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PLANT ANIMAL INTERACTIONS

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Piriformospora indica and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*

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Abstract A Sebacinales species was recovered from a clone library made from a pooled rhizosphere sample of *Nicotiana attenuata* plants from 14 native populations. Axenic cultures of the related species, *Piriformospora indica* and *Sebacina vermifera*, were used to examine their effects on plant performance. Inoculation of *N. attenuata* seeds with either fungus species stimulated seed germination and increased growth and stalk elongation. *S. vermifera* inoculated plants flowered earlier, produced more flowers and matured more seed capsules than did non-inoculated plants. Jasmonate treatment during rosette-stage growth, which slows growth and elicits herbivore resistance traits, erased differences in vegetative, but not reproductive performance resulting from *S. vermifera* inoculation. Total nitrogen and phosphorous contents did not differ between inoculated and control plants, suggesting that the performance benefits of fungal inoculation did not result from improvements in nutritional status. Since the expression of trypsin proteinase inhibitors (TPI), defensive proteins which confer resistance to attack from *Manduca sexta* larvae, incur significant growth and fitness costs for the plant, we examined the effect of *S. vermifera* inoculation on herbivore resistance and TPI activity. After 10 days of feeding on *S. vermifera*-inoculated plants, larval mass was 46% higher and TPI activity was 48% lower than that on non-inoculated plants. These results suggest that *Sebacina* spp. may interfere with defense signaling and allow plants to increase growth rates at the expense of herbivore resistance mediated by TPIs.

Keywords Herbivory · *Manduca sexta* · *Nicotiana attenuata* · *Piriformospora indica* · *Sebacina vermifera*

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Introduction

Arbuscular mycorrhizal fungi (AM) and ectomycorrhizal fungi (EC) form symbiotic associations with plants in which both partners benefit from an improved nutritional status. However, some plant species realize an enhanced performance from fungal associations while other species may be negatively affected by the same associations (Grime et al. 1987; Read 1998). Several studies have shown that associations with mycorrhizal fungi influence plant fitness in complex ways which are not directly related to the improved nutritional status of mycorrhizal-plants. Mycorrhizal fungi were found to increase plant fitness by increasing tolerance of extreme drought conditions (Ruiz-Lozano and Azcón 1995; Ruiz-Lozano et al. 1996, 2001; Marulanda et al. 2003) and heavy metals (Kaldorf et al. 1999). Other studies have found that mycorrhizal associations resulted in reduced resistance to plant pathogens (Norman et al. 1996; Trotta et al. 1996; Borowicz 2001) and nematodes (Little and Maun 1996; Borowicz 2001). The effects of fungal associations on herbivore-resistance are complex and highlight the interplay of below- and above-ground processes in plants (Wardle et al. 2004).

Mycorrhiza can have indirect effects on plant performance by influencing aboveground interactions of plants with herbivores. A study of 1,058 species from 37 plant families of the British flora concluded that taxa, which associated with mycorrhizal fungi, have a higher proportion of specialist insects (Gange et al. 2002) and that generalist herbivores are negatively affected by mycorrhizal associations (Gange 2001). In contrast,

Goverde et al. (2000) showed that survival and weight of the oligophagous *Polyommatus icarus* larvae fed on *Lotus corniculatus* was significantly higher on plants inoculated with either separate inoculums or a mixture of two *Glomus* spp. in comparison to non-inoculated plants. Fungicide application that reduced the percentage of mycorrhizal colonization in *Cirsium arvense* increased the number and weight of larvae of the monophagous gall forming *Urophora cardui* (Gange and Nice 1997). Both reports associated the changes in herbivore performance with the change in the nutritional value of the plant (Goverde et al. 2000) or galls (Gange and Nice 1997). Vicari et al. (2002) studied the effect of the inoculation of *Lolium perenne* with the mycorrhizal fungus *G. mosseae* and the endophyte *Neotyphodium lolii*, both in a mixture and separately, and showed that *G. mosseae* had negative effects on the survivorship of *Phlogophora meticulosa* larvae, independently of phosphorous (P) application. On the other hand, other reports suggested that plant–mycorrhiza associations could be affected by herbivores (Gehring and Whitham 1991, 1995; Kula et al. 2005).

Although more than 80% of plant families are associated with mycorrhizal symbiosis, only 3% are associated with ectomycorrhizae (Smith and Read 1997). However, in comparison to AM fungi, a large number of fungal species form ectomycorrhizal associations, mainly among the basidiomycetes and ascomycetes (Smith and Read 1997). Within the basidiomycetes, members of the Sebacinaceae family have a wide distribution (Weiss et al. 2004), and are known to form different types of mycorrhizal association (ecto, orchid and ericoid) with a wide range of host–plant species (Glen et al. 2002; Selosse et al. 2002a, 2002b; Allen et al. 2003; Kottke et al. 2003; Taylor et al. 2003; Urban et al. 2003). The nuclear rDNA was used for phylogenetic studies of ectomycorrhizal Sebacinales fungi (Verma et al. 1998; Glen et al. 2002; Urban et al. 2003; Weiss et al. 2004). Among these mycorrhizal species, *Piriformospora indica*, which was first isolated from the rhizophere of *Prosopis julifora* and *Zizyphus nummularia* Rajasthan, India (Verma et al. 1998), has been shown to colonize roots and increase the biomass of both roots and shoots of numerous plant species, including cultivated tobacco and *Arabidopsis thaliana* (Sahay and Varma 1999; Varma et al. 1999; Rai et al. 2001; Kumari et al. 2003; Peškan-Berghöfer et al. 2004). A closely related species, *Sebacina vermifera* forms ectomycorrhiza on a wide range of plant species (Warcup 1988).

The native tobacco, *Nicotiana attenuata* L. (Solanaceae), is a post-fire annual which synchronizes its germination with smoke-related cues to time growth in the nitrogen rich soils that follow wild fires in the Great Basin Desert of the SW USA (Lynds and Baldwin 1998). This species and its specialist herbivore *Manduca sexta* (Sphingidae) have been developed as a model system for the study of plant–herbivore interactions (Baldwin and Preston 1999; Kessler and Baldwin 2002). In response to attack from *M. sexta* larvae, which is recognized when larval oral secretions (OS) are introduced into wounds during feeding (McCloud and Baldwin 1997; Halitschke et al. 2001; Schittko et al. 2001; Roda et al. 2004), the plant dramatically increases the accumulation of trypsin proteinase inhibitors (TPI) which slow the growth of larvae (Zavala et al. 2004a; Zavala and Baldwin 2004), presumably by inhibiting the function of larval digestive proteases. TPI production, however, comes at a significant fitness cost for the plant as TPI-producing genotypes are out-competed and produce less seed than genotypes in which TPI production has been genetically silenced (Glawe et al. 2003; Zavala et al. 2004b).

In this study, we examined whether fungi of the Sebacinales influenced the performance of *N. attenuata*. A library of ITS clones of fungal species from the rhizosphere of 14 native plant populations was sequenced and a clone with sequence similarity to the Sebacinales was recovered. Axenic cultures of two related fungi, *P. indica* and *S. vermifera*, were acquired, and their effects on germination, growth, reproductive output and herbivore resistance of *N. attenuata* were evaluated.

Material and methods

Plant, fungal and insect species

N. attenuata seeds, collected in Utah, USA, and shelved for 13 or 17 generations, were germinated as described by Krügel et al. (2002). After 7 days of growth, seedlings were transferred to pots and grown in a potting soil mixture in a glasshouse at 16 h light 28°C/8 h dark 24°C. To study the effect of the fungi on plant performance, two plants were grown together with a size-matched conspecific in 2 l pots. Two to three-week-old rosette-stage *N. attenuata* plants were used in all experiments.

P. indica and *S. vermifera* were received from A. Varma, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India, and P. Franken, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany. Fresh cultures were routinely grown on $1/10^{th}$ strength Käfer's medium (Käfer 1977). For inoculation, seeds of *N. attenuata* were placed on plates of Gamborg's B5 (GB5) medium (Duchefa Biochemie) that had been previously inoculated with axenic mycelia of the fungi and incubated in the dark at 26°C for 10 days.

M. sexta larvae were from a culture maintained at North Carolina State University, Raleigh, NC, USA.

DNA sequencing and fungal phylogenetic analyses

Soil samples were collected from the rhizosphere of 14 *N. attenuata* populations across a 50,000 km^2 area in SW Utah (USA) and 100 g of each sample were mixed. Total DNA was isolated from the mixed sample and a fungal clone library based on the ITS regions was

established by NADICOM (Marburg, Germany). The 96 resulting clones were amplified and sequenced using ABI Prism 3100 Genetic Analyzer (Hitachi). Using the NCBI-BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), the sequences were compared with known fungal sequences.

Fungal DNA of the *P. indica* and *S. vermifera* clones was isolated from axenically cultured hyphae following the method described in Bubner et al. (2004). Universal primers, ITS1F and ITS4 (Gardes and Bruns 1993), were used to amplify the 5.8s rRNA and the flanking ITS1 and ITS2 spacer region by PCR. DNA templates were amplified in a Mastercycler gradient PCR (Eppendorf Inc.) under the following conditions: 94°C, 1 min; 38 cycles of 94°C 20 s, 51°C 40 s, 72°C 1 min; 72°C 3 min. Amplified bands were eluted from the gel using GFX PCR DNA and gel band purification kit (Amersham), and sequenced.

A phylogenetic tree was constructed on the basis of 5.8s rDNA and homologous parts of the flanking ITS1 and 2 sequences of an unknown Utah clone, several Sebacinales and representative members of the two other orders within the *Heterobasidiomycetidae* (from the Genbank databases). Sequence alignment was performed by progressive alignment (Hogeweg and Hesper 1984) with Clustal X (v 1.81) (Thompson et al. 1997). Distance estimation was calculated according to Tajima and Nei (1984), taking all alignment positions into account. Tree topology was inferred by neighbor-joining using Treecon software (v 1.3b) (van der Peer and De Watcher 1994). The data set was subjected to 1,000 bootstrappings with *Tremella aurantia* (Order Tremellales; subclass *Tremellomycetidae*) as an outgroup.

N. attenuata performance

N. attenuata seeds were germinated on either fungus-inoculated and non-inoculated GB5 plates. Plates were maintained at 26°C in an incubator with a 11/13 h day/night cycle, and germination was assessed every 12 h. For each fungal species, 25 seeds were sown on each of four replicate plates. Seven day-old seedlings were transferred to Teku pots and 10 days later, they were transferred to 2 l pots and grown with a size-matched conspecific to provide intra-specific competition. Forty days after germination, after plants reached the elongation stage, stalk length was measured every 5 days and the start of flowering was recorded for each plant. Counts of the number of flower insertions and seed capsules, as well as total seed biomass were used as proxies of plant fitness. Each non-inoculated and *P. indica* or *S. vermifera* inoculated treatments consisted of 20 pots with two plants in each pot. An additional 20 pots from each of the three inoculation treatments received methyl jasmonate (MeJA) at 150 µg applied in 20 µl lanolin to the second (+2) and third (+3) fully developed leaves of the rosette stage-plants.

Root staining

Roots of 20 days old seedlings grown on GB5 plates pre-inoculated with *S. vermifera* were immersed for 4 h in a 2% KOH solution at 80°C. The roots were then immersed in a staining solution of 0.05% trypan blue in lactic acid:glycerol:H_2O (1:1:1 v:v:v). Roots were shaken gently in the staining solution for 30 min at 30 rpm, and destaining was performed twice in a lactic acid:glycerol:H_2O solution (1:1:1 v:v:v). Roots were observed under visible light on a fluorescent-light microscope (Zeiss Axioskop model HBO50, Carl-Zeiss Jena, Germany).

Analysis of total N and P content

Leaves of 37–38 day-old *N. attenuata* plants inoculated with *P. indica*, *S. vermifera* or non-inoculated control pots, as well as leaves from MeJA-treated or non-treated plants (see above) were collected, oven-dried at 60°C, and dry mass was determined. Total nitrogen (N) and phosphorous (P) content were analyzed in pooled leaves from six replicate plants from each treatment by the Thüringer Landesanstalt für Landwirschaft, Jena, Germany. The analyses were done according to the standardized method of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) for total N and according to Deutsche Industrienorm (DIN) 38406-E22 for total P.

M. sexta performance

Freshly hatched *M. sexta* larvae were placed in polyethylene boxes and fed on freshly collected leaves of *N. attenuata*. Two days prior to collection, leaves were wounded and treated with a 1:10 dilution of OS and regurgitates of mature *M. sexta* larvae, which provides a highly reproducible elicitation of defense responses (Halitschke et al. 2001). Leaves were collected from 16 *S. vermifera*-inoculated and 16 non-inoculated WT plants and one leaf from each plant was placed in a polyethylene box. In each box, one freshly hatched caterpillar was placed. Fresh leaves were collected every second day and the old leaf was replaced in each of the plant's corresponding box. After 10 days, larval mass was recorded.

Analysis of TPIs, nicotine and phenolics

Fully developed leaves at nodal position (+1) of rosette stage-plants from each of five *S. vermifera* inoculated and non-inoculated pots were wounded and treated with a 1:5 dilution of OS from mature *M. sexta* larvae. Leaves were collected 3 days after elicitation, weighed and immediately frozen at −80°C until analysis. Trypsin

proteinase activity (TPI) was analyzed by radial diffusion activity as described in van Dam et al. (2001). Alkaloids and phenolics were extracted and quantified by HPLC as described in Keinänen et al. (2001).

Statistical analysis

SYSTAT version 10 (SPSS Inc. 2000) software was used for all statistical analyses.

Results

Phylogenetic analysis of fungi

Soil rhizosphere samples collected from 14 native populations of *N. attenuata* were used to establish a fungal clone library and to assess whether mycorrhizal fungi interact with the plant in its natural environment in Utah, USA. By comparing the sequences of the fungal clones with Genbank database (http://www.ncbi.nlm.nih.gov), a *Sebacina*-like fungus was identified. Its phylogenetic position within the Sebacinales was determined by a comparison to seven different members of the Sebacinales and five representatives of the two other orders within the subclass *Heterobasidiomycetidae* (Fig. 1). The resulting phylogenetic tree clearly represents the three orders of the *Heterobasidiomycetidae* as monophyletic branches. The Utah clone was found to be nearly identical to *S. allantoidea* (Fig. 1). Since *P. indica* and *S. vermifera* are basal to the *S. allantoidea*/Utah-clone branch (Fig. 1) and can be cultured axenically, we used these two species to study the interactions of Sebacinales fungi with *N. attenuata* and examine their possible effects on plant performance.

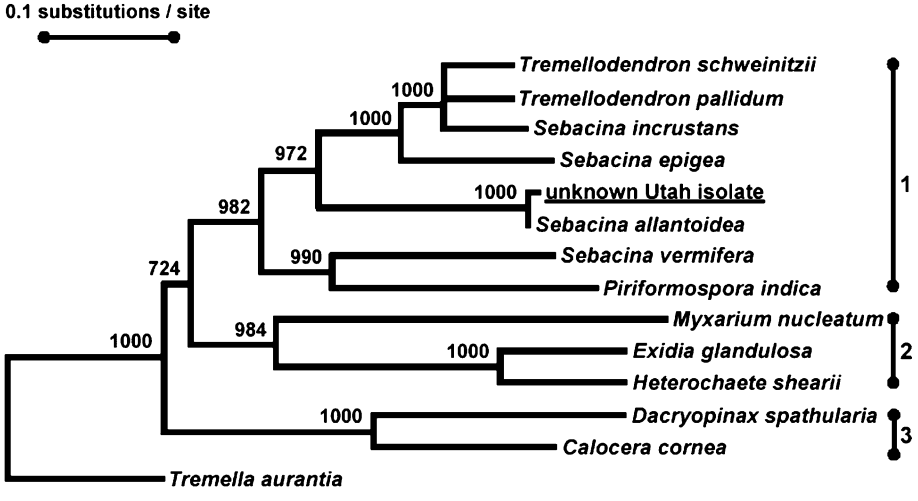

Fig. 1 Neighbor-joining tree of different members of the *Heterobasidiomycetidae*: (1) Sebacinales; (2) Auriculariales; (3) Dacrymycetales. The tree was constructed on the basis of the sequence of the ITS region of *Sebacina vermifera* and *Piriformaspora indica* clones used in this study, on a fungal clone extracted from *Nicotiana attenuata* rhizosphere samples (Utah) and sequences derived from Genbank (http://www.ncbi.nlm.nih.gov). Distance estimation was calculated according to Tajima and Nei (1984) using 1,000 bootstraps. *Tremella aurantia* was used as an outgroup

Effect of *P. indica* and *S. vermifera* on growth and fitness parameters of *N. attenuata*

Surface-sterilized *N. attenuata* seeds started to germinate on pre-inoculated and sterile Gamborg's B5 (GB5) medium 4 days after sowing, and germination was complete after 7–8 days (Fig. 2). During this time, fungal inoculated seeds germinated significantly faster (repeated measures ANOVA; $F_{2,9} = 9.99$; $P < 0.01$). After 84 h, 32 and 26% of the seeds germinated on the *P. indica* and *S. vermifera* inoculated plates, while only 8% of the seeds had germinated on the non-inoculated plates. Twelve hours later, the germination rate on the *P. indica* inoculated plates (66%) was twice that on the control, non-inoculated plates (34%) (Fig. 2).

After staining with trypan blue, microscopic observations of inoculated *N. attenuata* roots revealed a close association of root and hyphae (Fig. 3). However, typical structures of ectomycorrhizas such as a Hartig Net or endomycorrhizal structures (e.g. arbuscules and intracellular vesicles) could not be detected. Staining of fungal structures in older roots from plants grown in soil failed.

MeJA treatments were used to elicit herbivore resistance traits and we evaluated the effect of the two fungal species on fitness parameters in elicited and unelicited plants. In unelicited plants, both *P. indica* and *S. vermifera* significantly (repeated measures ANOVA, $F_{2,82} = 21.58$; $P < 0.01$) increased stalk elongation in comparison to non-infected plants (Fig. 4a). Forty days after germination, stalk lengths of plants inoculated with *S. vermifera* were 54% higher than of non-inoculated plants, and 26% higher than that of plants inoculated with *P. indica*. After 50 days of growth, all plants had attained similar sizes (Fig. 4a). Differences among the three treatments were also found in the day of first

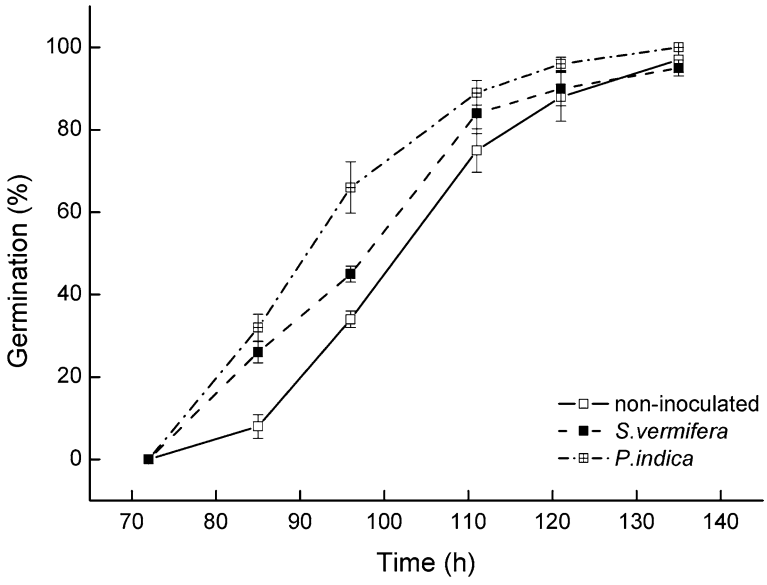

Fig. 2 The effect of preinoculation with *Piriformospora indica* and *Sebacina vermifera* on germination rates. Mean germination rate (±SEM) from four Petri dishes/treatment each with 25 seeds/plate of *Nicotiana attenuata* seeds on *P. indica*-, *S. vermifera*-inoculated, and on control, non-inoculated Gamborg's B5 medium. Petri dishes were inoculated with *P. indica* and *S. vermifera* and incubated for 10 days at 26°C before seeds were added to the plates for germination. Plates were incubated at 26°C and germination, defined by the splitting of seed coat, assayed every 12 h

flowering. Plants inoculated with *S. vermifera* started to flower 45 days after germination, 2 days earlier than plants inoculated with *P. indica* and 3 days earlier than non-inoculated plants (Fig. 4a). In addition, inoculation with *S. vermifera* significantly (one way ANOVA, $F_{1,41\text{-}56} \geq 13.35$; $P < 0.01$) increased the number of flowers and mature capsules produced per plant as well as mean seed mass/capsule (Fig. 5a). The average number of flower insertions of the *S. vermifera* and *P. indica* inoculated *N. attenuata* plants (126 and 96, respectively) was 88 and 43% higher in comparison to control non-inoculated plants (67 flower insertion/plant). *S. vermifera* increased the number of seed capsules by 29.7%, and both fungi increased the average seed mass/capsule by 50% in comparison to non-inoculated plants (Fig. 5a).

As expected, MeJA elicitation of non-inoculated plants decreased all growth related parameters: stalk length was significantly reduced by 7% (one way ANOVA $F_{1,60} = 9.03$; $P < 0.01$); and the number of capsules per plant of the elicited plants was 27% lower compared to the control non-elicited plants (one way ANOVA $F_{1,56} = 14.54$; $P < 0.01$) (Figs. 4a, b and 5a, b). In addition, in comparison to non-elicited plants, MeJA reduced stalk length in both *P. indica* and *S. vermifera* inoculated plants by 13% and the number of capsules per plant by ca 17% (Figs. 4a, b and 5a, b), but no significant differences between the inoculated and control plants were found in stalk elongation, the beginning of flowering (47–48 days after germination), and in the seed mass produced per capsule (Figs. 4b and 5b). However, when *S. vermifera*-inoculated plants were elicited, the number of flower insertions (one way ANOVA, $F_{1,56} = 109.95$; $P < 0.01$) and the number of seed capsules per plant (one way ANOVA, $F_{1,56} = 25.10$; $P < 0.01$) were 71.8 and 48.5% higher than those of elicited non-inoculated plants (Fig. 5b). These results demonstrate that without MeJA elicitation, both fungi increased plant growth performance, however, when plants were MeJA elicited, only plants inoculated with *S. vermifera* realized a fitness benefit of being associated with a fungus.

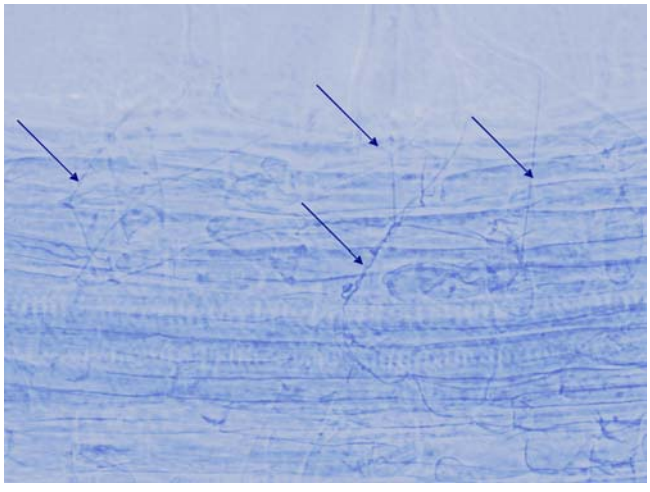

Fig. 3 Trypan blue-stained *Nicotiana attenuata* roots 20 days after inoculation with *Sebacina vermifera*. Seedlings were grown for 3 weeks on Gamborg's B5 medium inoculated with *Sebacina vermifera*. Roots were stained with trypan blue and observed under a light microscope (400× magnification). *Arrows* indicate hyphae; note the lack of classical mycorrhizal structures

Effect on total leaf P and N

To determine if the growth-promoting effects of the inoculation with *S. vermifera* and *P. indica* were associated with an enhanced nutrient status of the plants, total leaf P and N contents were determined. Inoculation with *P. indica* and *S. vermifera* significantly increased the average aboveground dry mass of *N. attenuata* plants by 28% (one way ANOVA, $F_{1,10} = 20.59$; $P < 0.01$) and

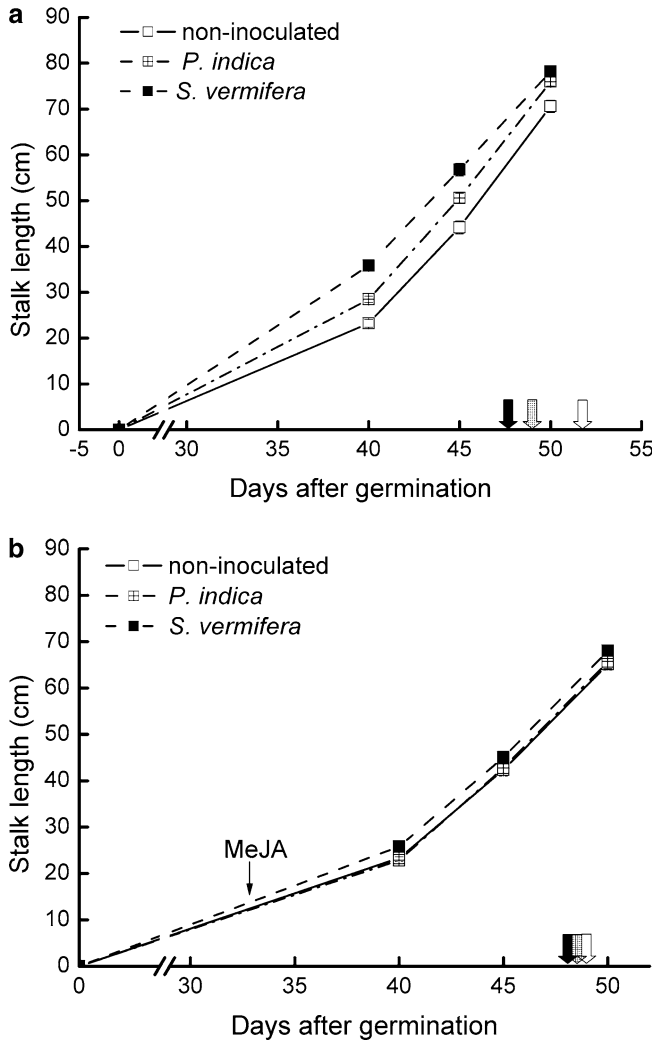

Fig. 4 **a** and **b** The effect of *Piriformospora indica* and *Sebacina vermifera* on growth of *Nicotiana attenuata*. *N. attenuata* plants inoculated with *P. indica* (*hatched bars*), *S. vermifera* (*black bars*) or non-inoculated (*open bars*) were grown in 2 l pots and were either not elicited (**a**) or elicited with methyl jasmonate (MeJA) (**b**) at the time indicated by the *arrow*. Mean (±SEM) stalk lengths during elongation period, with time of first flower production indicated by *arrows*

23% (one way ANOVA, and $F_{1,10} = 19.66$; $P < 0.01$). However, the two fungi had no effect on plant biomass after elicitation with MeJA (4.9 ± 0.16 g in the control, 4.9 ± 0.07 and 5.4 ± 0.11 g in *P. indica* and *S. vermifera* inoculated plants, respectively). Interestingly, the increase in plant biomass after inoculation was not related to an increase in total N and P content. Total N (4.97 ± 0.19%) and P (5.33 ± 0.31 mg/g) contents were not increased by *P. indica* (4.80 ± 0.03% and 5.37 ± 0.30 mg/g, respectively) or *S. vermifera* (4.45 ± 0.21% and 4.92 ± 0.08 mg/g, respectively) associations in unelicited plants. Moreover, when unelicited, N contents of *S. vermifera* inoculated plants was 10.5% lower than in non-inoculated plants. In comparison to unelicited plants, MeJA application did not have any effect on N and P contents, neither in non-inoculated plants (4.73 ± 0.11% and 5.25 ± 0.37 mg/g, respectively) nor in *P. indica*- (4.78 ± 0.03% and 5.71 ± 0.17 mg/g, respectively) and *S. vermifera*- (4.92 ± 0.08% and 5.27 ± 0.37 mg/g, respectively) inoculated plants.

M. sexta performance

To study the effect of *S. vermifera* on aboveground interactions of *N. attenuata* with *M. sexta*, no-choice feeding experiments were conducted. After 10 days of growth, the average weight of caterpillars fed on OS-induced leaves from inoculated plants was 318.50 ± 20.59 mg, significantly heavier (one way ANOVA, $F_{1,40} = 9.58$; $P < 0.01$) than those fed on OS-induced leaves of non-inoculated plants (218.80 ± 23.80 mg).

Effect of *S. vermifera* on direct defense metabolites and TPI activity

To determine whether the increased performance of larvae feeding on *S. vermifera* inoculated plants was associated with changes in defense metabolites in the inoculated plants, the concentration of three secondary metabolites nicotine (NI), rutin (RT) and chlorogenic acid (CA) known to function as direct defenses were measured. HPLC analysis of NI, RT and CA did not reveal significant differences (one way ANOVA, $F_{1,11\text{-}12} \geq 0.22$; $P > 0.05$) between the inoculated and non-inoculated plants. Concentrations of NI in the leaves were 7.4 ± 0.4 and 7.7 ± 0.5 μg/g FW, respectively. Similarly, no differences were found in the concentrations of RT (8.2 ± 0.6 μg/g FW; 8.8 ± 0.7 μg/g FW) and CA (1.8 ± 0.4 μg/g FW; 2.2 ± 0.5 μg/g FW) between the treatments. However, after elicitation with larval OS, TPI activity was significantly higher (one way ANOVA, $F_{1,12} = 4.84$; $P = 0.04$) in leaves of the control non-inoculated plants compared to plants associated with *S. vermifera* (0.68 ± 0.11 nmol/mg protein and 0.35 ± 0.08 nmol/mg protein, respectively).

Discussion

The results presented in this study demonstrate that *N. attenuata* plants realize a fitness benefit from associating with Sebacinales fungi and suggest that the enhanced fitness comes at the expense of a reduction in resistance against herbivores. *P. indica* (Verma et al. 1998) and *S. vermifera* (Warcup 1988) were used in this study as model organisms to test the fitness consequences of beneficial-fungal associations on *N. attenuata*. Both fungi are closely related to a *Sebacina* sp. clone that was detected in soil samples collected from the rhizosphere of *N. attenuata* in its natural habitat (Fig. 1). Several reports have shown the ability of *P. indica* to colonize roots of different plants and demonstrated its

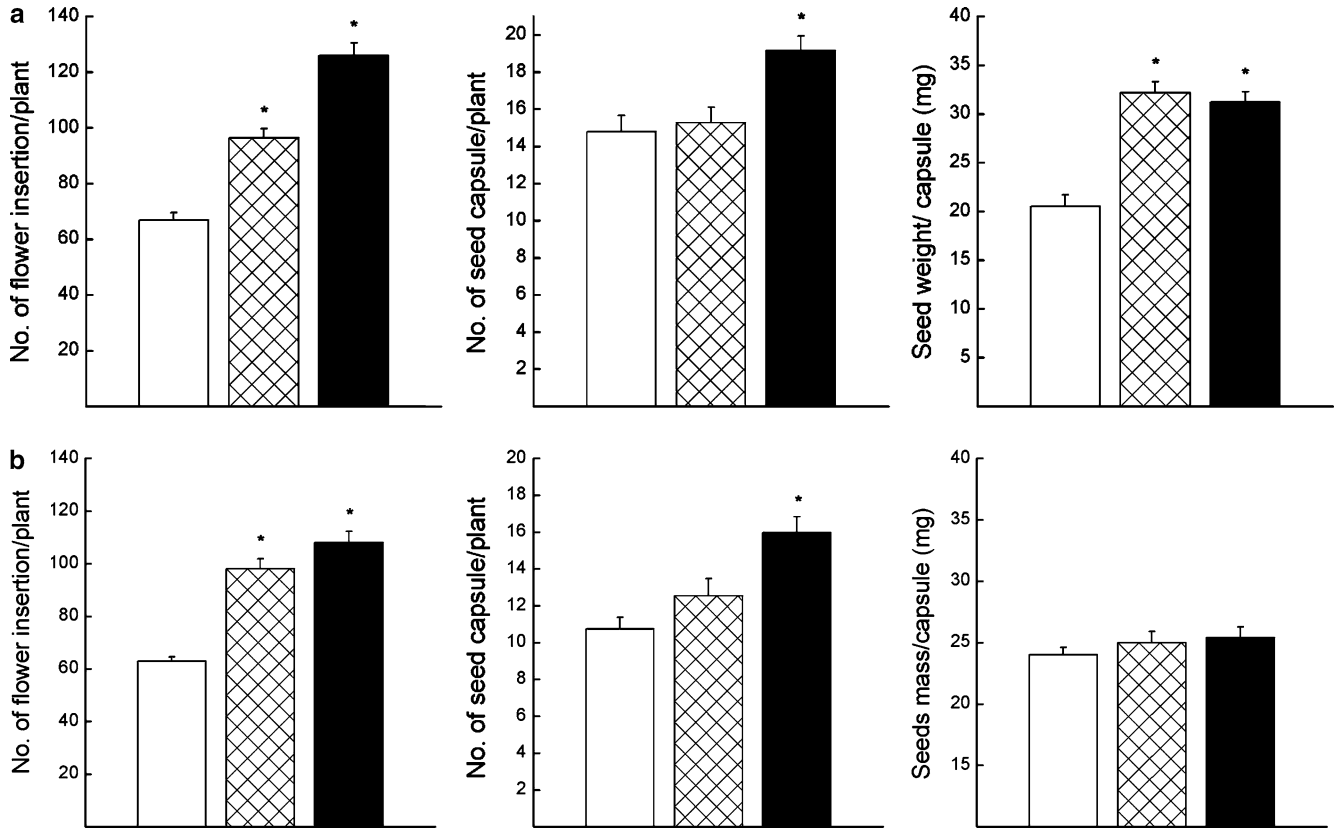

Fig. 5 **a** and **b** The effect of *Piriformospora indica* and *Sebacina vermifera* on fitness parameters of *Nicotiana attenuata*.*N. attenuata* plants inoculated with *P. indica* (*hatched bars*), *S. vermifera* (*black bars*) or non-inoculated (*open bars*) were grown in 2 l pots and were either not elicited (**a**) or elicited with methyl jasmonate (MeJA) (**b**). Mean (±SEM) of number of flower insertions, seed capsules and seed mass/capsule (mg) of 20 plants per treatment. *Asterisks* signify statistically significant differences from non-inoculated plants at $P < 0.05$

growth-promoting effects (Sahay and Varma 1999; Rai et al. 2001; Kumari et al. 2003; Peškan-Berghöfer et al. 2004). Microscopic observations of *N. attenuata* roots revealed a close association of the roots with the fungus (Fig. 3). However, in contrast to the results of Peškan-Berghöfer et al. (2004), the analysis did not reveal the typical features of either ecto- or endo-mycorrhizal fungi; only a very thin layer of mycelia was found on the inoculated roots (Fig. 3). Remarkably, this barely detectable interaction had strong beneficial effects for the plants for all measured growth parameters, from seed germination through seed production (Figs. 2, 4a and 5a). In AM symbiosis, fitness benefits for the plant can partly be attributed to an increased P uptake by the fungus (Smith and Read 1997). Recently, the phosphate transporter gene (StPT3) was identified in *Solanum tuberosum* (Rausch et al. 2001) and expressed in roots colonized with *Glomus intraradices* but not with *P. indica* (Karandashov et al. 2004). In accordance with these results, the analysis of the total N and P content in leaves of inoculated and control non-inoculated *N. attenuata* plants did not indicate an enhanced nutrient status of inoculated plants, suggesting that the beneficial effect is not due to an increased nutrient uptake.

In its natural habitat, germination of *N. attenuata* from long-lived seed banks is synchronized after fire events when water availability is high, which leads to intense intra-specific competition (Baldwin 2001). Under such conditions, the association with the fungi may facilitate rapid growth of *N. attenuata* and lead to a fitness advantage over non-inoculated plants. However, since inoculation with *S. vermifera* increased the plant's susceptibility to herbivore attack, this fitness advantage may become a liability if plants are attacked. Analysis of various defensive chemicals, i.e., NI, RT and CA, did not show significant differences between inoculated and control plants. However, TPI activity in inoculated plants was significantly lower than in control non-inoculated plants. The total protein content in leaves of inoculated plants was not significantly different from non-inoculated plants (data not shown). Moreover, both inoculated and non-inoculated plants were grown in nutrient-rich soil, suggesting that the increase in herbivore performance was not due to a higher nutritional value of the plants. Recently, it was shown that trypsin protease inhibitors (TPI) function as direct defenses in *N. attenuata* against its native herbivores *M. sexta* and *Tupiocoris notatus* (Glawe et al. 2003; Zavala et al. 2004a, 2004b), presumably by inhibiting digestive proteases in insect midguts. Therefore, we propose that *S. vermifera* increases the performance of the caterpillars by inhibiting the expression of TPI defenses. It is still not

clear how the fungi increased plant growth and fitness, but it is possible that the effect is indirectly mediated by decreasing TPI production. How TPI production interferes with plant growth remains an open question. Zavala et al. (2004b) hypothesized that high TPI production might suppress growth of plants by using resources that could otherwise be used for growth, or through the direct inhibition of enzymes that support growth.

The expression of direct defenses can be costly when plants are grown in environments without their natural enemies (Karban and Baldwin 1997) and the expression of TPIs in *N. attenuata* represents one of the best-documented examples of the fitness costs of defense (Zavala et al. 2004b). Zavala and Baldwin (2004) studied the costs and benefits of TPI production in two ecotypes of *N. attenuata*, both of which were transformed to silence or enhance TPI production (Zavala et al. 2004b). They showed that plants with low TPI production had a higher fitness than TPI-expressing genotypes when plants were not attacked, but when attacked, plants with high TPI production had an advantage over TPI-silenced plants (Zavala and Baldwin 2004). Hence, it is likely that reduced TPI production in mycorrhizal plants will have fitness costs when plants are attacked. However, when treated with MeJA, which elicits the production of TPIs, plants inoculated with *S. vermifera* still had a fitness advantage over non-inoculated plants (Fig. 5b). Gehring and Whitham (1991) found the level of ectomycorrhization in resistant pine trees (*Pinus edulis*) to be higher than in trees susceptible to attack from larvae of *Dioryctria albovitella*. Moreover, removal of the herbivores from susceptible trees increased mycorrhization rates. In another study of prairie grasses, the opposite pattern was observed; attack from the two-striped grasshopper (*Melanoplus bivittatus*) increased mycorrhizal colonization (Kula et al. 2005). Thus, it is possible that herbivore attack alters the association between *N. attenuata* and fungi either by reducing the association and increasing herbivore resistance, or by increasing it and promoting rapid growth. Whether the benefits of the association outweigh their costs is best determined in the plant's natural environment with its complete complement of ecological interactions.

The wound signal, jasmonic acid (JA), which is known to elicit herbivore resistance traits in many plant species, has also been implicated in plant–mycorrhizal associations. Treatment with JA stimulates ectomycorrhiza development in *Allium sativum* and *Picea abies* (Regvar et al. 1996, 1997). Hause et al. (2002) suggested that expression of JA biosynthesis genes occur after *G. intraradices* fully colonize root cortex cells of *Hordeum vulgare* and not during the infection process. In *N. attenuata*, plants transformed to express the gene for the first committed enzyme in JA biosynthesis, lipoxygenase 3, in an antisense orientation, had attenuated JA levels, reduced TPI activity and increased susceptibility to *M. sexta* larvae attack (Halitschke and Baldwin 2003). It is therefore possible that *S. vermifera* inhibits TPI production through a manipulation of the oxylipin pathway that leads to JA production. Examinations of JA contents of inoculated plants and performance tests with transgenic *N. attenuata* plants, silenced in JA elicitation, should cleanly falsify this hypothesis.

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References

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

Baldwin IT (2001) An ecologically motivated analysis of plant–herbivore interactions in native tobacco. Plant Physiol 127:1449–1458

Baldwin IT, Preston CA (1999) The eco-physiological complexity of plant responses to insect herbivores. Planta 208:137–145

Borowicz VA (2001) Do arbuscular mycorrhizal fungi alter plant–pathogen relations? Ecology 82:3057–3068

Bubner B, Gase K, Baldwin IT (2004) Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. BMC Biotechnol 4:1–11

van Dam NM, Horn M, Mares M, Baldwin IT (2001) Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata*. J Chem Ecol 27:547–568

Gange AC (2001) Species-specific responses of a root- and shoot-feeding insect to arbuscular mycorrhizal colonization of its host plant. New Phytol 150:611–618

Gange AC, Nice HE (1997) Performance of the thistle gall fly, *Urophora cardui*, in relation to host plant nitrogen and mycorrhizal colonization. New Phytol 137:335–343

Gange AC, Stagg PG, Ward LK (2002) Arbuscular mycorrhizal fungi affect phytophagous insect specialism. Ecol Lett 5:11–15

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

Gehring CA, Whitham TG (1991) Herbivore-driven mycorrhizal mutualism in insect-susceptible pinyon pine. Nature 353:556–557

Gehring CA, Whitham TG (1995) Duration of herbivore removal and environmental-stress affect the ectomycorrhizae of pinyon pines. Ecology 76:2118–2123

Glawe GA, Zavala JA, Kessler A, Van Dam NM, Baldwin IT (2003) Ecological costs and benefits correlated with trypsin protease inhibitor production in *Nicotiana attenuata*. Ecology 84:79–90

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

Goverde M, van der Heijden MGA, Wiemken A, Sanders IR, Erhardt A (2000) Arbuscular mycorrhizal fungi influence life history traits of a lepidopteran herbivore. Oecologia 125:362–369

Grime JP, Mackey JML, Hillier SH, Read DJ (1987) Floristic diversity in a model system using experimental microcosms. Nature 328:420–422

Halitschke R, Baldwin IT (2003) Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata*. Plant J 36:794–807

Halitschke R, Schittko U, Pohnert G, Boland W, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host

Nicotiana attenuata. III. Fatty acid–amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. Plant Physiol 125:711–717
Hause B, Maier W, Miersch O, Kramell R, Strack D (2002) Induction of jasmonate biosynthesis in arbuscular mycorrhizal barley roots. Plant Physiol 130:1213–1220
Hogeweg P, Hesper B (1984) The alignment of sets of sequences and the construction of phylogenetic trees: an integrated method. J Mol Evol 20:175–186
Käfer E (1977) Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv Genet 19:33–131
Kaldorf M, Kuhn AJ, Schroder WH, Hildebrandt U, Bothe H (1999) Selective element deposits in maize colonized by a heavy metal tolerance conferring arbuscular mycorrhizal fungus. J Plant Physiol 154:718–728
Karandashov V, Nagy R, Wegmuller S, Amrhein N, Bucher M (2004) Evolutionary conservation of a phosphate transporter in the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 101:6285–6290
Karban R, Baldwin IT (1997) Induced responses to herbivory. The University of Chicago Press, Chicago
Keinanen M, Oldham NJ, Baldwin IT (2001) Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. J Agric Food Chem 49:3553–3558
Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annu Rev Plant Biol 53:299–328
Kottke I, Beiter A, Weiss M, Haug I, Oberwinkler F, Nebel M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while *Aneura pinguis* (Metzgeriales) is associated with a *Tulasnella* species. Mycol Res 107:957–968
Krügel T, Lim M, Gase K, Halitschke R, Baldwin IT (2002) *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. Chemoecology 12:177–183
Kula AAR, Hartnett DC, Wilson GWT (2005) Effect of mycorrhizal symbiosis on tallgrass prairie plant–herbivore interactions. Ecol Lett 8:61–69
Kumari R, Kishan H, Bhoon YK, Varma A (2003) Colonization of cruciferous plants by *Piriformospora indica*. Curr Sci India 85:1672–1674
Little LR, Maun MA (1996) The ''Ammophila problem'' revisited: a role for mycorrhizal fungi. J Ecol 84:1–7
Lynds GY, Baldwin IT (1998) Fire, nitrogen, and defensive plasticity in *Nicotiana attenuata*. Oecologia 115:531–540
Marulanda A, Azcon R, Ruiz-Lozano JM (2003) Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress. Physiol Plantarum 119:526–533
McCloud ES, Baldwin IT (1997) Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. Planta 203:430–435
Norman JR, Atkinson D, Hooker JE (1996) Arbuscular mycorrhizal fungal-induced alteration to root architecture in strawberry and induced resistance to the root pathogen *Phytophthora fragariae*. Plant Soil 185:191–198
van der Peer Y, De Watcher R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 10:569–570
Peškan-Berghöfer T, Shahollari B, Giong PH, Hehl S, Markert C, Blanke V, Kost G, Varma A, Oelmüller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plantarum 122:465–477
Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128
Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, Amrhein N, Bucher M (2001) A phosphate transporter expressed in arbuscule-containing cells in potato. Nature 414:462–466
Read DJ (1998) Biodiversity: plants on the web. Nature 396:22–23
Regvar M, Gogala N, Zalar P (1996) Effects of jasmonic acid on mycorrhizal *Allium sativum*. New Phytol 134:703–707
Regvar M, Gogala N, Znidarsic N (1997) Jasmonic acid effects mycorrhization of spruce seedlings with *Laccaria laccata*. Trees Struct Funct 11:511–514
Roda A, Halitschke R, Steppuhn A, Baldwin IT (2004) Individual variability in herbivore-specific elicitors from the plant's perspective. Mol Ecol 13:2421–2433
Ruiz-Lozano JM, Azcón R (1995) Hyphal contribution to water uptake in mycorrhizal plants as affected by the fungal species and water status. Physiol Plantarum 95:472–478
Ruiz-Lozano JM, Azcón R, Palma JM (1996) Superoxide dismutase activity in arbuscular mycorrhizal *Lactuca sativa* plants subjected to drought stress. New Phytol 134:327–333
Ruiz-Lozano JM, Collados C, Barea JM, Azcón R (2001) Cloning of cDNAs encoding SODs from lettuce plants which show differential regulation by arbuscular mycorrhizal symbiosis and by drought stress. J Exp Bot 52:2241–2242
Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181:297–302
Schittko U, Hermsmeier D, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. II. Accumulation of plant mRNAs in response to insect-derived cues. Plant Physiol 125:701–710
Selosse MA, Bauer R, Moyersoen B (2002a) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol 155:183–195
Selosse MA, Weiss M, Jany JL, Tillier A (2002b) Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) LCM Rich. and neighbouring tree ectomycorrhizae. Mol Ecol 11:1831–1844
Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, San Diego, CA, USA
Tajima F, Nei M (1984) Estimation of evolutionary distance between nucleotide-sequences. Mol Biol Evol 1:269–285
Taylor DL, Bruns TD, Szaro TM, Hodges SA (2003) Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. Am J Bot 90:1168–1179
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882
Trotta A, Varese GC, Gnavi E, Fusconi A, Sampo S, Berta G (1996) Interactions between the soilborne root pathogen *Phytophthora nicotianae* var *parasitica* and the arbuscular mycorrhizal fungus *Glomus mosseae* in tomato plants. Plant Soil 185:199–209
Urban A, Weiss M, Bauer R (2003) Ectomycorrhizas involving sebacinoid mycobionts. Mycol Res 107:3–14
Varma A, Verma S, Sudha, Sahay N, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744
Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:896–903
Vicari M, Hatcher PE, Ayres PG (2002) Combined effect of foliar and mycorrhizal endophytes on an insect herbivore. Ecology 83:2452–2464
Warcup JH (1988) Mycorrhizal associations of isolates of *Sebacina vermifera*. New Phytol 110:227–231
Wardle DA, Bardgett RD, Klironomos JN, Setala H, van der Putten WH, Wall DH (2004) Ecological linkages between aboveground and belowground biota. Science 304:1629–1633

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

Zavala JA, Baldwin IT (2004) Fitness benefits of trypsin proteinase inhibitor expression in *Nicotiana attenuata* are greater than their costs when plants are attacked. BMC Ecol 4:11 (DOI 10.1186/1472-6785-4-11)

Zavala JA, Patankar AG, Gase K, Hui DQ, Baldwin IT (2004a) Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. Plant Physiol 134:1181–1190

Zavala JA, Patankar AG, Gase K, Baldwin IT (2004b) Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*. Proc Natl Acad Sci USA 101:1607–1612