---|---|---|
| Ingrid | - | - | 51.83 | 48.74 | 1.07 | 44.50 | 21.61 | 1.03 | 48.05 |
| Ingrid | - | + | 54.71 | 50.40 | 1.09 | 47.67 | 23.80 | 1.13 | 47.44 |
| Ingrid | + | - | 50.36 | 48.26 | 1.04 | 46.33 | 20.87 | 0.99 | 45.69 |
| Ingrid | + | + | 54.09 | 49.82 | 1.09 | 48.17 | 21.52 | 1.01 | 46.13 |
| Annabell | - | - | 56.70 | 44.37 | 1.28 | 44.50 | 24.45 | 1.21 | 49.31 |
| Annabell | - | + | 59.73 | 45.32 | 1.32 | 48.83 | 24.06 | 1.23 | 49.99 |
| Annabell | + | - | 55.04 | 45.28 | 1.22 | 45.33 | 22.74 | 1.16 | 48.80 |
| Annabell | + | + | 58.33 | 46.82 | 1.25 | 46.00 | 22.96 | 1.16 | 48.78 |

* TGW Thousand grain weight, P values lower than 0.05 are depicted in bold letters.

Table 4b. Analysis of variance. P values for the factors '*P. indica*', 'cultivar' and 'pathogen (*F. graminearum*)' and all possible interactions calculated according to the general linear model (GLM).

| Factors | Grain yield per pot | Straw yield per pot | Harvest index | Ears per pot | Grains per ear | Grain weight per ear | TGW* |
|---|---|---|---|---|---|---|---|
| *P. indica (P.i.)* | **<0.001** | **0.038** | **0.029** | **0.011** | 0.129 | 0.217 | 0.776 |
| Cultivar (cv.) | **<0.001** | **<0.001** | **<0.001** | 0.598 | **0.001** | **<0.001** | **<0.001** |
| *F. graminearum (F.g.)* | 0.137 | 0.613 | **0.011** | 0.930 | **0.002** | **0.020** | **0.002** |
| *P.i. x Cv.* | 0.931 | 0.788 | 0.890 | 1.000 | 0.089 | 0.411 | 0.617 |
| *Cv. x F.g.* | 0.781 | 0.200 | 0.062 | 0.256 | 0.903 | 0.764 | 0.244 |
| *P.i. x F.g.* | 0.747 | 0.858 | 0.851 | 0.191 | 0.598 | 0.375 | 0.838 |
| *P.i. x Cv. x F.g.* | 0.862 | 0.793 | 0.620 | 0.539 | 0.223 | 0.587 | 0.297 |

* TGW Thousand grain weight, P values lower than 0.05 are depicted in bold letters.

**Influence of *P. indica* on the photosynthetic rate of barley leaves**

To analyze possible mechanisms underlying the faster early development of *P. indica*-colonized plants, the impact of *P. indica* on photosynthesis was evaluated. The photosynthetic rate was measured in third leaves of barley plants 3 weeks after inoculation with the endophyte. Compared to non-inoculated control plants we found a 49 % increase of the photosynthetic rate at low light (200 µmol photons $m^{-2}$ $s^{-1}$), while no effect of the endophyte was recorded at saturating light conditions (800 and 1200 µmol photons $m^{-2}$ $s^{-1}$) (Fig. 3).

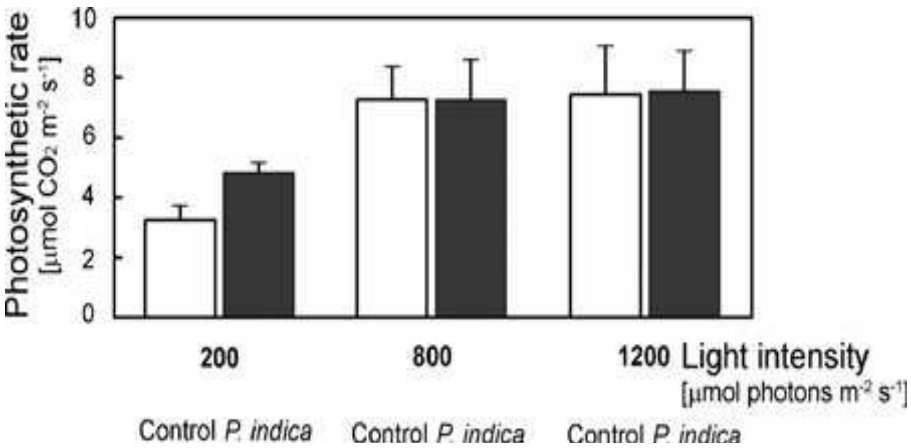


**Figure 3 Photosynthetic rate of barley plants inoculated and non-inoculated with *Piriformospora indica*.** The photosynthetic rate of barley (cv. Ingrid) was measured in *P. indica* colonized (black bars) and in non-colonized control plants (white bars). Values are means of three to four plants measured at three different light intensities. Error bars indicate standard deviation. Statistically significant differences between control and *P. indica* inoculated plants were detected only for the light intensity of 200 µmol photons $m^{-2}$ $s^{-1}$ (Students t-test $p < 0.05$).

## Discussion

Colonization with *Piriformospora indica* positively influences host plant growth (Varma et al. 1999; Rai et al., 2001; Peskan-Berghöfer et al. 2004) and induces higher grain yield and resistance against local and systemic pathogen infection in the crop plant barley (Waller et al. 2005).

Our results presented here confirm the induction of higher grain yield by *P. indica,* mainly due to an increase in the number of ears per plant (Table 3a). While average grain weight and the number of grains per ear are genetically determined to a large extent, the number of tillers and resulting number of ears is influenced during specific phases of plant development by cues from the different plant organs, such as photosynthetic activity, source-sink relationships, and nutrient supply (Borgmann 2000). To elucidate possible mechanisms leading to enhanced grain yield, we analyzed plant development, yield parameters under different fertilization conditions, and photosynthetic activity. Furthermore, we compared P and N content of barley plants colonized with an arbuscular mycorrhizal fungus and with *P. indica*.

We first tested the hypothesis that *P. indica* might improve grain yield only when P or N supply are low, as described for symbiotic interactions of arbuscular mycorrhizal (AM) fungi and their host plants (e.g. Smith and Read 1997; Govindarajulu et al. 2005; Bucher 2007). Indeed, P and N fertilization were able to increase barley grain yield in our experiments by up to 55 %, compared to control fertilization (Table 3a). This increase was to a large extent due to an increase in the number of ears per plant. Because this correlation was observed also for the *P. indica* effect in all experiments, it was feasible to suggest that *P. indica* might elevate grain yield by increasing P and N supply. In addition, it was reported that *P. indica* induces increased P levels in Arabidopsis (Shahollari et al 2005). However, all results obtained in this study suggest a different mechanism for *P. indica* colonized plants: (1) The endophytes' positive influence on grain yield was independent of the factor 'fertilization' (Table 3) and was observed in a similar magnitude when P and N supply was high, indicating that *P. indica*-induced yield increase does not depend on low P or N supply. In contrast, AM fungi are influencing host biomass mainly at suboptimal P availability in the soil (Smith and Read 1997). (2) Direct comparison of P and N contents of barley plants inoculated with *P. indica* and with the AM fungus *G. mosseae* revealed that the latter increased P content of shoots and roots significantly, while no effect of *P. indica* on P or N content could be detected (Fig. 2). The effect of the AM fungus was therefore based on increasing host plant nutrient concentrations. In line with this result, Barazani et al. (2005) showed that *P. indica* did not

induce a significant increase of leaf P and N content in the dicotyledonous plant *Nicotiana attenuata*. (3) mRNA levels of the barley phosphate transporter *HvPht1;8* which is specifically expressed in barley roots colonized by AM fungi (Glassop et al. 2005), were not significantly influenced by *P. indica (*Deshmukh, Sharma and Baltruschat, unpublished results). The latter finding resembles results with the potato phosphate transporter *StPT3*, which is specifically expressed in arbuscular mycorrhiza-colonized root regions (similar to *HvPht1;8*), but is not responding to inoculation with *P. indica* (Karandashov et al. 2004).

Taken together, we show that *P. indica* effects on yield in the crop plant barley are not dependent on low P supply, do not lead to an elevated total P or N content, and are therefore mechanistically different from effects of AM fungi. This is in line with marked morphological differences of AM fungi and *P. indica* colonized roots: The latter is forming inter- and intracellular hyphae in the root and requires host cell death, while it is not inducing arbuscule-like interaction structures in the host tissues (Deshmukh et al. 2006). Despite the lack of specialized interaction structures, the intimate contact and crosstalk between the symbiotic partners (e.g. Deshmukh et al. 2006; Schäfer et al. 2009) results in a number of beneficial effects on the host plant which could contribute to the observed higher grain yield. Three main effects, (1) biotic stress resistance, (2) higher assimilation at low light intensities and (3) faster development are discussed below for their putative role in enhancing grain yield.

*P. indica* induces enhanced resistance of the host against fungal pathogens, e.g. powdery mildew (*Blumeria graminis*), root rot (*Fusarium culmorum, F. graminearum*) and the fungal stem base pathogen *Pseudocercosporella herpotrichoides* (Waller et al. 2005; Deshmukh and Kogel 2007; Serfling et al. 2007). Increased grain yield could therefore –at least in part- result from an increased biotic stress resistance, as observed for some, but not all, pathogens in AM colonized plants (Dehne 1982; Cordier et al. 1998; Gernns et al. 2001; Pozo et al. 2002). Root inoculation with *F. graminearum* decreased several yield parameters, while *P. indica* was able to increase yield under these conditions of elevated biotic stress to a similar extent as without stress. Furthermore, no statistically significant interaction (with respect to yield parameters) between the pathogen and the endophyte was observed (Table 4). This shows that positive *P. indica* effects on yield can be independent of low levels of pathogen pressure, and enhanced biotic stress resistance alone cannot explain the observed grain yield increase.

*P. indica* enhanced the host plants` photosynthetic rate under low light conditions (Fig. 3). The tested low light condition of 200 µmol photons $m^{-2}$ $s^{-1}$ corresponds to the light intensity during dusk and dawn, but also at noon if the sky is overcast in temperate regions (e.g. Humbeck et al. 1994). Therefore, increased assimilation of *P. indica* colonized plants could

significantly contribute to faster development and higher yield. In contrast, plants colonized by AM fungi show enhanced photosynthetic activity at both low and high light intensities (Mathur and Vyas 1995; Caravaca et al. 2003), which was shown to be independent of leaf phosphate concentrations (Fay et al. 1996; Wright et al. 1998). This could indicate that the mechanism of *P. indica* influencing the rate of photosynthesis is different from AM fungi. A reason for a higher photosynthetic rate at low light could be higher chlorophyll content, which would be in line with an observed darker green pigmentation of *P. indica* colonized plants up to the age of 8 weeks. Determination of relative chlorophyll content between 8 and 12 weeks in the two barley cultivars under outdoor conditions did not indicate consistently higher relative chlorophyll contents of *P. indica* colonized plants (Achatz 2006). A higher photosynthesis rate of host plants without differences in chlorophyll content was also observed in experiments analysing the effects of AM fungi (e.g. Paradi et al. 2008).

*P. indica* is not only inducing a faster development of plants (Fig. 1), but is also enhancing growth early in development: Three weeks after inoculation total plant fresh weight, leaf length and root fresh weight were significantly elevated in barley (Waller et al. 2005; Achatz 2006; Baltruschat et al. 2008). At this stage of development, *P. indica* colonized roots are further developed, as suggested by earlier expression of developmentally regulated genes (Waller et al. 2008). Such enhancement of root growth could be based either on fungal production of phytohormones like the auxin indole acetic acid, as shown in the interaction of *P. indica* with *Arabidopsis* (Sirrenberg et al. 2007) and as discussed for the effect of *P. indica* on the rooting of cuttings (Druege et al. 2007). Recent results of barley root transcriptome analysis, on the other hand, suggest a rather complex interplay of *P. indica* with the host plant, including transiently altered expression levels of gibberelic acid biosynthesis genes and abscisic acid responsive genes, but not a strong broad-scale induction of auxin-induced genes in the early phases of root colonization (Schäfer et al. 2009). The molecular details of *P. indica*-induced enhanced growth early in development therefore remain to be clarified.

## Conclusions

We show here that two beneficial effects induced by *P. indica* –faster development and higher grain yield of barley- are independent of biotic stress, independent of P and N fertilization, and are based on mechanisms different from AM fungi, which specifically improve P supply of their host plants. Our current working hypothesis for enhanced yield and development of *P. indica* colonized barley plants can be summarized as follows: *P. indica* colonization enhances growth of roots and shoots from about three weeks after inoculation. This enhanced early root

growth could support enhanced shoot growth and is accompanied by a higher photosynthetic activity under low light conditions. These initial advantages of *P. indica* colonized plants lead not only to a faster emergence of ears, but also to an increased number of tillers and ears which, finally, result in higher grain yield.

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# Chapter 6

# General discussion

# 6. General Discussion

The present thesis deals with the characterization of a number of root-endophytic fungi isolated from tomato plants of four crop sites of Colombia and presents experiments concerning the interaction of the model root endophyte *Piriformospora indica* with the crop plants tomato and barley. In the subsequent chapter four important aspects of this study will be discussed:

1. The isolation, the taxonomic characterization and the root colonization of new root endophytic fungi
2. The impact of three new fungal isolates and *Piriformospora indica* on tomato plant growth and fruit yield and quality
3. The impact of three new fungal isolates and *Piriformospora indica* on plants protection against *Verticillium dahliae*
4. The comparison of *Piriformospora indica* with an arbuscular mycorrhizal fungus concerning uptake of mineral nutrients

## 6.1 Isolation, taxonomic characterization and root colonization of new root endophytic fungi.

Tomato plants from different sites in Colombia showed to be hosts of root fungal endophytes of different taxa among the phylum Ascomycota also including some Dark Septate Endophytes (DSEs). According to the general classification proposed by Rodriguez *et al*. (2009), they correspond to the non-clavicipetaceous endophytes. These class 4 endophytes colonize only root tissues of a broad range of plant hosts, seem to be highly diverse and are horizontally transmitted. Some of the isolates of the order Hypocreales (E9, E27 and E67), however, might belong to the class 2 endophytes which colonize not only roots but also shoots. Class 2 endophytes can be horizontally and vertically transmitted and correspond to the genera *Fusarium*, *Bionectria* and *Trichoderma*. They show a high ecological diversity. Some species are saprotrophs or facultative biotrophs and species of the genera *Fusarium* and *Trichoderma* have been reported as endophytes also in leaves and stems (Schroers, 2001; Shoresh *et al*., 2010).

The other endophytic isolates belong to different orders among the Ascomycota such as Phyllachorales (E20), Sordariales (E22), Pleosporales (DSE36, DSE41, DSE48, DSE49, DSE63 and E52) Chaetosphaeriales (E133) and Helotiales (DSE131 and DSE135). It is

interesting that these fungi represented a highly diverse taxonomy in spite of the low frequency of recovering from disinfected tomato root fragments. The Pleosporales were dominant and four of them were related to unknown fungi. This opens a view on the potential fungal community existent in roots of vegetable crops being different from the forest ecosystem where usually DSEs of the order Helotiales (e.g *Phialocephala* spp., *Phialophora finlandica*, *Leptodontidium orchidicola*) were recovered (Jumpponen and Trappe, 1998; Mandyam and Jumpponen, 2005). Moreover, the results also point to the need assessing the diversity and function of uncultured fungal endophytes across field crops by molecular techniques (Arnold and Lutzoni, 2007). 33% of the isolates from tomato showed such a limited growth and could not be sufficiently recovered for subsequent characterization indicating their dependence on the plant host.

The commercial cultivation of tomato seemed to have an impact on the number and population of endophytes. More root fungal isolates were obtained from plants of semi-commercial- and organic-grown crops (18 and 22 fungi, respectively) than from conventional farming systems (< 7 isolates). Especially the DSEs were only recovered from sites where no mineral fertilizers and pesticides were applied. Analysis of the *Zea mays* root endophytic community with group-specific primers differentiated between organic and mineral agricultural practices for some groups of bacteria. In contrast, the number of cultivable root endophytic fungi was not affected and the use of herbicides did not change their population structure (Seghers *et al*., 2004). It has taken, however, into account that the number of samples in the present study did not allow a statistical analysis of the distribution among the sites. Further analyses using also culture-independent methods are necessary to confirm the suggested impact of crop management on fungal communities inside of roots.

An *in vitro* system on agar plates was used to evaluate colonization patterns in roots of tomato seedlings under controlled conditions. The endophytes showed different types of root contact such as appressoria-like structures (E27, DSE63 and DSE135), swollen cells (DSE49 and DSE63), only single adhesion tips (E20, E67 and DSE135) and single and branched adhesion tips (DSE41, DSE48, DSE49, DSE63 and E133). Colonization patterns for DSEs have been described for *Heteroconium chaetospira* (Narisawa *et al*., 2000) and *Phialocepala fortinii* (Currah *et al*., 1993) showing that these endophytes form appressoria, but other kind of structures at the first contact with the root has been here reported for the first time. The occurrence of these different root contact types with one host opens the question, if these types are characteristic for the fungus and independent of the plant. They neither seem to be

typical for a particular type of endophyte nor do they predict the outcome of the interaction. Appressoria are formed by DSEs and non-DSEs endophytes and are followed by interactions involving a plant response (DSE63) or not (E27 and DSE135). These results indicate that such structures might be characteristic for a particular plant-interacting isolate, but not for a certain mode of interaction. Further studies using the same isolates but different plant hosts should be carried out to prove this hypothesis.

Hyphal penetration occurred in most cases through the intercellular space. Subsequently, fungal growth further progressed inter- and intracellular in the epidermal cell layer and in most cases in the cortex, but with different intensities. Variations in the colonization patterns was observed before: Depending on the host and the species root-interacting fungi stayed on the root surface entered the epidermal layer or grew through the cortex (Nariwasa *et al*., 2004; Peterson *et al*., 2008; Wu *et al*., 2010). Degradation of cortical and vascular parenchyma cells has been reported for some DSEs (Peterson *et al*., 2008), but this could be observed for none of the endophytes in the current analysis.

Particular structures were hyphal constrictions at sites where the fungus traverses the cell wall (see DSE41, DSE48, DSE49 and E133). Formation of hyphal coils or vesicles being characteristic for the fungus-host combination (Abdellatif *et al*., 2009) were, however, rarely observed. Only the DSE49 and the putative pathogen E63 formed intracellular microsclerotia-like structures or hyphal coils. Microsclerotia were frequently found in roots colonized by *P fortinii*, where they were typically melanized (Jumpponen and Trappe, 1998). They also can be associated with root hairs of dicotyledonous and monocotyledonous species (Peterson *et al*., 2008). In tomato roots, however, these structures were fluorescent by the labelled chitin-antibody and did not show melanization. They could be therefore interpreted as hyphal knots formed by growing mycelium in living root cells (Abdellatif *et al*., 2009). In such cells, colonization pattern was irregular. In dead root cells, however, hyphae grow regularly in a linear direction as it was observed for DSE63 and E133. Hence, regular growth was correlated with the pathogenicity of the two fungi. These two endophytes probably possess necrotrophic features killing numerous root cells they colonized without any barriers, while all other endophytes were forced by the living cells to a more irregular growth pattern.

In summary, roots of field-grown tomatoes are exposed to and colonized by diverse endophytic fungi which belong to the phylum Ascomycota and present different ways of colonization. Some of them seem to be new species or new anamorphic stages of known teleomorphs, others are new isolates from already described plant-interacting fungi. Phylogenetic linkage between these new endophytes and fungi previously identified does not

necessarily indicate the same ecological function as has been shown before (Freeman *et al*., 2001). In some cases, strains were identified as belonging to plant pathogenic species e.g. *Colletotrichum* sp. These isolates could simply represent avirulent races or they might act as endophytes or pathogens depending on the host as it has been shown for *Colletotrichum acutatum* (Freeman *et al*., 2001). Alternatively, putative pathogenic features might appear only at very late stages of the development as described for some fungi that belong to *Botryosphaeriaceae* (Slippers and Wingfield, 2007). The plant host and the genetic background of the fungus play important roles to adopt certain lifestyle during colonization (Freeman and Rodriguez, 1993; Redman *et al*., 1999; Kogel *et al*., 2006; Peterson *et al*., 2008).

### 6.2 The impact of three new fungal isolates and *Piriformospora indica* on tomato plant growth and fruit yield and quality.

It has been suggested by a number of researchers that naturally occurring endophytes are much more effective in the rhizosphere than other biocontrol agents are. Due to their colonization capacity and permanence in the plant they are much better adapted to this hostile environment (Hoitink and Boehm, 1999; Narisawa *et al*., 2002). They might be therefore used to improve plant growth or to confer non-habitat/habitat–adapted benefits to the plant host (Saikkonen *et al*., 2004; Rodriguez *et al*., 2009). Hence, the effect of the new isolates on tomato performance was analyzed in pot cultures under greenhouse conditions. Among 14 pre-selected endophytes, E22, E52 and E133 could not be recovered from disinfected tomato roots and hyphae were difficult to observe inside the trypan blue-stained roots. This result shows at least for E133 that colonization capacity depends on the environmental conditions. The *in vitro* colonization of the root could not be confirmed in the pot cultures. One problem might be the disinfection procedures which could inhibit outgrowth of the endophyte, the other critical technique is the light microscopy where visualization of the fine hyaline hyphae of some isolates could be difficult. One alternative techniques is the PCR on DNA extracts from colonized roots as it has been used to quantify virulent and avirulent fungal isolates of *Verticillium dahliae* (Mercado-Blanco *et al*., 2003; Shittu *et al*., 2009) or the endophyte *Acremonium alternatum* (Jäschke *et al*., 2010). This can, however, not distinguish between surface and internal colonization. GFP-labelling of root colonizers has been successfully applied for the specific detection and observation of fungal development *in planta* and avoided any misinterpretations (Skadsen and Hohn, 2004; Grunewaldt-Stöcker *et al*., 2007). This tool helps to unequivocal visualization of tiny, inconspicuous root endophytes. It is

however very time-consuming as it includes genetic engineering of the fungus under investigation and therefore not suitable for screening numerous new isolates.

Significant negative effects on plant development were detected in the treatments with the isolates E9, E22, E27, DSE63 and E133, especially concerning the shoot weight. This corroborates the symptoms observed on plates for DSE63. The optimal growth conditions for the plants in pot cultures might have avoided the spread of the symptoms induced by E20 in the root to the whole plant. In case of E133, the defense reactions observed *in vitro* could have successfully prevented intense root colonization. This would explain the negative results of the recovery tests and the light microscopy, as well as the absence of any negative effects. In contrast, root symptoms were not evident for E27 in seedlings, but negative impact on whole plant development (shoots, roots and blooms) was obvious. Heavy colonization of the roots in vitro without any symptoms indicates the absence of any defense reaction by the plant. Such an intense hyphal growth might uses resources that are necessary for an optimal plant development. The results of this experiment show how important it is to evaluate colonization and pathogenicity or promoting effects not only *in vitro* but also *in vivo* for the selection of endophytes with potential use as biological agents.

Three of the fungal isolates (DSE48, DSE49 and DSE135, identified as *Leptodontidium orchidicola*) were selected for further analysis because they clearly colonized the roots of tomato and they never showed a negative effect on any of the plant growth parameters. They were investigated together with *Piriformospora indica* concerning their impact on early and late plant development and fruit yield and quality.

Inoculation of tomato plants with DSE49, *L. orchidicola* and *P. indica* influenced significantly plant fresh and dry weights at the early plant growth stage between 5-7 weeks. However, this positive effect was not evident when tomato growth was estimated after 22 weeks of cultivation. It is interesting to see that fungi of different phyla can have the same impact on growth of a particular plant. This indicates that the same mechanisms are underlying this phenomenon. Plant growth promotion by DSE was observed in some investigations, but either plants were relatively young (Wu *et al*., 2010) and/or effects were dependent on the host (Fernando and Currah, 1996), on environmental conditions (Alberton *et al*., 2010) or on the substrate (Jumpponen *et al*., 1998; Upson *et al*., 2009). That vegetative plant growth promotion by fungal endophytes is dependent on the developmental stage of the plant was shown here for the first time. The beneficial effect of the endophytes can be observed up to the stage around inflorescence emergence after 7 weeks of cultivation. Such a phenomenon was also observed in barley plants inoculated with *P. indica*. Shoot lengths were

increased at early stages, but biomass of colonized and non-colonized plants were similar at time of harvest (Achatz *et al*., 2010). Metabolic changes in the plants could influence the interaction with the endophytes. At later stages of the development of annual plants, sink strength relations changes, because fruit growth consumes more and more of the photoassimilates (Obiadalla-Ali *et al*., 2004). This was confirmed in tomato by studies on the gene *LIN5*. This gene encoding a cell wall invertase which is exclusively expressed at the floral-to-fruit transition and down-regulation of its expression leads to lower levels of sugars in the fruits (Zanor *et al*., 2009). This shift of carbohydrate distribution between different sink organs could alter the outcome of the plant-endophyte interaction and might lead to the reduction of the positive effects on the vegetative growth. Further investigations following the distribution of carbohydrates not only at a particular stage, but during the development from early to late vegetative growth will be necessary to clarify the metabolic changes underlying these observations. Another line of explanation concerns phytohormone production and sensing. Acceleration of plant growth could be influenced directly by fungal auxin-like substances as it has been described for *P. indica* (Sirrenberg *et al*., 2007) and for the DSE *Colletotrichum* sp. (Lu *et al*., 2000). Additional, *P. indica* have shown to influence hormone signal transduction and/or hormone-regulated gene expression (Barazani *et al*., 2007; Vadassery *et al*., 2008; Schäfer *et al*., 2009). The growth of younger plants might be more sensitive for such hormonal doses or changes. It will be interesting to analyze if the newly isolated DSEs also produce auxin-like substances and other phytohormones.

Promotion of plant performance was also observed during generative development. Plants inoculated with *L. orchidicola* and *P. indica* showed accelerated flowering and significantly higher fruit biomass at the beginning of the harvests. Hence, when the plant switches from its vegetative to the generative phase of development, the promoting effect by the endophytes was also transferred from the one type to the other type of organs. Comparing colonized and non-colonized plants, it turned, however, out that these differences could not be observed any more at later dates of harvest. Finally, total yields of tomato fruits were not significantly different. In contrast, *P. indica* was able to increase total yield in barley up to 11%. This seems to be not a general difference between mono-and dicotyledonous plants. A higher number of total seeds as a result of inoculation with *P. indica* could be obtained in tobacco (Barazani *et al.*, 2005) and Arabidopsis (Shahollari *et al.*, 2007), while total yield of winter wheat was not influenced (Serfling *et al.*, 2007). The reasons for these differences are not clear up to know. Where inoculation with endophytes increases yield and can be therefore

recommended for application in plant production, has probably to be therefore tested for each crop and each management system separately.

Fruit quality of tomato plants inoculated with the DSEs or with *P. indica* did generally not change. In one experiment, however, *L. orchidicola*-colonized plants showed significantly higher levels of glucose. Glucose concentration depends among others on enzymatic activity of the acid invertase (Johnson *et al*., 1998; Zanor *et al*., 2009) which is also regulated by phytohormones. High levels of gibberellic acid, auxins and abscisic acid induce the expression of the corresponding genes (Roitsch *et al*., 2003). Hence, higher glucose content in colonized plants might be also a consequence of the endophyte interfering with the phytohormone balance of the plant. This must be, however, specific for *L. orchidicola*, dependent on colonization intensity and only effective at early stages of harvest.

### 6.3 The impact of three new fungal isolates and *Piriformospora indica* on plants protection against *Verticillium dahliae*.

In addition to the impact on plant development, experiments were carried out for estimating the ability of the 3 DSEs and of *P. indica* to reduce disease symptoms in tomato plants after challenge with the fungal pathogen *V. dahliae*. At a low pathogen pressure (2 x $10^5$ conidia $mL^{-1}$), expression of disease symptoms were dependent on environmental conditions and the endophytes DSE49, *L. orchidicola* and *P. indica* were able to reduce the impact of *V. dahliae*. At higher pathogen concentrations (7 x $10^6$ conidia $mL^{-1}$) disease symptoms were always visible, but only *P. indica* reduced the negative effect on plant growth (Appendix 11) and reduced the disease index together with DSE49 in one out of two experiments.

These experiments revealed at first that the environmental conditions play an important role for disease expression by *V. dahliae* as it has been already shown before (Robb, 2007). At low humidity, disease symptoms were expressed in plants inoculated even with low concentrations of the pathogen. Vessels are occluded by the pathogen leading to a higher stem resistance of water flow resulting in lower water content in wilted leaves (Pegg and Brady, 2002). Additionally, light might play a role. Leaf photosynthesis is greatly impaired by *V. dahliae* infection, with lower net assimilation rates (Pegg and Brady, 2002). Higher radiation could therefore minimize the impact by the pathogen.

The protective effect against the pathogen probably depended on the pathogen pressure, but the environmental conditions might have also influenced the outcome of these tripartite interactions. In the first experiment with the lower concentrations of conidia, the substrate was not mixed with sand and had therefore a higher concentration of organic compounds. DSEs

facilitate the uptake of organic nitrogen, phosphate, sulphur compounds and carbon (reviewed in Mandyam and Jumpponen, 2005) and positive growth promotion by different DSEs was only observed when plants were grown in substrate amended with organic nitrogen (Upson *et al*., 2009). A second factor, which might have influenced the interactions, is light. The protective effect was most prominent in the experiment where the mean daily radiation was lowest. Since it was shown in barley that *P. indica* is able to increase photosynthetic rate at low light conditions (Achatz *et al*., 2010) one might speculate that this had also affected the protective abilities in the present experiments.

Different phytohormones as salicylic acid (SA), jasmonate (JA), ethylene (ET) and abscisisc cid (ABA) are involved in the mechanism underlying induced resistance in plants (see general introduction). Investigations in *Arabidopsis* show that SA and JA are not involved in the resistance of plants against *V. dahliae*, but a mutation in the receptor (*etr* 1-1) for ET perception induces up-regulation of the defense-related proteins PR1, PR2 and PR5 (Pantelides *et al*., 2010). It has been shown; however, for *P. indica* that jasmonate is involved in the induced resistance in Arabidopsis against powdery mildew (Stein *et al*., 2008). Which of the phytohormone signaling pathways is involved in the tripartite interaction (plant-endophyte-pathogen) can be in future analyzed by means of tomato mutants or transgenic tomato lines with alterations in the production or perception of JA, ET, SA and ABA.

## 6.4 **The comparison of *Piriformospora indica* with an arbuscular mycorrhizal fungus concerning uptake of mineral nutrients.**

*P. indica* improves grain yield in barley independent on low or high phosphate (P) and nitrogen (N) fertilization, since yield promotion was similarly high under different fertilizer treatments (Chapter 5.3). This was also shown for the interaction of the endophyte with tobacco (Barazani *et al.*, 2005). Additionally, while plants colonized with *Glomus mosseae* showed a clear enhanced uptake of P and N, this could not be observed for *P. indica*. This endophyte also induced mycorrhiza-regulated phosphate transporters neither in barley (Achatz *et al.*, 2010) nor in potato (Karandashov *et al*. 2004). Promotion of plant growth by *P. indica* seems not to be based on improved plant nutrition as it has been shown for mycorrhizal symbioses (Smith and Read, 1997). However recently, a phosphate transporter (**PiPT**) expressed in the external hyphae of *P. indica* was characterized and its activity is involved in P transfer from the fungus to maize plants (Yadav *et al*., 2010). This seems also to contribute to the plant growth promotion observed in this experimental system. In tomato plants *P. indica* promoted plant growth at low P concentrations slightly more than under optimal

fertilization (Chapter 5.1). This might indicate that the promoting effect in tomato could be at least partially consequence of P transfer from the fungus to the plant. The mechanism of transfer must be, however, very different from that shown for the biotrophic AM fungi, since *P. indica* only colonizes dead plant cells (Deshmukh *et al*., 2006).

### 6.5 Conclusions and outlook of the research

A number of new root-endophytic fungi are presented in this study. They are taxonomically described, as well as characterized for their colonization ability of tomato roots and their impact on tomato plant growth. Some of them could be new species or new anamorphic stages of known teleomorphs, others are new isolates from already described plant-interacting fungi. Better resolution of the phylogenetic relationships could be obtained in future by sequencing of other regions of their genomes as e.g. the complete 18s and 28s rDNA or genes encoding ß-tubulin or the translation factor EF-1alpha, as it has been done for some pathogenic fungi (Klosterman *et al*., 2009). Another line of research could be directed into the ecology and community structure of such root endophytes. This must include culture-independent methods as sequencing of the root metagenome from other vegetable crops among the *Solanaceae* from different agronomic sites and their wild relatives from natural ecosystems. More directed approaches to identify root endophytes useful for application, should be targeted to crop indigenous fungi belonging to the very wide-spread non-mycorrhizal members of the *Sebacinales* (Selosse *et al*., 2009) or to the *Trichoderma* species similar to the one identified in this study which are described to influence plant-pathogen interactions (Cordo *et al*., 2007).

The new tomato root endophytes and *P. indica* showed different effects on plant growth depending to the development stage of the plant. The interactions therefore cover a continuum from mutualism to commensalism that at least for *P. indica* may become even parasitic under particular nutrient regimes. Plant protective effects could also be only observed under distinct experimental constrains. More research is necessary to understand the mechanisms underlying these interactions and the requirements for applying these endophytes in horticulture without any risk. Experiments should be focused on the way how internal factors as phytohormones and external environmental conditions affect the distribution of resources especially carbohydrates between the partners of the symbiosis and between different organs of the plant.

# 7 Summary

Non-mycorrhizal fungal endophytes are able to colonize internally roots without causing visible disease symptoms establishing neutral or mutualistic associations with plants. These fungi known as non-clavicipitaceous endophytes have a broad host range of monocot and eudicot plants and are highly diverse. Some of them promote plant growth and confer increased abiotic-stress tolerance and disease resistance. According to such possible effects on host plants, it was aimed to isolate and to characterize native fungal root endophytes from tomato (*Lycopersicon esculentum* Mill.) and to analyze their effects on plant development, plant resistance and fruit yield and quality together with the model endophyte *Piriformospora indica*.

Fifty one new fungal strains were isolated from desinfected tomato roots of four different crop sites in Colombia. These isolates were roughly characterized and fourteen potential endophytes were further analyzed concerning their taxonomy, their root colonization capacity and their impact on plant growth. Sequencing of the ITS region from the ribosomal RNA gene cluster and in-depth morphological characterisation revealed that they correspond to different phylogenetic groups among the phylum Ascomycota. Nine different morphotypes were described including six dark septate endophytes (DSE) that did not correspond to the *Phialocephala* group. Detailed confocal microscopy analysis showed various colonization patterns of the endophytes inside the roots ranging from epidermal penetration to hyphal growth through the cortex. Tomato pot experiments under glass house conditions showed that they differentially affect plant growth depending on colonization time and inoculum concentration.

Three new isolates (two unknown fungal endophyte DSE48, DSE49 and one identified as *Leptodontidium orchidicola*) with neutral or positiv effects were selected and tested in several experiments for their influence on vegetative growth, fruit yield and quality and their ability to diminish the impact of the pathogen *Verticillium dahliae* on tomato plants. Although plant growth promotion by all three fungi was observed in young plants, vegetative growth parameters were not affected after 22 weeks of cultivation except a reproducible increase of root diameter by the endophyte DSE49. Additionally, *L. orchidicola* increased biomass and glucose content of tomato fruits, but only at an early date of harvest and at a certain level of root colonization. Concerning bioprotective effects, the endophytes DSE49 and *L. orchidicola*

decreased significantly disease symptoms caused by the pathogen *V. dahliae*, but only at a low dosis of the pathogen.

In order to analyze, if the model root endophytic fungus *Piriformospora indica* could be suitable for application in production systems, its impact on tomato was evaluated. Similarly to the new fungal isolates, significant differences for vegetative growth parameters were only observable in young plants and, but protection against *V. dahliae* could be seen in one experiment also at high dosage of the pathogen. As the DSE *L. orchidicola*, *P. indica* increased the number and biomass of marketable tomatoes only at the beginning of fruit setting, but this did not lead to a significant higher total yield. If the effects on growth are due to a better nutrition of the plant with mineral element was analyzed in barley in comparison to the arbuscular mycorrhizal fungus *Glomus mosseae*. While the mycorrhizal fungus increased nitrogen and phosphate uptake of the plant, no such effect was observed for *P. indica*.

In summary this work shows that many different fungal endophytes can be also isolated from roots of crops and, that these isolates can have positive effects on early plant development. This does, however, not lead to an increase in total yield or in improvement of fruit quality of tomatoes under greenhouse conditions.

# 8 Zusammenfassung

Endophyten, die nicht zu den Mykorrhizapilzen gehören, können das Innere von Wurzeln ohne sichtbare Krankheitssymptome besiedeln und bilden so mit der Pflanze neutrale oder mutualistische Wechselwirkungen. Diese Pilze, auch als nicht-clavicipetale Endophyten bekannt, haben ein breites Wirtsspektrum von mono- und dikotyledonen Pflanzen und weisen eine hohe Diversität auf. Einige von ihnen fördern Pflanzenwachstum und erhöhen Resistenz und Toleranz gegenüber biotischem und abiotischem Stress. Ausgehenden von diesen möglichen Effekten auf ihre Wirtspflanzen war das Ziel der vorliegenden Arbeit die Isolierung und Charakterisierung neuer pilzlicher Wurzelendophyten der Tomate (*Lycopersicon esculentum* Mill.) und die Analyse ihres Einflusses auf Pflanzenentwicklung und Pflanzenresistenz, sowie auf Ertrag und Fruchtqualität unter Einbeziehung des Modellendophyten *Piriformospora indica*.

Aus vier verschiedenen Anbaugebieten in Kolumbien konnten 51 neue Pilzstämme von oberflächensterilisierten Tomatenwurzeln isoliert werden. Diese Isolate wurden vorcharakterisiert und 14 potentielle Endophyten bezüglich ihrer Taxonomie, ihrer Besiedlungsmuster und ihres Einfluss auf das Pflanzenwachstum näher untersucht. Sequenzierung der ITS Region des ribosomalen RNA Genclusters und genaue morphologische Charakterisierung zeigten, dass sie zu verschiedenen phylogenetischen Gruppen innerhalb der Ascomycota gehören. Neun Morphotypen ließen sich beschreiben, wobei sechs zu den ‚Dark Septate Endophytes' (DSEs) gehören, aber nicht mit der bekannten *Phialocephala* Gruppe verwandt waren. Ausführliche konfokale mikroskopische Untersuchungen ergaben sehr verschiedene Besiedelungsmuster der Wurzelendophyten vom Endringen in die Epidermis bis zum Hyphenwachstum durch den Kortex. Topfexperimente unter Gewächshausbedingungen zeigten dass die Isolate in Abhängigkeit von der Inokulumkonzentration und der Zeit der Besiedlung das Wachstum der Tomaten sehr unterschiedlich beeinflussten.

Drei neue Isolate (die beiden unbekannte pilzlichen Endophyten DSE48 und DSE49 und eines identifiziert als *Leptodontidium orchidicola*) mit neutralen oder positiven Effekten wurden für weitere Versuche ausgewählt. In mehreren Experimenten sollte ihr Einfluss auf das vegetative Wachstum, auf Ertrag und auf Fruchtqualität untersucht werden, sowie ihre Fähigkeit die Auswirkungen des Pathogens *Verticillium dahliae* auf Tomatenpflanzen zu vermindern.

Obwohl wachstumsfördernde Effekte durch alle drei Pilze in jungen Pflanzen beobachtet wurden, waren vegetative Wachstumsparameter nach 22 Wochen der Besiedlung nicht mehr beeinflusst bis auf ein signifikante Erhöhung des Wurzeldurchmessers durch den Endophyten DSE49. *L. orchidicola* dagegen erhöhte die Biomasse und den Glukosegehalt der Früchte, aber nur zu frühen Ernteterminen und bei einer bestimmten Intensität der Wurzelbesiedelung. Hinsichtlich eines schützenden Effekts, konnten die Endophyten DSE49 und *L. orchidicola* die Krankheitssymptome, die durch *V. dahliae* verursacht wurden, vermindern, aber nur bei einem geringen Pathogendruck.

Um zu überprüfen, ob der Modellendophyt *P. indica* in Produktionssytemen eingesetzt werden kann, wurde seine Auswirkungen auf Tomaten untersucht. Ähnlich wie die neuen pilzlichen Isolate, zeigte aber auch er seinen fördernden Einfluss nur auf das frühe vegetative Wachstum. Schützende Effekte gegen *V. dahliae* konnten ebenfalls nur bei niedrigem Pathogendruck konstant beobachtet werden. Wie *L. orchidicola* erhöhte *P. indica* die Biomasse an marktfähigen Tomaten am Anfang des Fruchtansatzes, was nicht zu einem insgesamt höheren Ertrag führte. Ob die beobachteten Effekte auf ein verbesserte Nährstoffversorgung der Pflanze zurückzuführen seien, wurde in Gerste im Vergleich mit dem arbuskulären Mykorrhizapilz *Glomus mosseae* untersucht. Während der Mykorrhizapilz sowohl Phosphat wie Stickstoffaufnehme der Pflanze erhöhte, konnte dies für *P. indica* nicht festgestellt werden.

Zusammenfassend zeigt diese Arbeit, dass auch aus Wurzeln von Kulturpflanzen viele verschiedene pilzliche Endophyten isoliert werden können, und dass einige von diesen durchaus einen positiven Effekt auf die frühe Pflanzenentwicklung aufweisen. Zumindest für Tomate unter Gewächshausbedingungen führen diese Effekte aber nicht zu einer Erhöhung des Gesamtertrags oder einer nachhaltigen Verbesserung der Fruchtqualität.

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# 10 Appendices

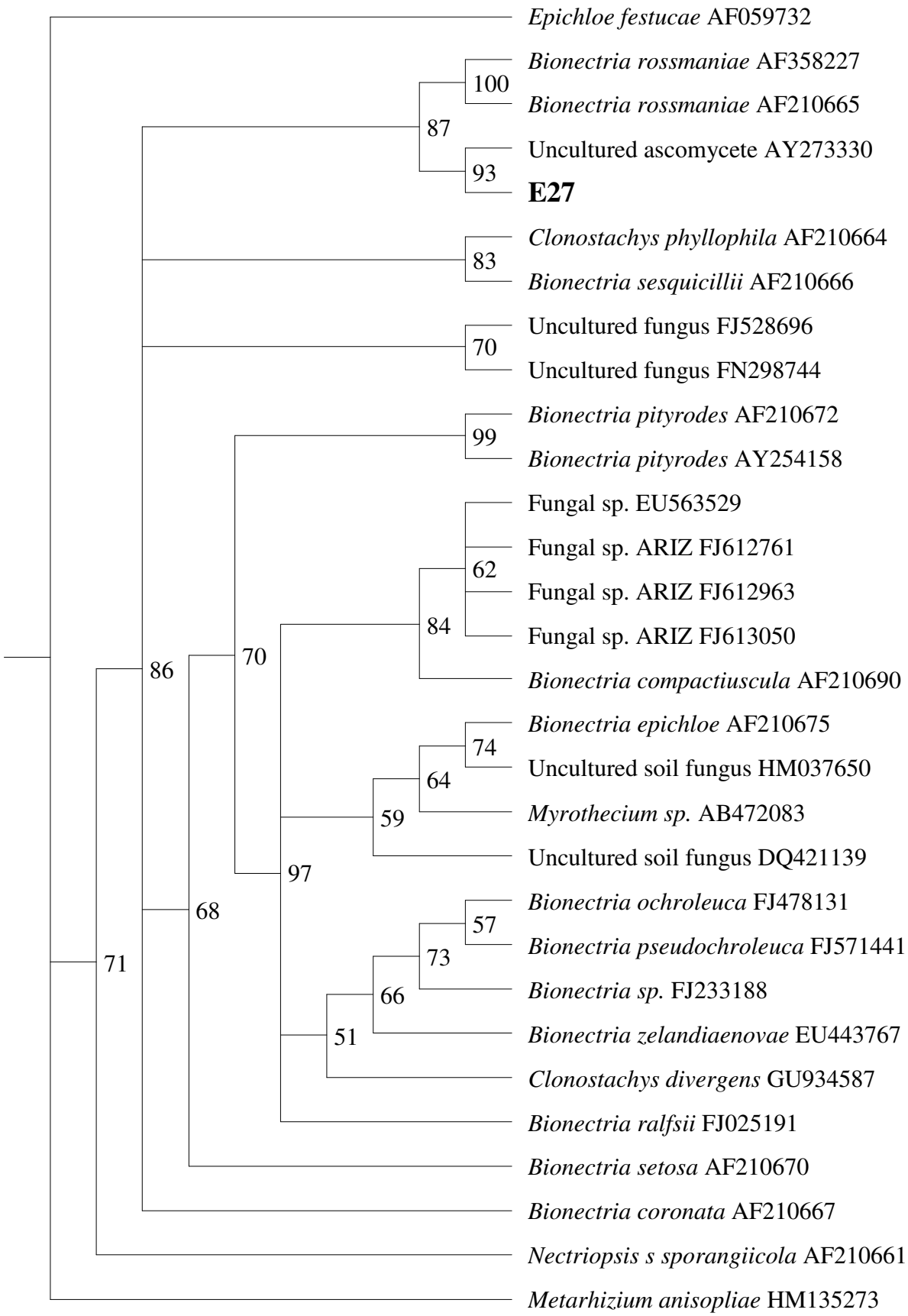
## APPENDIX 1

Phylogenetic analysis of endophytic fungi isolated from tomato roots. Sequences of the internal transcribed spacer (ITS) regions from the endophytes were aligned using the program MUSCLE with ITS sequences from related genera.

As outgroups, the ITS sequence from *Epichloe festuca* was used for the endophytes E27 (a) and E67 (b), *Endomeliola dingleyae* for E20 (c), *Preussia polymorpha* for E36, E41 and E48 (d), *Triplosphaeria maxima* for E49 (e), *Paraphoma chrysanthemicola* for E63 (f), *Blumeria graminis* for E131 and 135 (g) and *Pilidiella granati* for E133 (h).

Based on the alignment, phylogeny was reconstructed by maximum-likelihood with the program PUZZLE and the distance tree was displayed by the program TREEVIEW. Quartet puzzling support values of 1000 replicates are indicated.

**APPENDIX 1a**


*Epichloe festucae* AF059732
*Bionectria rossmaniae* AF358227
*Bionectria rossmaniae* AF210665
Uncultured ascomycete AY273330
**E27**
*Clonostachys phyllophila* AF210664
*Bionectria sesquicillii* AF210666
Uncultured fungus FJ528696
Uncultured fungus FN298744
*Bionectria pityrodes* AF210672
*Bionectria pityrodes* AY254158
Fungal sp. EU563529
Fungal sp. ARIZ FJ612761
Fungal sp. ARIZ FJ612963
Fungal sp. ARIZ FJ613050
*Bionectria compactiuscula* AF210690
*Bionectria epichloe* AF210675
Uncultured soil fungus HM037650
*Myrothecium sp.* AB472083
Uncultured soil fungus DQ421139
*Bionectria ochroleuca* FJ478131
*Bionectria pseudochroleuca* FJ571441
*Bionectria sp.* FJ233188
*Bionectria zelandiaenovae* EU443767
*Clonostachys divergens* GU934587
*Bionectria ralfsii* FJ025191
*Bionectria setosa* AF210670
*Bionectria coronata* AF210667
*Nectriopsis s sporangiicola* AF210661
*Metarhizium anisopliae* HM135273
100
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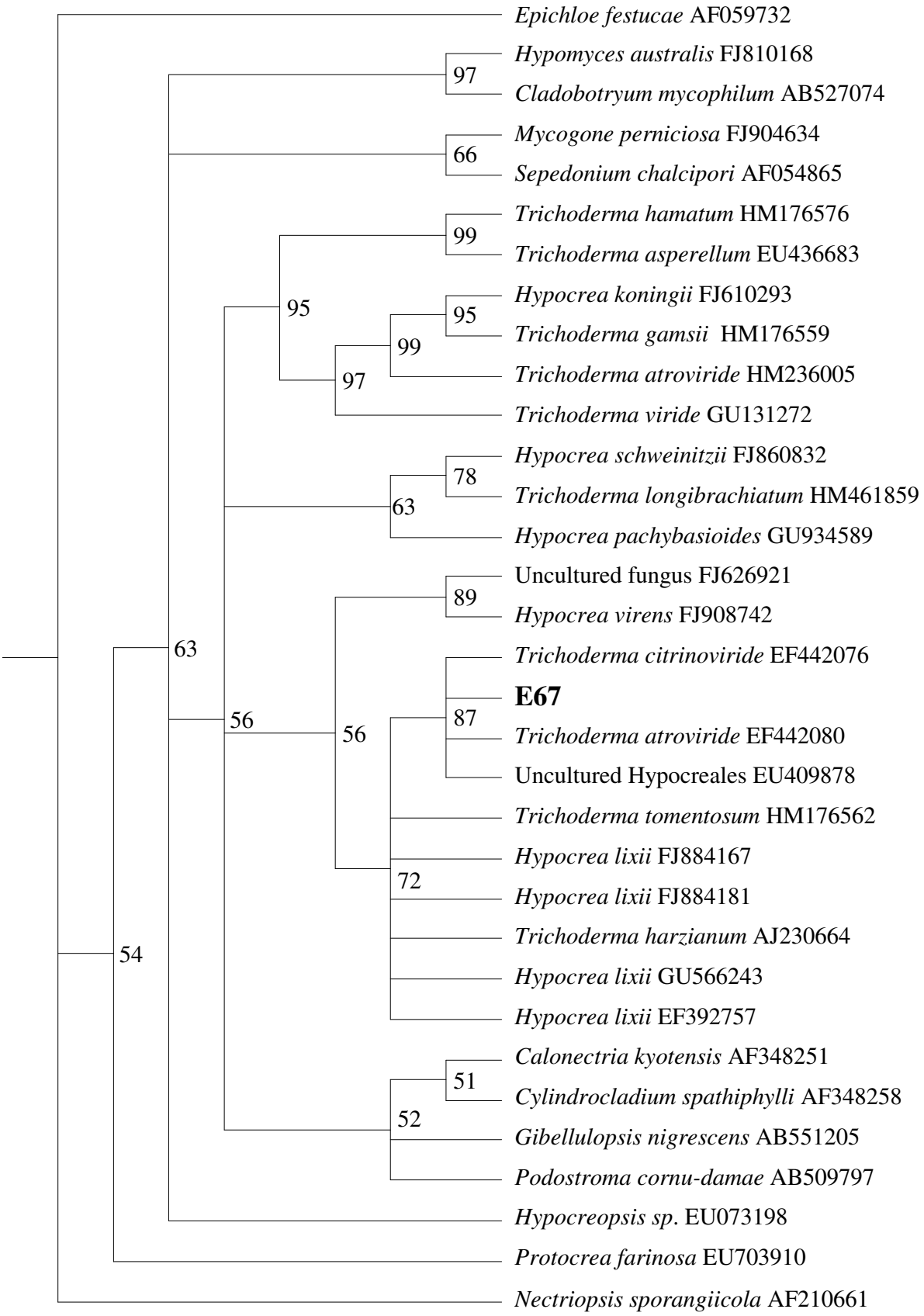
**APPENDIX 1b**


*Epichloe festucae* AF059732
*Hypomyces australis* FJ810168
97
*Cladobotryum mycophilum* AB527074
*Mycogone perniciosa* FJ904634
66
*Sepedonium chalcipori* AF054865
*Trichoderma hamatum* HM176576
99
*Trichoderma asperellum* EU436683
*Hypocrea koningii* FJ610293
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95
*Trichoderma gamsii* HM176559
99
*Trichoderma atroviride* HM236005
97
*Trichoderma viride* GU131272
*Hypocrea schweinitzii* FJ860832
78
*Trichoderma longibrachiatum* HM461859
63
*Hypocrea pachybasioides* GU934589
Uncultured fungus FJ626921
89
*Hypocrea virens* FJ908742
63
*Trichoderma citrinoviride* EF442076
**E67**
56
87
56
*Trichoderma atroviride* EF442080
Uncultured Hypocreales EU409878
*Trichoderma tomentosum* HM176562
*Hypocrea lixii* FJ884167
72
*Hypocrea lixii* FJ884181
*Trichoderma harzianum* AJ230664
54
*Hypocrea lixii* GU566243
*Hypocrea lixii* EF392757
*Calonectria kyotensis* AF348251
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*Cylindrocladium spathiphylli* AF348258
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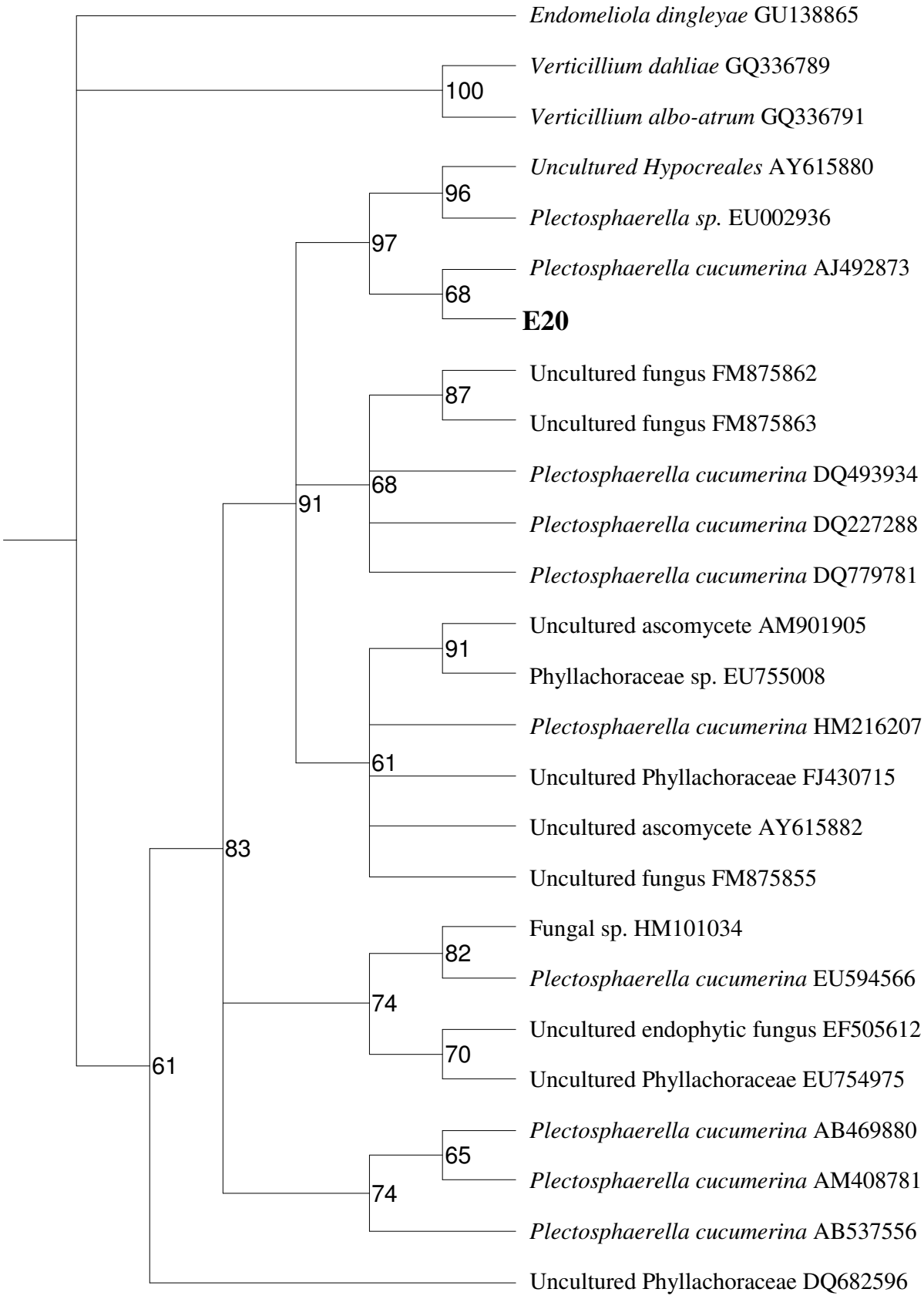
*Gibellulopsis nigrescens* AB551205
*Podostroma cornu-damae* AB509797
*Hypocreopsis sp*. EU073198
*Protocrea farinosa* EU703910
*Nectriopsis sporangiicola* AF210661

**APPENDIX 1c**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*Verticillium dahliae* GQ336789
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*Verticillium albo-atrum* GQ336791
*Uncultured Hypocreales* AY615880
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*Plectosphaerella sp.* EU002936
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*Plectosphaerella cucumerina* AJ492873
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**E20**
Uncultured fungus FM875862
87
Uncultured fungus FM875863
*Plectosphaerella cucumerina* DQ493934
68
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*Plectosphaerella cucumerina* DQ227288
*Plectosphaerella cucumerina* DQ779781
Uncultured ascomycete AM901905
91
Phyllachoraceae sp. EU755008
*Plectosphaerella cucumerina* HM216207
61
Uncultured Phyllachoraceae FJ430715
Uncultured ascomycete AY615882
83
Uncultured fungus FM875855
Fungal sp. HM101034
82
*Plectosphaerella cucumerina* EU594566
74
Uncultured endophytic fungus EF505612
70
61
Uncultured Phyllachoraceae EU754975
*Plectosphaerella cucumerina* AB469880
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*Plectosphaerella cucumerina* AM408781
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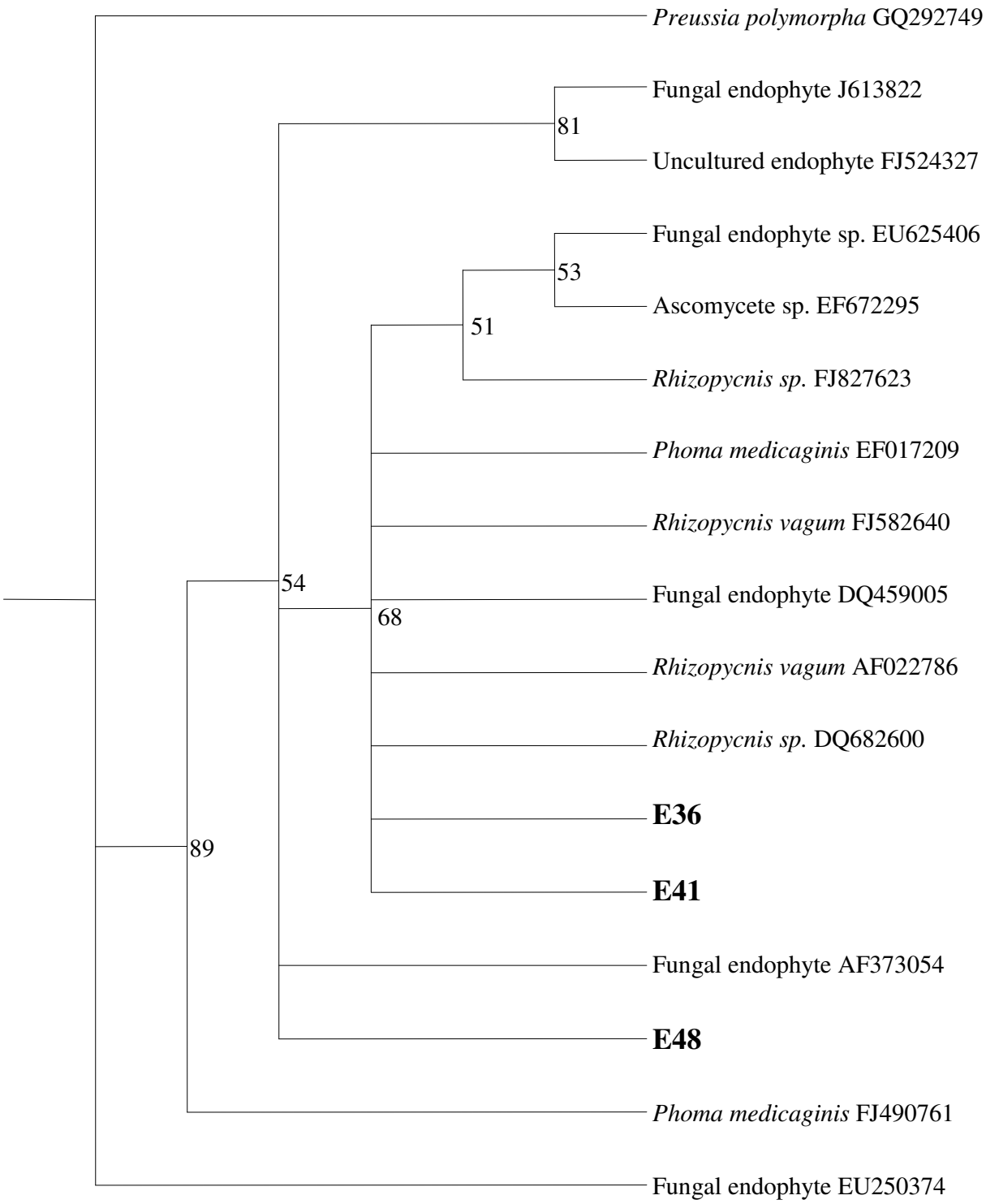
*Plectosphaerella cucumerina* AB537556
Uncultured Phyllachoraceae DQ682596

**APPENDIX 1d**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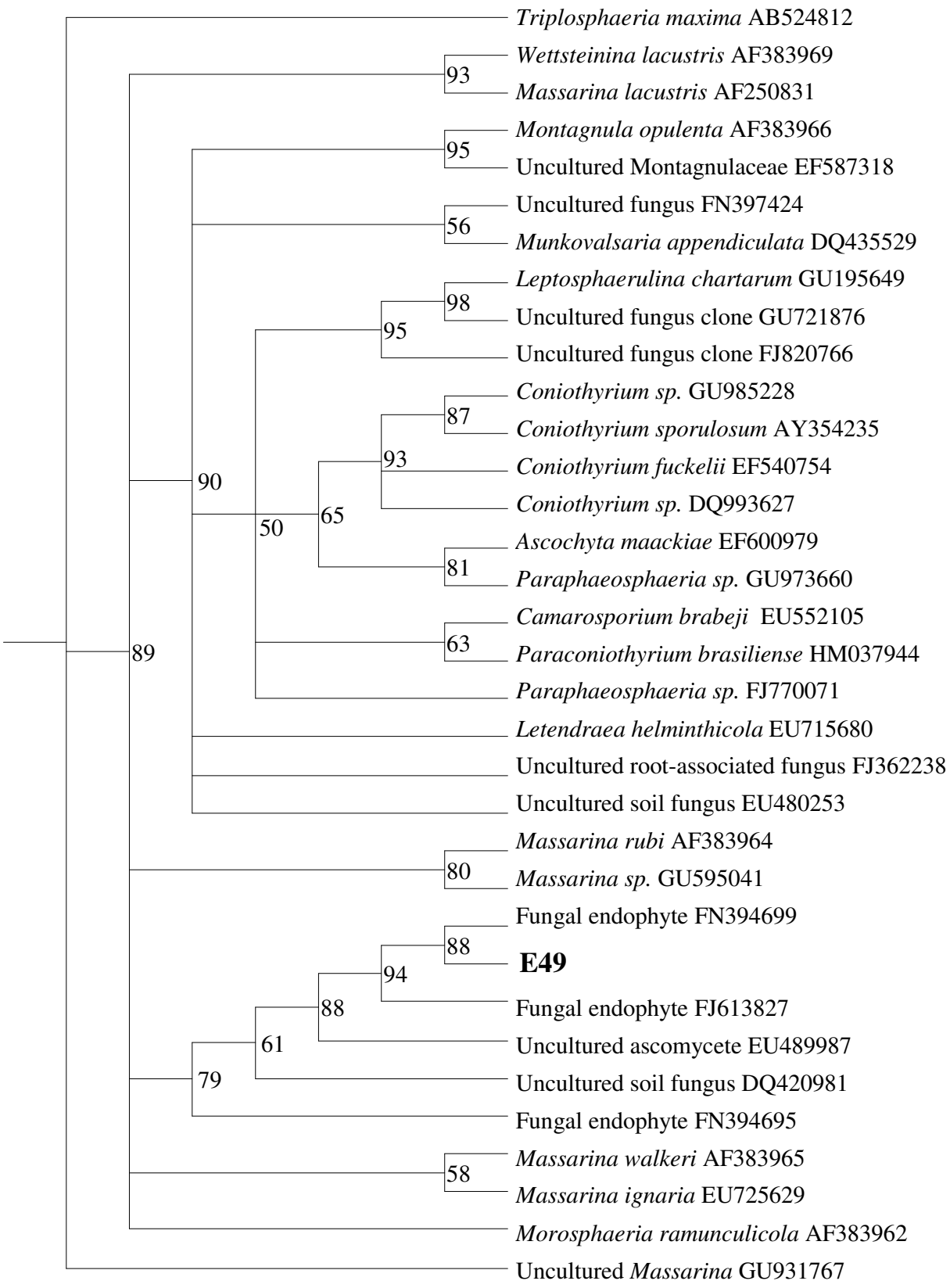
Fungal endophyte J613822
81
Uncultured endophyte FJ524327
Fungal endophyte sp. EU625406
53
Ascomycete sp. EF672295
51
*Rhizopycnis sp.* FJ827623
*Phoma medicaginis* EF017209
*Rhizopycnis vagum* FJ582640
54
Fungal endophyte DQ459005
68
*Rhizopycnis vagum* AF022786
*Rhizopycnis sp.* DQ682600
**E36**
89
**E41**
Fungal endophyte AF373054
**E48**
*Phoma medicaginis* FJ490761
Fungal endophyte EU250374

**APPENDIX 1e**


*Triplosphaeria maxima* AB524812
*Wettsteinina lacustris* AF383969
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*Massarina lacustris* AF250831
*Montagnula opulenta* AF383966
95
Uncultured Montagnulaceae EF587318
Uncultured fungus FN397424
56
*Munkovalsaria appendiculata* DQ435529
*Leptosphaerulina chartarum* GU195649
98
Uncultured fungus clone GU721876
95
Uncultured fungus clone FJ820766
*Coniothyrium sp.* GU985228
87
*Coniothyrium sporulosum* AY354235
93
*Coniothyrium fuckelii* EF540754
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*Coniothyrium sp.* DQ993627
65
50
*Ascochyta maackiae* EF600979
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*Paraphaeosphaeria sp.* GU973660
*Camarosporium brabeji* EU552105
63
89
*Paraconiothyrium brasiliense* HM037944
*Paraphaeosphaeria sp.* FJ770071
*Letendraea helminthicola* EU715680
Uncultured root-associated fungus FJ362238
Uncultured soil fungus EU480253
*Massarina rubi* AF383964
80
*Massarina sp.* GU595041
Fungal endophyte FN394699
88
**E49**
94
88
Fungal endophyte FJ613827
61
Uncultured ascomycete EU489987
79
Uncultured soil fungus DQ420981
Fungal endophyte FN394695
*Massarina walkeri* AF383965
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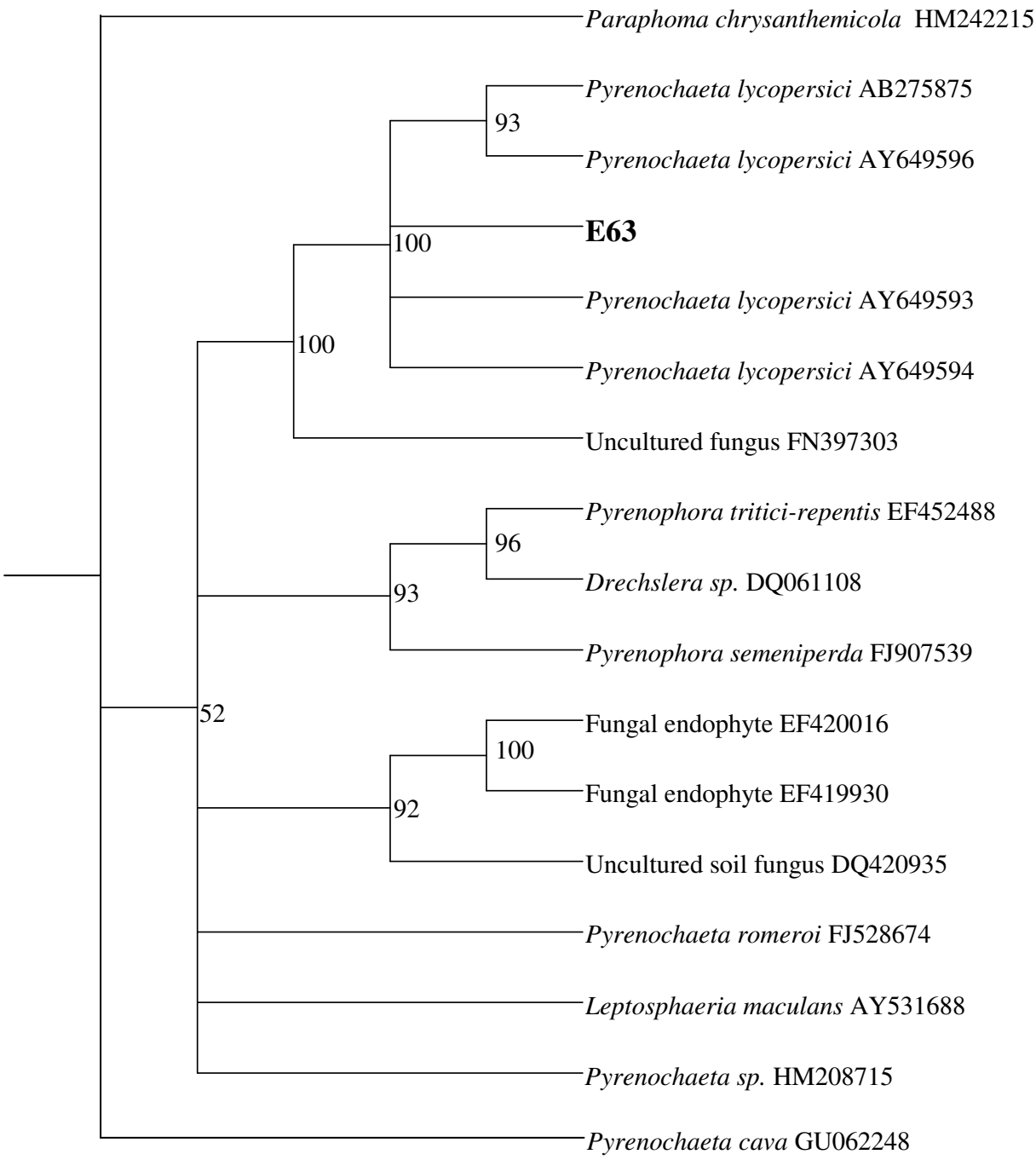
*Massarina ignaria* EU725629
*Morosphaeria ramunculicola* AF383962
Uncultured *Massarina* GU931767

**APPENDIX 1f**


*Paraphoma chrysanthemicola* HM242215
*Pyrenochaeta lycopersici* AB275875
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*Pyrenochaeta lycopersici* AY649596
**E63**
100
*Pyrenochaeta lycopersici* AY649593
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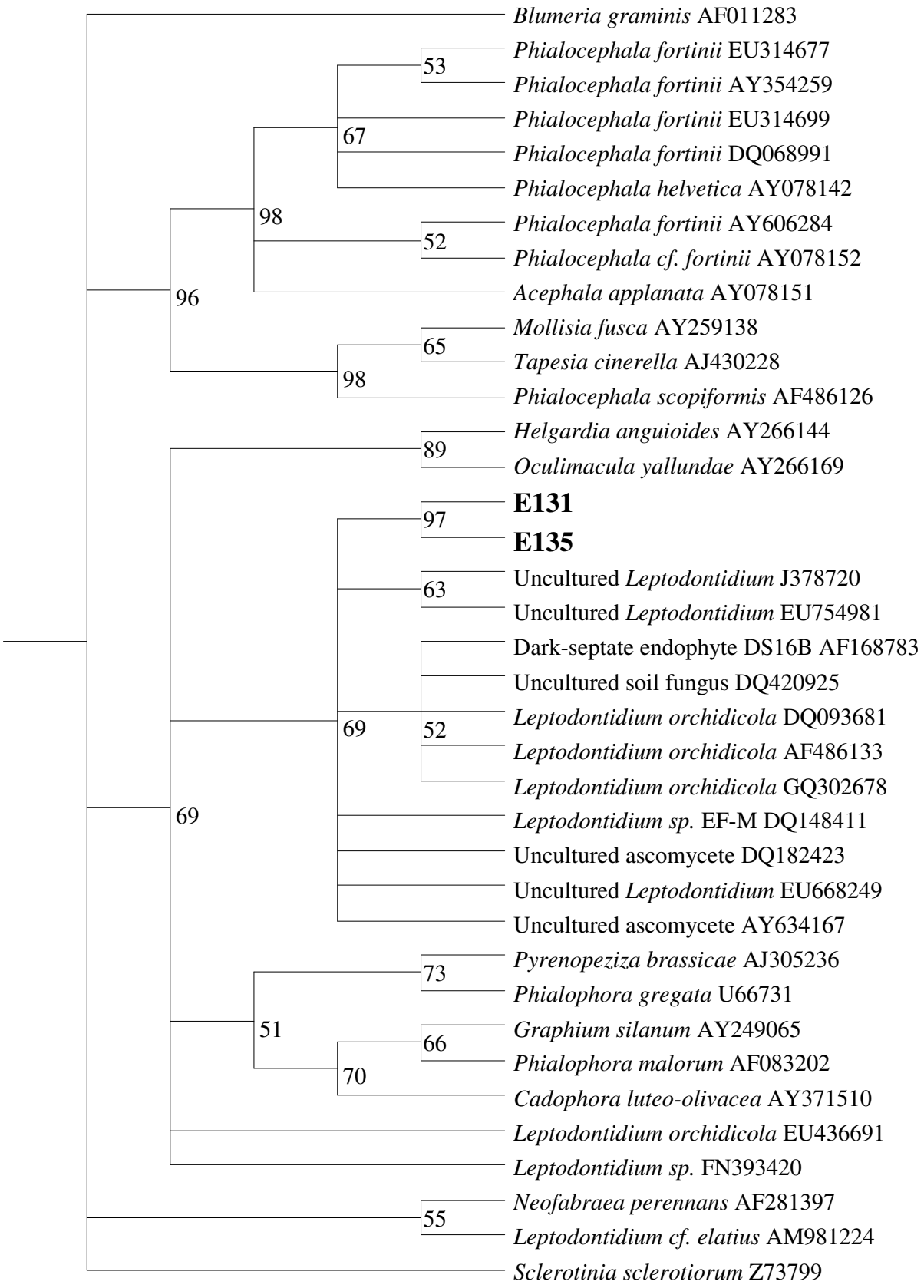
*Pyrenochaeta lycopersici* AY649594
Uncultured fungus FN397303
*Pyrenophora tritici-repentis* EF452488
96
*Drechslera sp.* DQ061108
93
*Pyrenophora semeniperda* FJ907539
52
Fungal endophyte EF420016
100
Fungal endophyte EF419930
92
Uncultured soil fungus DQ420935
*Pyrenochaeta romeroi* FJ528674
*Leptosphaeria maculans* AY531688
*Pyrenochaeta sp.* HM208715
*Pyrenochaeta cava* GU062248

**APPENDIX 1g**


*Blumeria graminis* AF011283
*Phialocephala fortinii* EU314677
*Phialocephala fortinii* AY354259
*Phialocephala fortinii* EU314699
*Phialocephala fortinii* DQ068991
*Phialocephala helvetica* AY078142
*Phialocephala fortinii* AY606284
*Phialocephala cf. fortinii* AY078152
*Acephala applanata* AY078151
*Mollisia fusca* AY259138
*Tapesia cinerella* AJ430228
*Phialocephala scopiformis* AF486126
*Helgardia anguioides* AY266144
*Oculimacula yallundae* AY266169
**E131**
**E135**
Uncultured *Leptodontidium* J378720
Uncultured *Leptodontidium* EU754981
Dark-septate endophyte DS16B AF168783
Uncultured soil fungus DQ420925
*Leptodontidium orchidicola* DQ093681
*Leptodontidium orchidicola* AF486133
*Leptodontidium orchidicola* GQ302678
*Leptodontidium sp.* EF-M DQ148411
Uncultured ascomycete DQ182423
Uncultured *Leptodontidium* EU668249
Uncultured ascomycete AY634167
*Pyrenopeziza brassicae* AJ305236
*Phialophora gregata* U66731
*Graphium silanum* AY249065
*Phialophora malorum* AF083202
*Cadophora luteo-olivacea* AY371510
*Leptodontidium orchidicola* EU436691
*Leptodontidium sp.* FN393420
*Neofabraea perennans* AF281397
*Leptodontidium cf. elatius* AM981224
*Sclerotinia sclerotiorum* Z73799
53
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73
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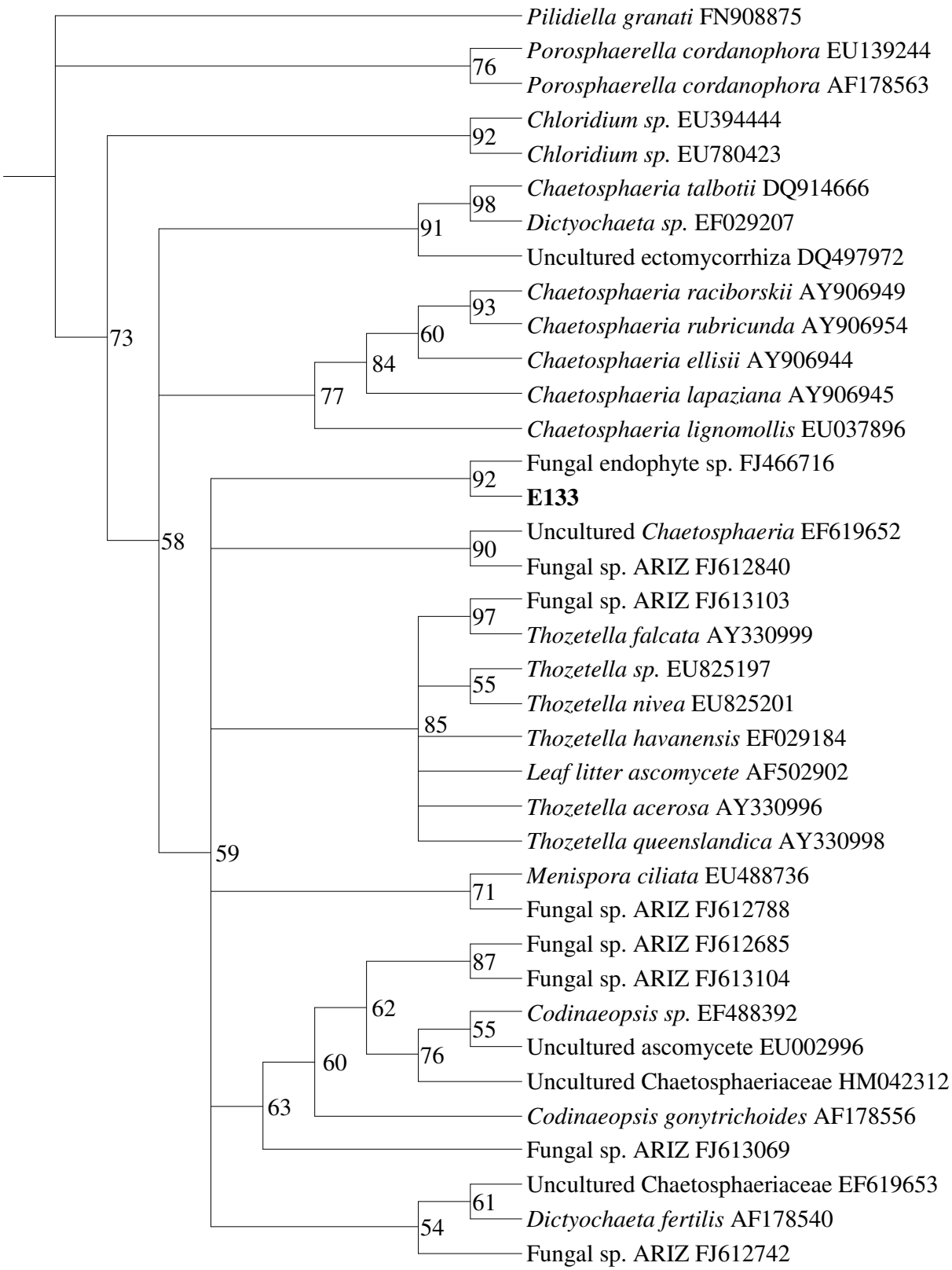
55

**APPENDIX 1h**


*Pilidiella granati* FN908875
*Porosphaerella cordanophora* EU139244
*Porosphaerella cordanophora* AF178563
*Chloridium sp.* EU394444
*Chloridium sp.* EU780423
*Chaetosphaeria talbotii* DQ914666
*Dictyochaeta sp.* EF029207
Uncultured ectomycorrhiza DQ497972
*Chaetosphaeria raciborskii* AY906949
*Chaetosphaeria rubricunda* AY906954
*Chaetosphaeria ellisii* AY906944
*Chaetosphaeria lapaziana* AY906945
*Chaetosphaeria lignomollis* EU037896
Fungal endophyte sp. FJ466716
**E133**
Uncultured *Chaetosphaeria* EF619652
Fungal sp. ARIZ FJ612840
Fungal sp. ARIZ FJ613103
*Thozetella falcata* AY330999
*Thozetella sp.* EU825197
*Thozetella nivea* EU825201
*Thozetella havanensis* EF029184
*Leaf litter ascomycete* AF502902
*Thozetella acerosa* AY330996
*Thozetella queenslandica* AY330998
*Menispora ciliata* EU488736
Fungal sp. ARIZ FJ612788
Fungal sp. ARIZ FJ612685
Fungal sp. ARIZ FJ613104
*Codinaeopsis sp.* EF488392
Uncultured ascomycete EU002996
Uncultured Chaetosphaeriaceae HM042312
*Codinaeopsis gonytrichoides* AF178556
Fungal sp. ARIZ FJ613069
Uncultured Chaetosphaeriaceae EF619653
*Dictyochaeta fertilis* AF178540
Fungal sp. ARIZ FJ612742
76
92
98
91
73
93
60
84
77
92
58
90
97
55
85
59
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## APPENDIX 2

### HYPOCREALES

*E27.*—The endophytic isolate E27 produced after 5 weeks growth on PDA a beige to pale pink colony of 6.5 cm in diameter with little aerial mycelium. After 12 weeks the diameter was around 7 cm and appearance became powdery due to the mass of spores. The reverse of the colony was unpigmented. Perithecia were not observed on PDA and malt extract agar (MEA) after 14 and 16 weeks respectively. The conidiophores were Sesquicillium-like, diverticillate with branches terminating in flask-shaped phialides. These characteristics and the production of unicellular, symmetrical and ellipsoidal conidia (5.7 µm x 3.4 µm) correspond to *Clonostachys rossmaniae* Schroers which is the anamorph of *Bionectria rossmania* (Schroers 2001).

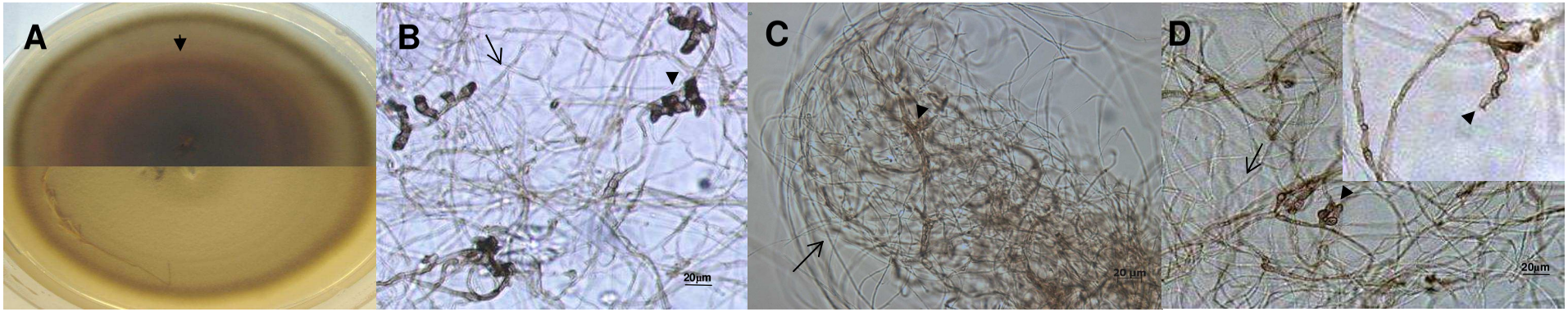
*E67.*—The isolate E67 produced after 7 days growth on PDA a yellowish green colony covering the whole agar plate (8 cm). The mycelium was hyaline bearing repeatedly divergent branched conidiophores in tufts. Colony produced flask-shaped phialides, subglobose unicellular hyaline conidia with smooth cell walls forming slimy masses, which were observed after two weeks with an immersion lent on PDA, MEA and CM. Moreover, intercalar and terminal hyaline chlamydospores were found. A characteristic odour was not detected. These features correspond to *Trichoderma harzianum* Rifai (Dodd et al 2003).

### PHYLLACHORALES

*E20.*—The endophyte E20 produced on PDA after 2 weeks growth at 25 C a moist yellowish white colony with little aerial mycelium. After eight weeks the maximum diameter of colony was approximately 7 cm. If grown on diluted PDA (0.5%) for 8 weeks, beige perithecia were formed. Ovoid hyaline 1- or 2-celled conidia (5.5 µm x 2.4 µm) with a slightly truncate base were produced on elongate flask-shaped phialides with widest diameter close to base. These characters fit well with the anamorph of *Plectosphaerella cucumerina* named *Plectosporium tabacinum*,(J.F.H.Beyma) M.E.Palm, W.Gams & Nierenberg, which was known before as *Fusarium tabacinum* (J.F.H. Beyma) W. Gams (Palm et al 1995).

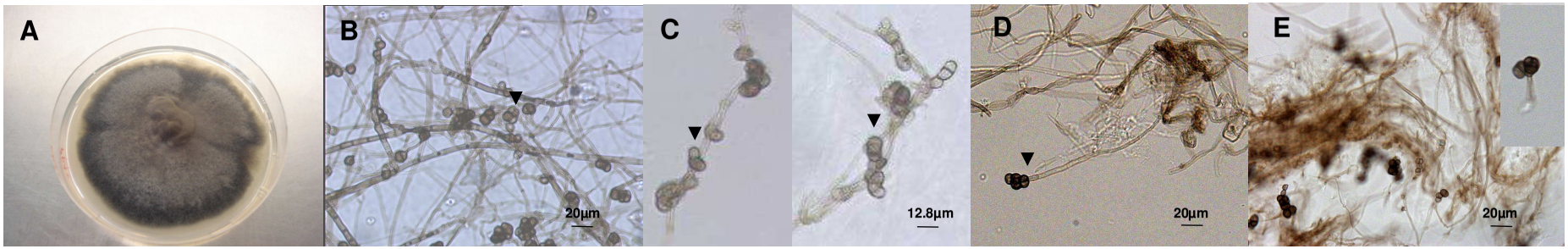
**PLEOSPORALES**

*E36/E41*.—The endophyte E41 (as E36) produced after 2 weeks growth on PDA an olive green colony of 4 cm diameter. The maximum diameter of colony was 6.5-7 cm after 12 weeks and it became grey with white-grey aerial mycelium (FIG. A). The dark septate hyphae did not produce any conidia or pycnidia on PDA, half diluted PDA, cornmeal agar (CMA), water agar (WA), V8 agar or Oat agar (OA, data not shown). A purple pigment on colony reverse was produced in a 7-week-old culture which was incubated on PDA with tomato roots at 23 C at 16/8h day/night (FIG. B). Dark pigmented chlamydospore-like cells were observed in 28-week-old PDA cultures at room temperature (FIG. C, D).



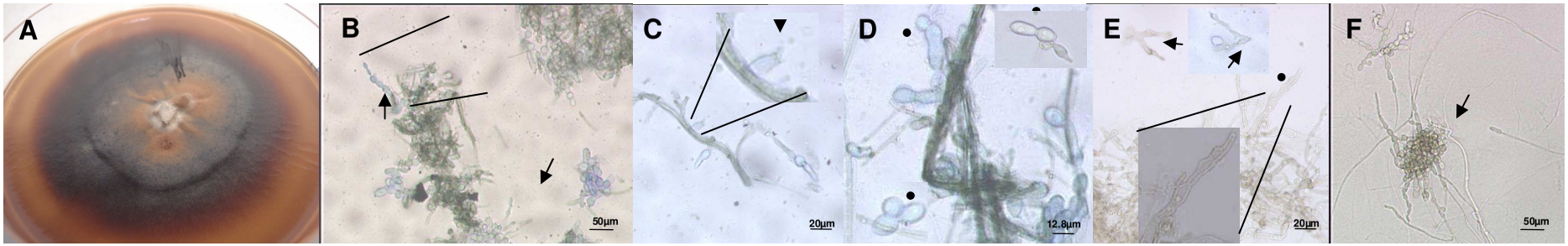

APPENDIX 2a. Morphology of E41. Colonies grown after 7 weeks on PDA at 23 C (A) produced a purple pigment on reverse (▼). Dematiaceous hyphae (→) on PDA, showing mycelia of 7 months old (B) on V8 agar (C) and CM agar after 8 weeks with chlamydospore-like structures in formation (D,▼).

*E48.*—The endophyte E48 produced a dark grey velvet colony of 7 cm diameter on PDA after 5 weeks of growth (FIG. A). Seven-month old cultures produced on PDA dark pigmented chlamydospore-like cells (FIG. B - E) either intercalary or in short lateral branches or dense groups of about eight cells (FIG. B and C). These spores were also formed on WA, CMA (FIG. D, E) and half diluted PDA, but not on V8 agar. Dark pycnidia, conidia and microsclerotia were not present on all media. A purple pigment on the colony reverse was produced after 7 weeks of inoculation on OA (data not shown).




APPENDIX 2b. Morphology of E48. The endophyte E48 produced a dark grey velvety colony (A). Seven-month old cultures produced on PDA dematiaceous chlamydospore-like structures (B) in succession (▼) directly from the hyphae (C) and also forming groups of about eight cells (B and C). These spores were also formed on WA (D) and CMA (E).

*E49.*—The endophyte E49 produced after 5 weeks growth on PDA a reddish brown flat colony of 5 cm in diameter (FIG. A). After 7 weeks the maximum diameter of colony was 6-7 cm. The growing colony produced a diffusible red pigment in PDA. Dematiaceous hyphae were observed on PDA, V8, MEA and CMA. After 7 to 8 weeks on V8 agar, hyaline conidia-like cells in acropetal succession (FIG. B), were observed. This was also observed on WA, CMA and MEA. Phialide-like cells presented collarette (FIG. C) and blastic conidia-like cells were observed (FIG. D).The hyphae grown on PDA and V8 produced bifurcate or poliphialide-like cells (FIG. E). Irregularities along the hyphae at sites of putative conidia formation were observed on PDA (FIG. E). There was formation of melanised hyphal strands on CMA, MEA and V8 agar (FIG. B and D) and dark sclerotia-like structures were developed in the media (CMA, MEA WA) at the margin of the colony (FIG. F).




APPENDIX 2c. Morphology of E49. The isolate E49 on PDA produced a diffusible red pigment (A). Hyaline conidia-like cells (→) in acropetal succession (B), phialide-like cells (▼) with collarette (C) and blastic conidia-like cells (●) developed as buds (D). Hyphae grown on PDA and V8 produced bifurcate or poliphialide-like cells (→) (E). Sympodial-like growing (●) was observed on PDA (E), melanized hyphal strands on V8 agar (B). Dark sclerotia-like structures (→) precipitated on water agar (F).

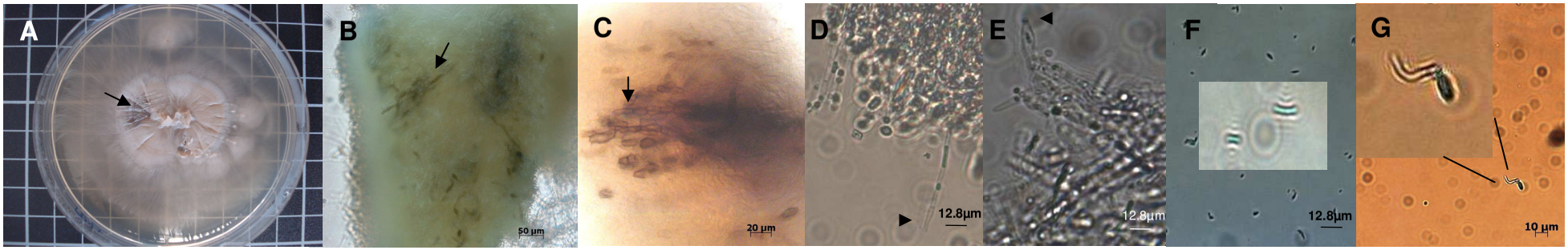
*E63.*—The isolate E63 produced after 4 weeks growth on PDA a grey silky colony of 4 cm-diameter. After 5-7 weeks the maximum diameter of colony became 7 cm. After *8 weeks production of conidia was not observed. Dark microsclerotia-like structures were formed after 7 weeks on PDA*. Typical conidia of Pyrenochaeta lycopersici were not induced by culture on V8 Juice agar at 22 C with a 16 h black light photoperiod.

**HELOTIALES**

*E131/E135.*—The endophyte E135 (as E131) produced after 5 weeks growth on PDA at 22 C a grayish brown velvety colony with diffusible dark red pigment and 5-6 cm in diameter. After 8 weeks the maximum diameter of the colony became around 8 cm. Scarce production of conidia (≤1µm) was observed on PDA. The conidia were produced directly from dematecеous hyphae and corresponded to *Leptodontidium orchidicola*. Production of microsclerotia-like structures inside of WA was observed after 8 weeks.

**CHAETOSPHAERIALES**

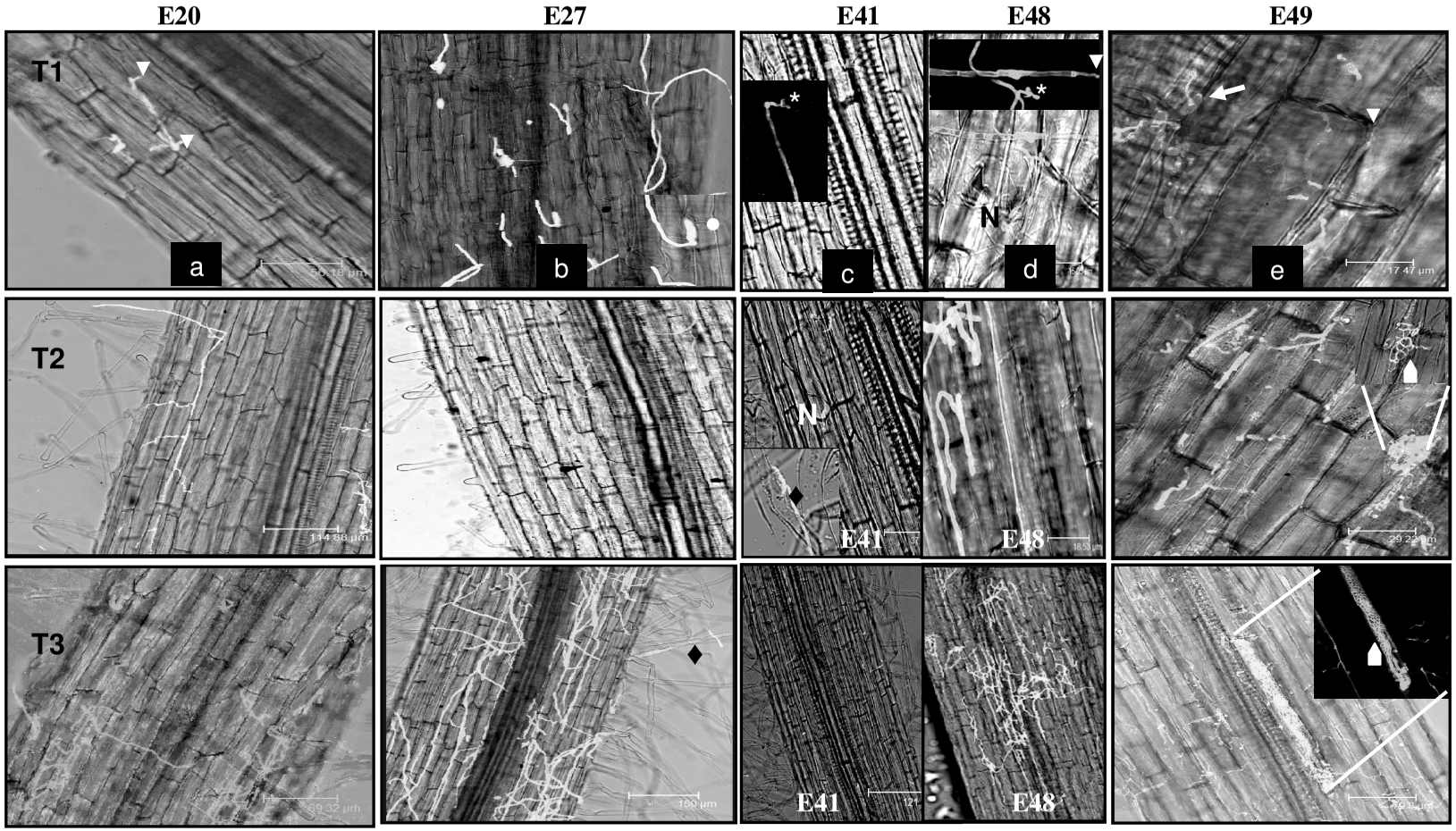
*E133.*—The endophyte E133 produced after 8 weeks growth on PDA at 22 C a colony whitish to beige of 5-6 cm diameter with a dark zone close to the center (FIG. A). The mycelium partly immersed in the agar with hyaline hyphae and brown conidiophores-like structures were observed on PDA and MEA after 4 months (FIG. B, C). Phialides with an apical funnel-shape collarette were terminally formed (FIG. D, E). The colony on PDA produced one-celled small thin hyaline cylindrical conidia without appendages (FIG. F) and microawns-like cells were observed (FIG. G).



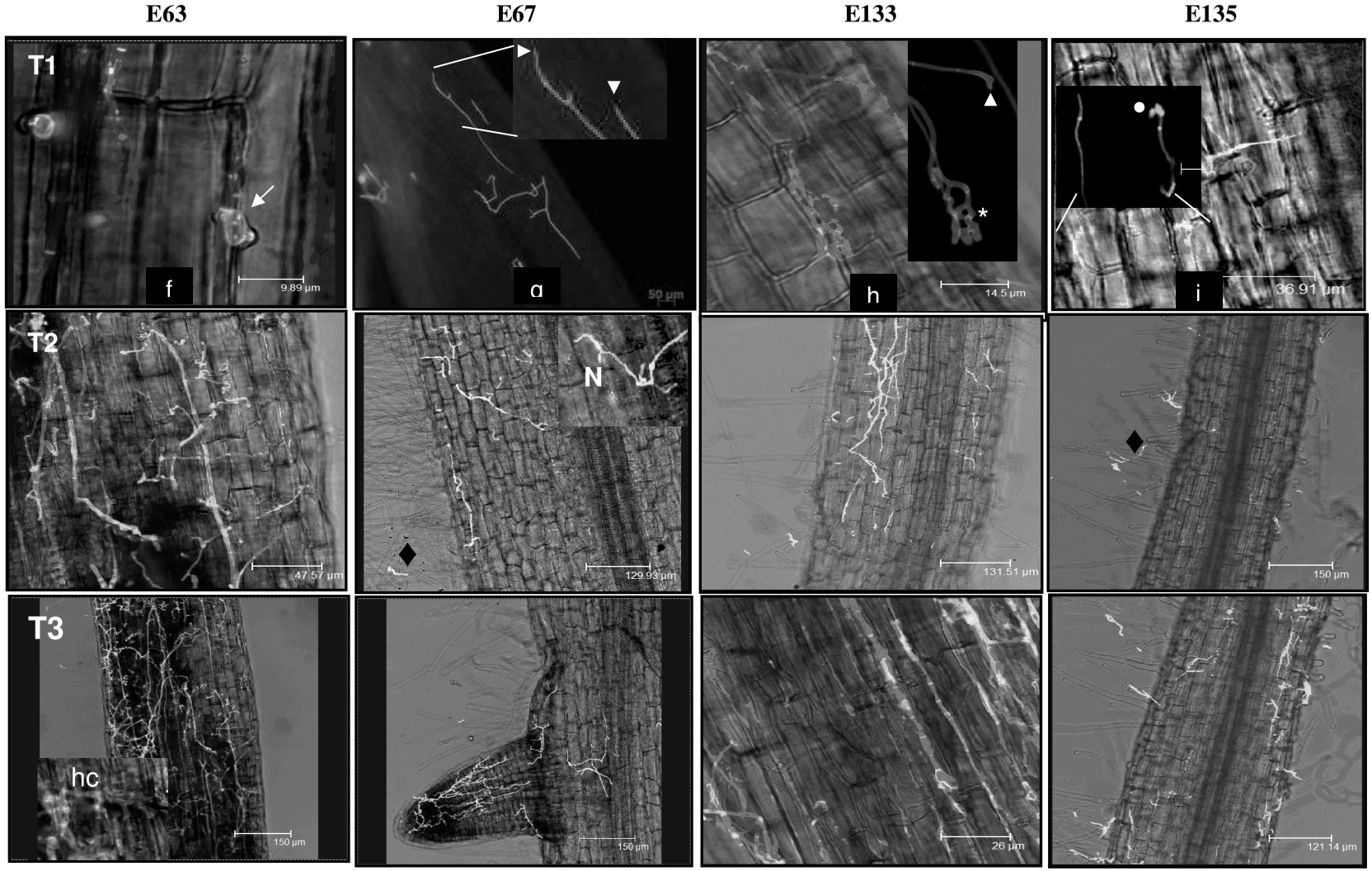

APPENDIX 2d. Morphology of E133. Whitish colony of the isolate E133 with an immature sporodochium-like (→) (A). Hyaline hyphae and brown conidiophores-like structures (→) on PDA and MEA after 4 Months (B, C). Conidiogenous cells-like (▼) (D) with an apical funnel-shaped collarette (▼) (E). Unicellular hyaline conidia cylindrical (F). Microawns-like structures (G).

## APPENDIX 3.

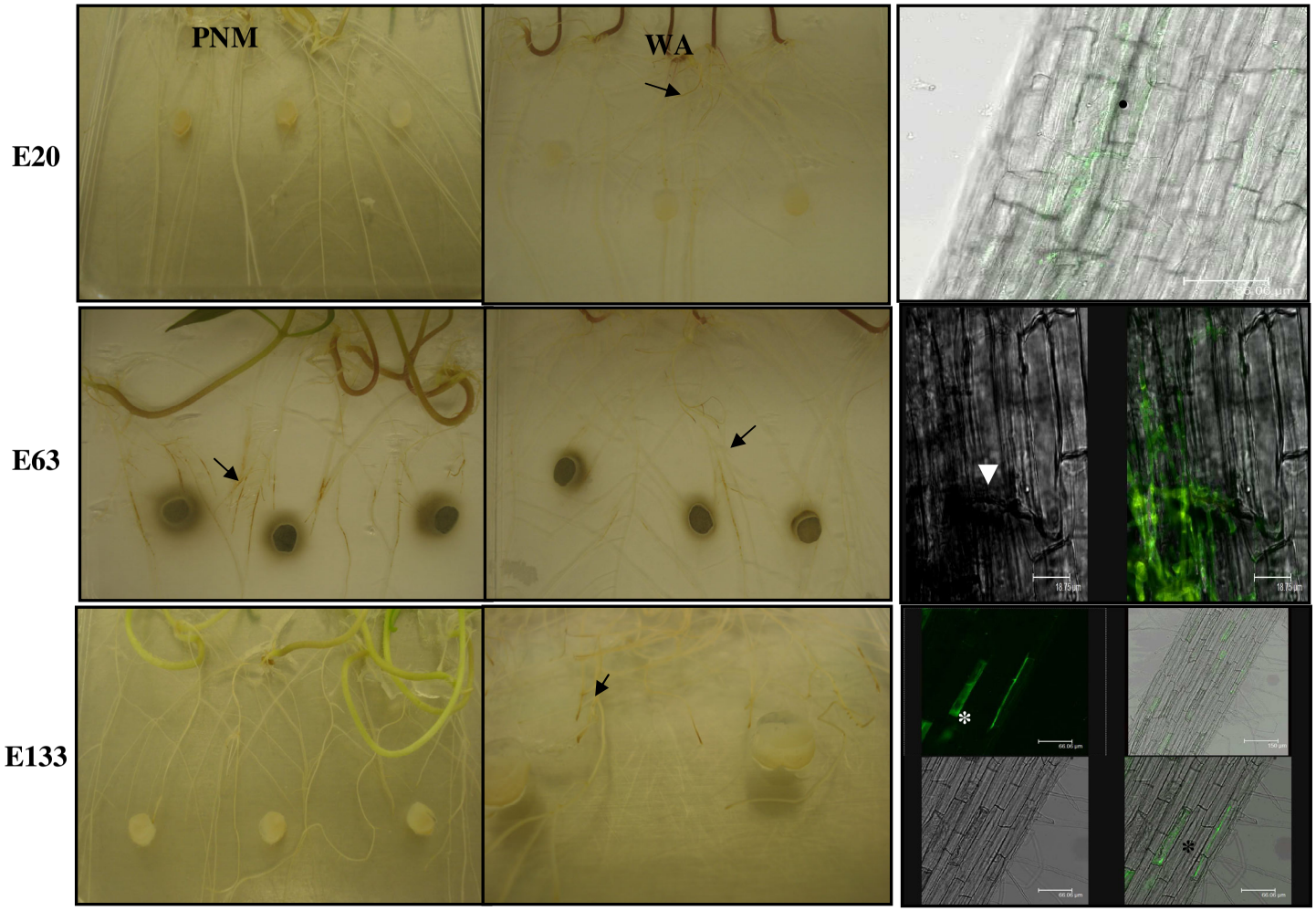
Endophytic colonization of roots. Tomato seedlings were inoculated on PNM together with each of the fungal endophytes, After first contact (T1), 2 weeks (T2) and 4 weeks (T3), tomato roots were stained with WGA-Alexa Fluor and Congo red and were evaluated by confocal microscopy. The Fig. shows an overlay picture of the two stains and bright field microscopy for the different endophytes. Hyphal single (▼) or branched tips (*) as the first contact with the root surface. Appressorium-like structures (●). Swelled cells (→) attached the intercellular space. Hyphal contact with root hair (♦). Hyphal narrowing (N). Formation of hyphal coils (hc). Microsclerotium of the isolate E49 (⌂).



**APPENDIX 3.** (continued). Endophytic colonization of roots. Legend see previous page**.**



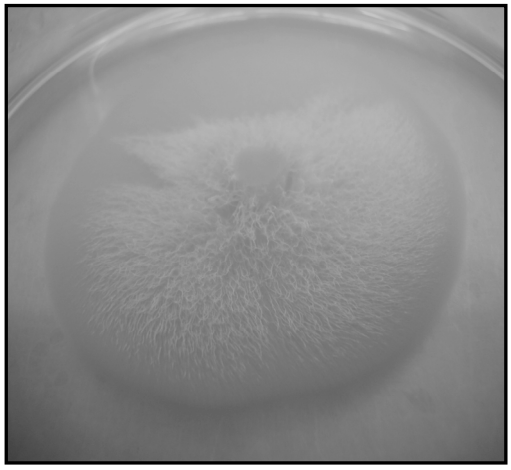
**APPENDIX 4** Symptoms in the roots. Tomato seedlings were placed on PNM and water agar in the presence of agar plugs of the endophytes E20, E63 and E133. After two weeks tomato roots were stained with WGA-Alexa Fluor and evaluated under the microscope. The figures show visual symptoms on roots as brown spots (◊). Microscopic observation of the root revealed defense responds of the colonized seedlings such as thickening of the cell wall (●), necrosis (▼) or localized fluorescence (*).



## APPENDIX 5

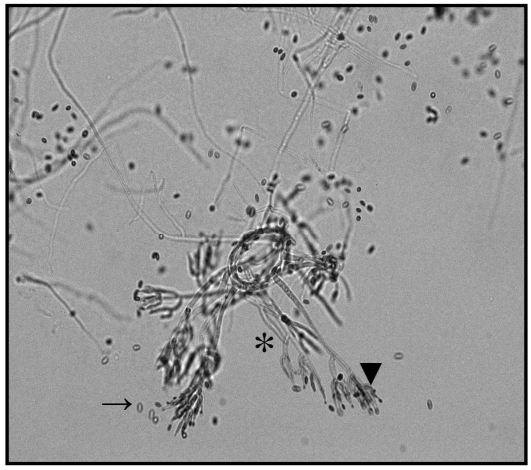
### E20: *Plectosphaerella cucumerina*- AJ492873 (99% similarity)

PDA 53 d. at 25°C colony diameter 7cm



Moist yellowish white colony with little aerial mycelium produced on PDA after 2 weeks in darkness at 25°C.

Blue lactophenol stain

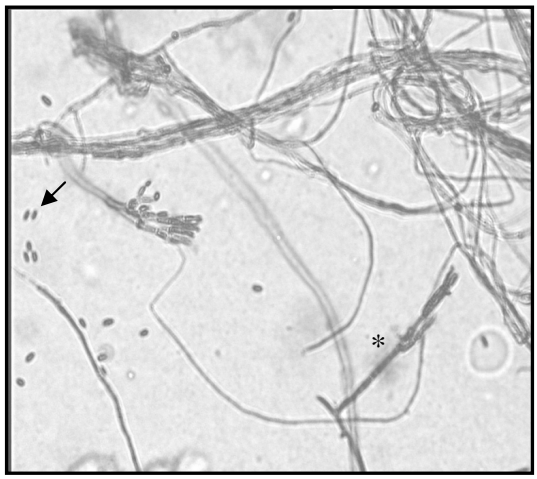



Conidiophores (*) and phialide (▼ )
Conidia ovoid and hyaline (→) (200 x).

### E27: *Bionectria rossmaniae* (similarity 97%)

PDA after 3 Months at 25°C.

Powdery colony with a diameter of 7 cm.
Colony reverse unpigmented.




Conidiophores of a penicillate type (*) and conidia one-celled, ellipsoidal symmetrical (→) (200 x).

# APPENDIX 6

Tabla 1. Impact of endophytes on tomato plants cv. Hildares. Plants from were harvested ten weeks after inoculation with 14 fungal endophytes isolated from 4 different sites in Colombia. Average values of plant grwth parameters ± standard deviations are shown. Values in bold denote significant differences between colonized and non-colonized control plants (one-way ANOVA according to LSD-test; $P = 0.05$; $n = 6$).

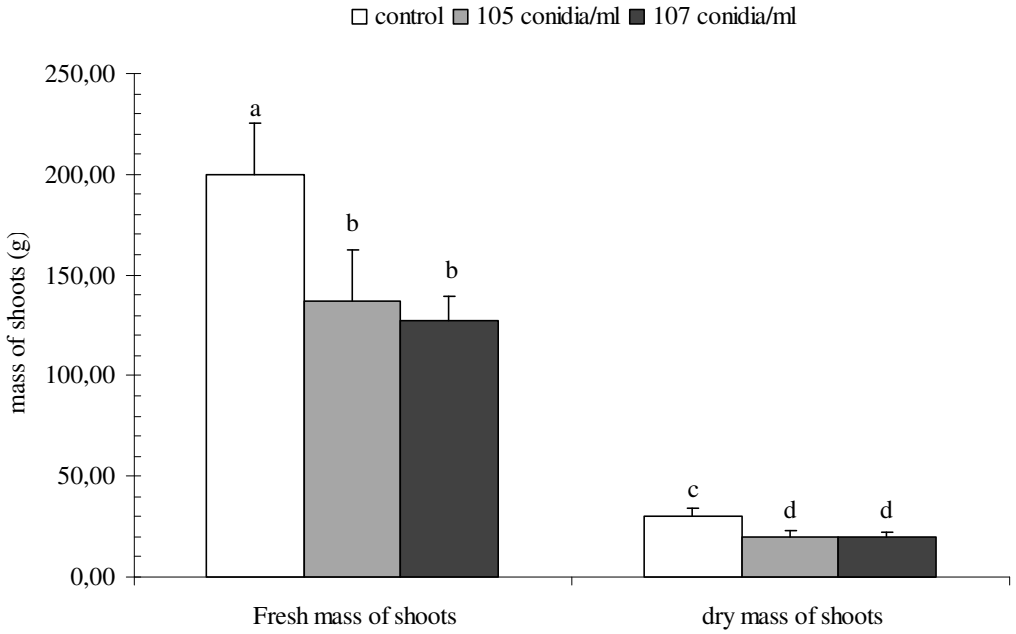
| Treatment | Fresh weight of shoot | Dry mass of shoot | Fresh weight of root | Leaf number | Leaf area | Bloom number |
|---|---|---|---|---|---|---|
| c001 | 53,12±3,78$^{ac}$ | 5,21±0,43$^{a}$ | 16,87±2,68$^{ac}$ | 12,50±0,84$^{ac}$ | 1262,1±87,97$^{a}$ | 1,33±0,82$^{acd}$ |
| E9 | 48,82±6,46$^{bc}$ | **4,25±0,79$^{b}$** | **11,19±2,71$^{b}$** | **11,40±1,14$^{b}$** | 1162,3±169,04$^{a}$ | **0,00±0,00$^{b}$** |
| E22 | 51,21±3,84$^{bcd}$ | **4,48±0,46$^{bc}$** | 12,71±4,53$^{bc}$ | 12,17±0,75$^{abc}$ | 1250,3±102,22$^{a}$ | **0,00±0,00$^{b}$** |
| E27 | **47,19±6,67$^{b}$** | **4,04±0,79$^{b}$** | **10,45±6,60$^{b}$** | 11,83±0,41$^{ab}$ | 1172,2±207,73$^{a}$ | **0,00±0,00$^{b}$** |
| E41 | 56,73±2,05$^{a}$ | 5,55±0,23$^{a}$ | 18,54±3,21$^{a}$ | 12,33±0,52$^{ac}$ | 1347,6±77,77$^{a}$ | 2,00±1,09$^{c}$ |
| E48 | 54,69±4,17$^{ad}$ | 5,06±0,51$^{ac}$ | 16,03±4,25$^{abc}$ | 12,83±0,75$^{c}$ | 1284,6±64,54$^{a}$ | 1,17±0,98$^{d}$ |
| E67 | 55,70±3,28$^{ad}$ | 5,48±0,18$^{a}$ | 16,29±3,79$^{abc}$ | 12,40±0,55$^{ac}$ | 1254,4±67,45$^{a}$ | 2,00±0,00$^{ac}$ |
| c002 | 80,21±4,22$^{a}$ | 7,58±0,76$^{a}$ | 10,92±2,01$^{a}$ | 13,00±0,63$^{a}$ | 1720,1±194,73$^{a}$ | 0,50±0,84$^{a}$ |
| E36 | 76,85±4,20$^{ab}$ | 7,09±0,49$^{ab}$ | 11,26±2,28$^{a}$ | 13,00±0,63$^{a}$ | 1650,1±186,98$^{ab}$ | 0,00±0,00$^{a}$ |
| E63 | **72,61±3,05$^{b}$** | **6,70±0,78$^{b}$** | 12,16±1,85$^{a}$ | 13,00±0,00$^{a}$ | **1517,4±78,9$^{b}$** | 0,00±0,00$^{a}$ |
| E20 | 77,60±3,81$^{a}$ | 7,08±0,4$^{ab}$ | 12,69±1,12$^{a}$ | 13,00±0,63$^{a}$ | 1643,6±141,68$^{ab}$ | 0,00±0,00$^{a}$ |
| c003 | 105,54±11,39$^{a}$ | 9,04±1,23$^{a}$ | 12,98±2,51$^{a}$ | 14,00±1,26$^{a}$ | 2148,1±178,97$^{a}$ | 1,83±1,60$^{a}$ |
| E49 | 99,09±10,80$^{ab}$ | 8,28±1,67$^{ab}$ | 14,93±4,15$^{a}$ | 13,67±0,82$^{a}$ | 2000,5±160,64$^{a}$ | 1,6±1,63$^{a}$ |
| E52 | 102,17±10,65$^{ab}$ | 8,85±2,26$^{a}$ | 13,93±2,91$^{a}$ | 14,83±1,47$^{a}$ | 1973±161,84$^{a}$ | 1,50±1,76$^{a}$ |
| E131 | **84,11±27,64$^{b}$** | **6,21±2,69$^{b}$** | 9,81±2,99$^{a}$ | 13,17±2,04$^{a}$ | 1791,6±523,09a | 1,00±1,55$^{a}$ |
| E133 | 99,24±10,43$^{ab}$ | 8,59±1,21$^{a}$ | 14,87±5,17$^{a}$ | 14,17±0,98$^{a}$ | 1998,4±167,44$^{a}$ | 1,83±1,60$^{a}$ |
| E135 | 98,17±23,78$^{ab}$ | 8,23±2,51$^{ab}$ | 13,17±2,17$^{a}$ | 14,00±1,67$^{a}$ | 2150,7±598,39$^{a}$ | 1,00±1,26$^{a}$ |

c00 : Control treatments : mock-inoculated tomato plants without fungal endophyte.

## APPENDIX 7

Results of the pathosystem interaction *Verticillium dahliae* and tomato cv. Hildares.

Preliminary experiments were carried out to evaluate symptom expression with two different concentrations of the pathogen ($10^5$ conidia/ml and $10^7$conidia/ml). Fresh weight (FW) and dry weight (DW) of ten plants for each 3 replicas per concentration were estimated. Different letters denote significant differences between colonized and non-colonized control plants (one-way ANOVA according to LSD-test; $P = 0.05$; $n = 30$).




Disease severity caused by *V. dahliae* was assessed (experiment A-D) on the following scale according to Morgan et al. (1992): 0 = no symptoms, 1 = slight yellowing of leaf, stunting or wilting, 2 = moderate yellowing of leaf, stunting or wilting, 3 = severe yellowing of leaf, stunting or wilting and 4 = leaf death at harvest

Symptoms caused by *Verticillium dahliae* infection

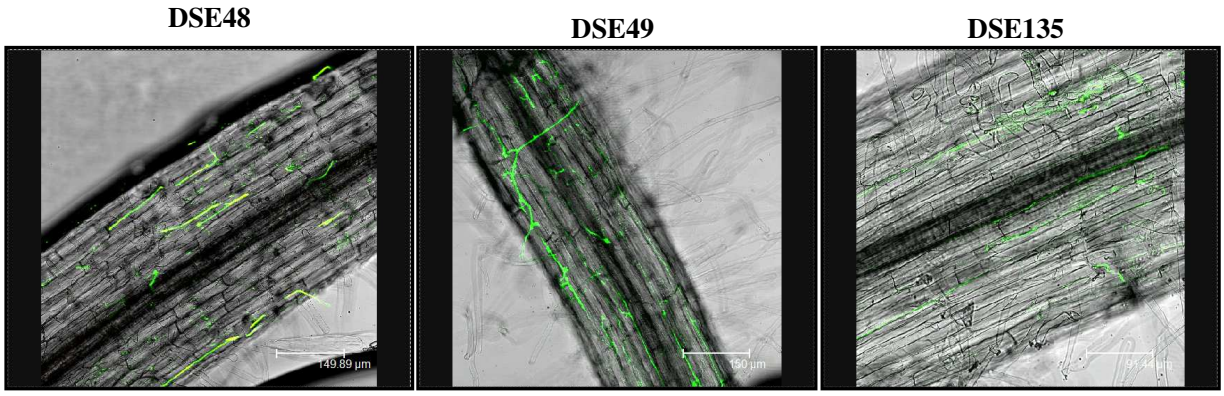
Symptom Score **0** **1** **2** **3** **4**

$$\textbf{Disease Index} = \frac{(\#\text{leaves}\mathbf{1}X1 + \#\text{leaves}\mathbf{2}X2 + \#\text{leaves}\mathbf{3}X3 + \#\text{leaves}\mathbf{4}X4)}{\text{leaves total}}$$

# APPENDIX 8

Colonization of tomato roots in pot experiments by DSE 48, DSE 49 and DSE 135 to evaluate the endophytic impact on growth after 22 weeks.

Figure 1. Tomato roots were stained with WGA-Alexa Fluor and Congo red and were evaluated by confocal microscopy after 2 and 3 weeks of inoculation with DSE48, DSE49 and DSE135. The Figure shows an overlay picture of the two stains and bright field microscopy for the different endophytes. Hyphal growth is visualized as green.



# APPENDIX 9

Table 1. Quantification of the root colonization by DSE135. after two weeks of its inoculation in tomato plants cv. Hildares grown in climate chamber until flowering. The quantification was based on the method of Trouvelot *et al*. (1986) for arbuscular mycorrhizal fungi. Average values ± standard error are shown. Different letters denote significant differences of colonization between both experiments (one-way ANOVA according to LSD-test; $P = 0.05$; $n = 3$).

| Experiment | F% | M% | m% |
|---|---|---|---|
| E | $68.1 \pm 18.1^{a}$ | $16.7 \pm 11.8^{a}$ | $22.6 \pm 10.2^{a}$ |
| F | $43.7 \pm 2.8^{a}$ | $3.4 \pm 0.9^{b}$ | $13.1 \pm 2.8^{a}$ |

F% : Percentage of the colonization frequency in the fragments analysed.

M%: Percentage of the colonization intensity in the complete root system.

m%: Percentage of the colonization intensity in the fragment analysed.

# APPENDIX 10

## MATERIALS AND METHODS

### 1. *Piriformospora indica* impact on vegetative growth 10 weeks after inoculation.

*Plant material*

Disinfected tomato seeds (cv. Hildares F1) were germinated on 0.8% water agar for five days at 24°C and seedlings were subsequently transplanted into pots (10 cm x 10 cm x 10 cm) containing humid sterilised sand (1:1 of EN12620:EN1339; Euroquarz, Dorsten, Germany). These pot cultures were placed in a green house (26±3°C, 60-70% relative humidity) for eight days and watered twice during this period with 40 ml of nutrient solution (De Kreij *et al.* 1997). After one week, the seedlings were used for inoculation with *P. indica*.

*Fungal material*

*P. indica* was grown in liquid complete medium (CM, Pontecorvo *et al.* 1953) for three to four weeks at 28°C under moderate speed agitation (100 rpm). The cultures were mixed by a blender (Model D72, Moulinex, Leipzig, Germany) for 15 seconds at minimal speed. The number of propagules (conidia and/or hyphal fragments) was estimated by counting in a Thoma chamber and their viability was checked by plating on PDA (VWR, Berlin, Germany). The suspensions were adjusted to a concentration of 5 x $10^5$ cfu/ml with sterile water containing 0.025% Tween 20 (Merck, Darmstadt, Germany).

*Inoculation of plants*

Three independent experiments were carried out. Eight seedlings per treatment were inoculated by root dipping in a fungal suspension ($10^5$ cfu/ml) for two to three hours and after transplanting with a final concentration of $10^5$ cfu/g substrate. After five weeks, two plants per treatment were harvested to evaluate colonization by staining with trypan blue and acid fuchsine. In addition, root fragments were disinfected for two to three minutes with 0.5% sodium hypochlorite and placed on PDA (VWR, Berlin, Germany) and water agar (1.2% w/v). Outgrowth and morphology of *P. indica* was controlled under the microscope. After 10 weeks of greenhouse cultivation, the remaining six plants per treatment were harvested to evaluate plant growth parameters (shoot and root fresh and dry weight, leaf and bloom number). Leaf areas were scanned with a LI-3100 Area Meter (LI-COR, Bad Homburg, Germany).

**2. *Piriformospora indica* impact on flowering.**

*Plant material*

The seeds and seedlings were prepared as above is described.

The tomato seedlings were transplanted into pots (1 L) at 1-2 leave stage and inoculated with the endophytes (see below). The pots were filled with sterilised substrate (Fruhstorfer Erde Typ P; Archut, Vechta, Germany; chemical analysis (mg per 100 g): N=75, P= 75, K= 125; pH 5.9). The plants were further cultivated under greenhouse conditions (temperature 23.9/18$^{o}$C day/night; relative humidity 63.3/82.4% day/night; light 40.3 Mol*m$^{-2}$*d$^{-1}$; $CO_2$ 466.8 ppm).

*Fungal material*

*P. indica* was grown in a 300-ml Erlenmeyer flask with 150 ml liquid medium (Pontecorvo et al. 1953) for three to four weeks at 25°C and 100 rpm agitation. Fresh mycelium was filtered and washed with sterile distilled water until the liquid became clear. The fresh mycelium was weighted and part of it was mixed with sterile tap water by a blender (Model D72, Moulinex, Leipzig, Germany) for 1 minute at minimal speed.

*Inoculation of plants*

Tomato plants were treated with the endophytes by root dipping (5 x $10^5$ cfu/ml) at 1-2 leaf stage before planting. The substrate was also drenched with fresh mycelium suspension of the endophytes (1% w/v).

Each treatment consisted of three replicates with six plants each. The pots were watered daily to maintain the substrate moisture and twice a week with 40 to 200 ml nutrient solution (De Kreij et al. 1997; EC = 2 dS m$^{-1}$; pH 5.5) dependent on the growth stage. Shoot fresh and dry weights of tomato plants were measured and number of flowers was calculated after a cultivation time of six weeks.

**3. *Piriformospora indica* impact on vegetative and generative growth 16 weeks after inoculation.**

*Cultivation of fungus*

The endophyte *P. indica* were inoculated in 300 ml-Erlenmeyer flasks with 150 ml liquid complete medium (CM, Pontecorvo *et al.* 1953) for 3-4 weeks at 25°C at 100 rpm. Fresh mycelium was filtered and washed with sterile distillate water. 500g fresh mycelium was weighted and mixed with 1 L sterile tap water by a blender (Model D72, Moulinex, Leipzig,

Germany) for 1 minute at minimal speed. Half of the suspension was autoclaved to use as negative control. The viability of the inocula was controlled by plating on PDA.

*Cultivation and inoculation of tomato plants*

4-week-old tomato plants (cv. Hildares) were carefully uprooted from sand/coarse (Euroquarz, Laußnitz, Germany) and inoculated by root dipping in 1% (w/v) of *P. indica* suspension or control solution (autoclaved suspension) during two hours. Inoculated plants and controls were transferred to buckets (10 plants per biological replica and 4 replicas per treatment), containing 10 L of nutrient solution according to the recipe of De Kreij *et al.* (1997) with an electric conductivity of 2 dS $m^{-1}$ and pH 5.5. Additionally, each bucket was inoculated with 10 ml of the same fungal or autoclaved suspensions for each treatment.

For evaluation of root colonization in both treatments, five disinfected root fragments (2 cm) per plant were plated on PDA. Colonization was determined by growth of the fungus from the root fragments and by light microscopy observing the characteristic spore morphology inside the root fragment.

**Statistical analyses of growth parameters for the trhee experiments**

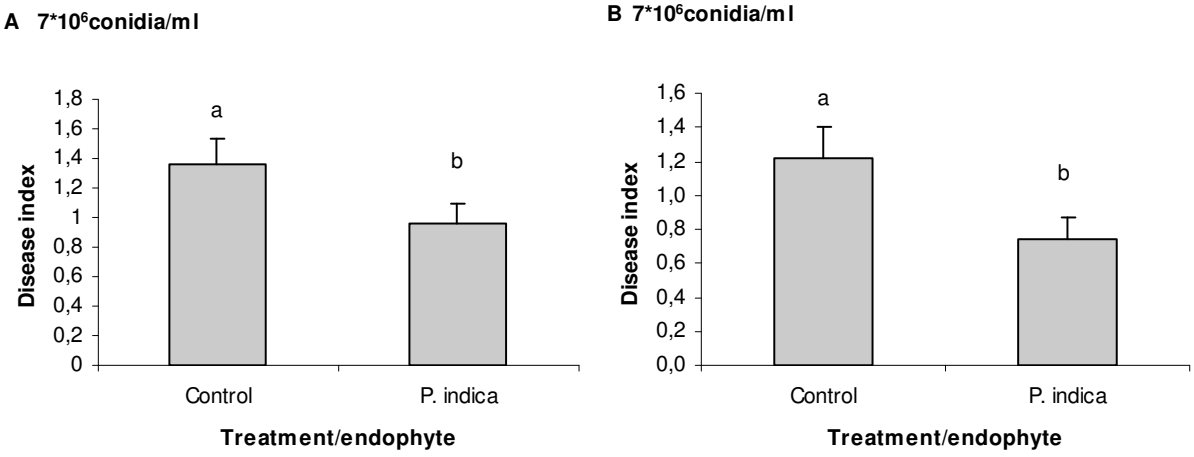
The STATISTICA program version 6.0 (StatSoft Inc., Tulsa, Oklahoma, USA) was used for the statistical analysis of fresh and dry weight of shoots, roots and fruits and number of flowers. Tomato growth parameters were analysed by one-way of variance (ANOVA) and the Fisher's protected LSD at $p = 0.05$.

# APPENDIX 11

Table 1. **Weight loss percentages of tomato plants after *Verticillium dahliae* infection.** Two weeks after inoculation with *Piriformospora indica*, plants were infected with 30 ml of $7x10^6$ conidia $mL^{-1}$ pathogen suspension. Fresh weights were measured and weight losses were calculated as the ratio of the values from pathogen-infected to non-infected plants. The data gives as percentage (shoot loss weight) were transformed to arcsin before one and two-way analysis of variance (ANOVA) and the Fisher's protected LSD test (p = 0.05; n=12).Values in bold denote statistically significant differences between endophyte-colonized and non-colonized plants (control).

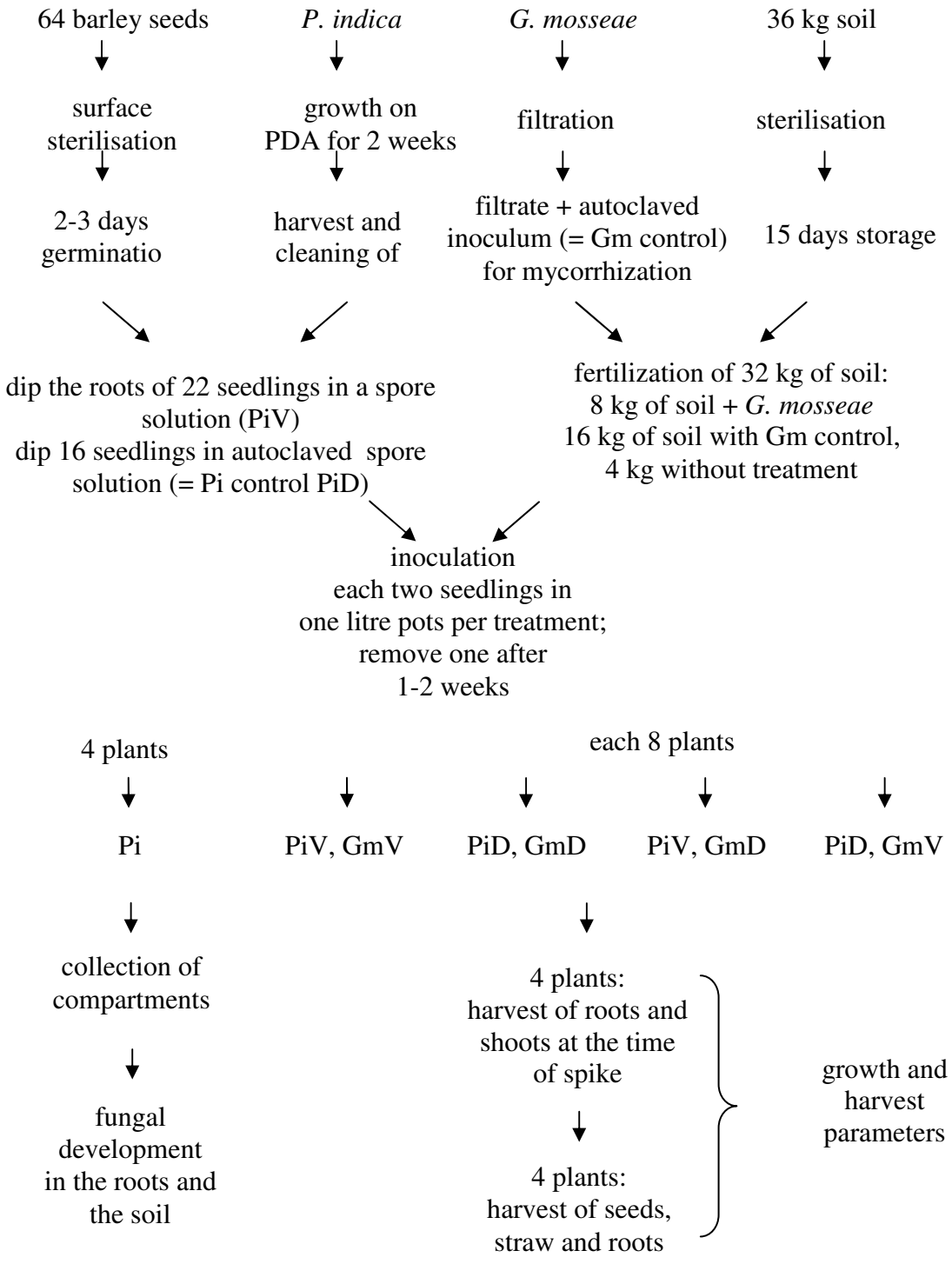
| Inoculum | $7X10^6$conidia/ml | | | |
|---|---|---|---|---|
| Endophytic Isolate | Weight loss percentage A | | Weight loss percentage B | |
| | FW | DW | FW | DW |
| control | 30,23[a] | 31,25[a] | 25,06[a] | 26,46[a] |
| *P. indica* | 21,8[a] | 24,8[a] | **14,51[b]** | 15,82[a] |
| *P. indica* x V. d | 0,195 | 0,297 | **0,013** | 0,059 |

**Fig. 1** Bioprotectice effects of *Piriformospora indica*. Disease indices of leaf symptoms caused by *Verticillium dahliae* infection in tomato control plants and plants colonized by the *P. indica* were estimated based on a 0 - 4 scale 5 weeks after inoculation with 30 ml of a $7x10^6$ conidia $mL^{-1}$-pathogen suspension (experiment A and B). Significant differences of the endophyte-colonized plants to the respective controls are indicated by different letters above the columns. Statistical comparisons between treatments were performed by Kruskal-Wallis test (*P* = 0.05; n=12).



## APPENDIX 12

**Methodology for inoculation of Barley plants with the fungi *Piriformospora indica* and *Glomus mossea*:**



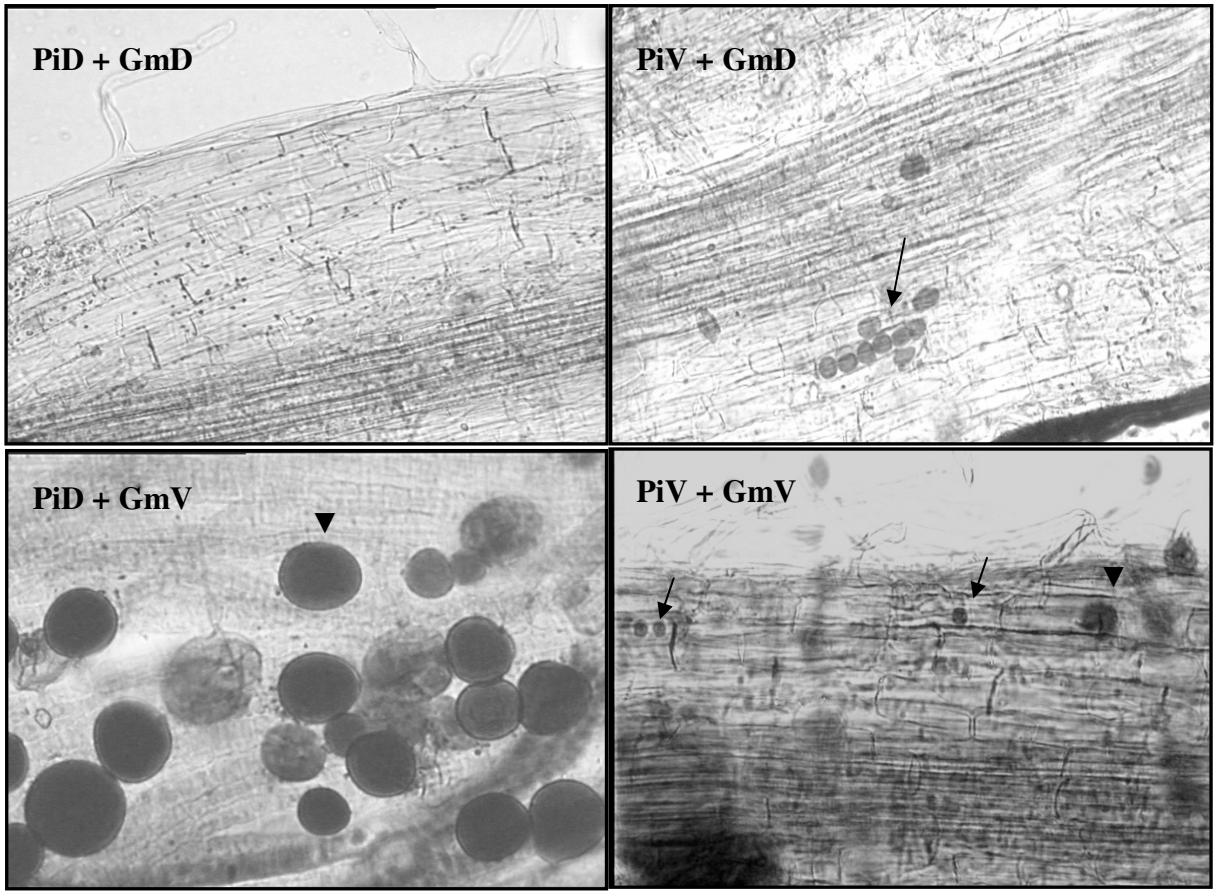
## APPENDIX 13

Trypan blue stain to confirm tomato root colonization by *Piriformospora indica* and *Glomus mosseae* in each treatment:

PiV= *P. indica*
PiD= *P. indica* autoclaved

GmV= *G. mosseae*
GmD = *G. mosseae* autoclaved

Figure 1. Visualization in barley roots of *P. indica* and *G. mosseae* colonization. After 2 and 4 weeks root fragments were stained with Trypan blue. Colonization was also controlled at the end of the experiment.




Colonization by *P. indica* (→). Spores are visualized inside root cells

Colonization by *G. mosseae* (▼). Spores on root surface and vacuoles inside the cells

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Diana Andrade

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