New Phytologist

# Suppression of plant defence response by a mycorrhiza helper bacterium

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Received: *27 October 2006*
Accepted: *22 December 2006*

## Summary

• The aim of the present study was to determine whether the mycorrhiza helper bacterium *Streptomyces* sp. AcH 505 could serve as a biocontrol agent against *Heterobasidion* root and butt rot.

• Bacterial influence on mycelial growth of *Heterobasidion* sp. isolates, on the colonization of wood discs and Norway spruce (*Picea abies*) roots was determined. The effect of AcH 505 on plant photosynthesis, peroxidase activity and gene expression, and needle infections were investigated.

• AcH 505 was antagonistic to 11 of 12 tested fungal *Heterobasidion* isolates. The antagonism resulted in a suppression of fungal colonization of Norway spruce roots and wood discs. Mycelial growth rate of the 12th strain, *Heterobasidion abietinum* 331 was not affected by AcH 505, and colonization of roots by this fungal strain was promoted by AcH 505. Bacterial inoculation led to decreased peroxidase activities and gene expression levels in roots.

• AcH 505 promotes plant root colonization by *Heterobasidion* strains that are tolerant to antifungal metabolites produced by the bacterium. This may result from unknown bacterial factors that suppress the plant defence response.

**Key words:** biocontrol, defence, *Heterobasidion*, plant pathogen, root infection, *Streptomyces*.

*New Phytologist* (2007) **174**: 892–903


**doi**: 10.1111/j.1469-8137.2007.02021.x

## Introduction

The establishment of mycorrhizal symbioses can be positively influenced by certain bacterial isolates, an effect exhibited by mycorrhiza helper bacteria (MHB, Garbaye, 1994). The MHB include a variety of Gram-negative (Barea *et al.*, 1998; Founoune *et al.*, 2002; Frey-Klett *et al.*, 2005) and Gram-positive (Budi *et al.*, 1999; Poole *et al.*, 2001; Schrey *et al.*, 2005) species. Because of their potential for increasing plant nutrition and health, the use of MHB in low-input agriculture and forestry has been addressed in several investigations (Duponnois *et al.*, 1993; Barea *et al.*, 1998; Becker *et al.*, 1999; Budi *et al.*, 1999; Schrey *et al.*, 2005). With increasing interest in safe and environmentally friendly alternatives for plant disease control compared with the application of conventional pesticides (Whipps, 2001), the MHB could be used in nurseries to promote controlled mycorrhization of seedlings and to prevent attacks by phytopathogens (Frey-Klett & Garbaye, 2005).

*Streptomyces* sp. AcH 505 promotes the growth of and mycorrhiza formation by symbiotic fungi *Amanita muscaria* and *Suillus bovinus*, but it is antagonistic against *Heterobasidion annosum* (Maier, 2003; Maier *et al.*, 2004; Schrey *et al.*, 2005). In earlier experiments conducted *in vitro*, we showed that the secondary metabolites of AcH 505 regulate these interactions: whereas the promoted fungi are tolerant against the antibiotic WS-5995 B produced by AcH 505, the suppressed ones are sensitive. Both promoted and suppressed fungal isolates respond in a similar way to the fungal growth promoter produced by AcH 505, a novel compound classified as auxofuran (Riedlinger *et al.*, 2006). Thus, in regard to biocontrol by AcH 505 against plant pathogenic fungi like *H. annosum*, the most critical aspect seems to be the tolerance of the fungus against the antibiotic WS-5995 B, and the bacterial

production of the antifungal metabolites on natural substrates.

Most conifer trees are susceptible to infection by the basidiomycete *Heterobasidion annosum* s.l., the causal organism of annosum root rot (Asiegbu *et al.*, 2005). It is common on forest trees in the northern hemisphere (Korhonen & Stenlid, 1998), and as a result of decayed wood and reduced tree growth this fungus is responsible for annual estimated losses of 790 million Euros (Woodward *et al.*, 1998). *Heterobasidion annosum* s.l. forms a species complex in Europe, consisting of three species with partly overlapping distributions and host specificities (Korhonen, 1978). These include *H. annosum*, *Heterobasidion parviporum* and *Heterobasidion abietinum*, with a preference, but not species-specificity, for pine, spruce and fir, respectively (Niemelä & Korhonen, 1998).

*Heterobasidion annosum* s.l. infects its host trees by two major routes. First, the fungus develops basidiocarps on infected trees that release masses of basidiospores (Redfern & Stenlid, 1998). These basidiospores infect exposed woody tissues such as wounds or stumps and, supported by the nutrients from the infected plant material, the fungal mycelia grow through root-to-root contacts or grafts to the next tree (Risbeth, 1951). Diverse plant defence responses have been developed in the host plants that minimize damage caused by the fungus (Asiegbu *et al.*, 2005). These include necrosis, formation of structural barriers, formation of antifungal enzymes and metabolites, and defence related gene expression responses (Karjalainen *et al.*, 1998; Hietala *et al.*, 2004; Nagy *et al.*, 2004a; Asiegbu *et al.*, 2005).

We have previously suggested that AcH 505 could be used for simultaneous promotion of symbiotic and suppression of plant pathogenic fungi (Schrey *et al.*, 2005). The aim of the present study was to determine whether the production of antifungal metabolites by AcH 505 would make it an efficient biocontrol agent against *H. annosum* s.l. in wood discs and in seedling roots of Norway spruce (*Picea abies*). Taking into consideration the potential variability among fungal isolates, we first used a large selection of Norway spruce pathogenic *H. annosum* s.l. isolates and then concentrated on two strains that were different in their responses against the bacterium. We show that while most fungal strains tested were suppressed by the bacterium, root infection with a WS-5995 B tolerant *H. abietinum* isolate was promoted by AcH 505.

## Materials and Methods

### Bacterial and fungal strains

*Streptomyces* sp. AcH 505, isolated from the hyposphere soil around Norway spruce mycorrhizas in Haigerloch, Germany (Maier *et al.*, 2004), was maintained on ISP2 agar medium (Shirling & Gottlieb, 1966). Twelve spruce pathogenic strains of *H. annosum* (Fr.) Bref., *H. abietinum* Niemelä & Korhonen or *H. parviporum* Niemelä & Korhonen (Table 1) were maintained on 1.5% malt agar. Spruce pathogenic grey mould *Botrytis cinerea* Pers. Fr, teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel isolate BcSjk1.1 (Petäistö *et al.*, 2004), originating from a diseased Norway spruce seedling from Suonenjoki, Finland, was maintained on potato dextrose agar.

**Table 1** *Heterobasidion* isolates

| Abbreviation | *Heterobasidion* isolate | Geographical origin |
|---|---|---|
| Hab 130 | *H. abietinum* 130 | Trentino, Italy |
| Hab 331 | *H. abietinum* 331 | Klein Kotterbachtal, Austria |
| Han 005 | *H. annosum* 005 | Kirkkonummi, Finland |
| Han 009 | *H. annosum* 009 | Kirkkonummi, Finland |
| Han 013 | *H. annosum* 013 | Jämsänkoski, Finland |
| Han F2120 | *H. annosum* F2120 | Tübingen, Germany |
| Hpa 005 | *H. parviporum* 005 | Oravainen, Finland |
| Hpa 007 | *H. parviporum* 007 | Kirkkonummi, Finland |
| Hpa 017 | *H. parviporum* 017 | Kirkkonummi, Finland |
| Hpa 062 | *H. parviporum* 062 | Trentino, Italy |
| Hpa 088 | *H. parviporum* 088 | Trentino, Italy |
| Hpa 107 | *H. parviporum* 107 | Trentino, Italy |

### Fungus–bacterium interaction assays on agar and on Norway spruce wood

The conditions for bacterium–fungus dual cultures on ISP2 agar medium, and the screening of bacterial effects on fungal growth were as described in Maier *et al.* (2004). Fungal growth was measured at day 10 post inoculation. Ten replicates were made.

Bilayer assays on wood were based on Schoeman *et al.* (1994), who observed that the results from bilayer assays correlate well with those from dual inoculations of nonsterile wood blocks. The bilayer assay was used here because of its simplicity and reproducibility. Stem discs, $6 \pm 2$ cm in diameter and 0.8 cm thick, were cut from a 10-yr-old Norway spruce tree. The bark was removed and the discs were sterilized by autoclaving. Streptomyces sp. AcH 505 was cultured in liquid ISP2 medium as described by Schrey *et al.* (2005). Actively growing bacterial cultures at an optical density at 600 nm ($OD_{600}$) of 0.6 were harvested by centrifugation at 500 ***g***, washed to remove nutrients and exudates, and blended with sterile distilled water. The concentration of the colony forming unit (CFU) suspension, composed of streptomycete hyphae, fragments and spores, was adjusted to $3 \times 10^5$ CFU $ml^{-1}$ of water. Four hundred microlitres of AcH 505 suspension ($4 \times 10^5$ cells) were spread uniformly on the surface of wood discs that were previously placed on top of a wet filter paper to maintain a high relative humidity. Control discs to be treated only with *H. annosum* were spread with 400 µl of sterile distilled water. The wood discs were incubated in the dark at 20°C. After an incubation period of 7 d, the discs were overlaid with a 1-ml aliquot of 2% water agar, cooled to 40°C. After the agar had set, one mycelial plug, cut with a 5-mm

**Table 2** Bacterial and fungal inoculations of Norway spruce (*Picea abies*) seedlings

| Sample | Seedling age 28 d Preinoculation | 35 d Postinoculation |
|---|---|---|
| Control | Water | Water |
| AcH 505 | AcH 505 | Water |
| Han 005 | Water | *H. annosum* 005 |
| AcH 505 + *Han* 005 | AcH 505 | *H. annosum* 005 |
| *Hab* 331 | Water | *H. abietinum* 331 |
| AcH 505 + *Hab* 331 | AcH 505 | *H. abietinum* 331 |
| Bc | Water | *Botrytis cinerea* |
| AcH 505 + Bc | AcH 505 | *B. cinerea* |

AcH 505, *Streptomyces* sp. AcH 505; *Han* 005, *Heterobasidion annosum* 005; *Hab* 331, *Heterobasidion abietinum* 331. AcH 505, *Han* 005 and *Hab* 331 were inoculated on the roots, and *B. cinerea* on the needles.

cork borer from actively growing rims of the *Heterobasidion* strains, was placed face down on the fresh agar surface of each wood disc. After a further 72 h incubation, *Heterobasidion* growth was evaluated with the aid of a binocular from 10 wood discs per treatment. Based on work carried out by Schoeman *et al.* (1994), the growth of *Heterobasidion* isolates was assessed according to six categories: (0) no mycelial growth; (1) presence of a few dispersed mycelial bristles (short filaments) extending from the plug; (2) continuous growth of mycelial bristles around the plug periphery; (3) plug completely covered with mycelium but no growth onto the adjoining agar; (4) mycelial growth on the plug and extending linearly up to 1 cm onto the bilayer surface; (5) mycelial growth extending to more than 1 cm beyond the plug onto the bilayer surface. The categories were used as values to obtain the means and standard deviations for the bilayer experiments.

## Seedling preparation, bacterial and fungal inoculations

Seeds of Norway spruce (*Picea abies* (L.) Karst) were obtained from the Staatsklenge Nagold (Nagold, Germany). Seedlings were cultivated until 4-wk-old according to Schaeffer *et al.* (1996). Bacterial and fungal inoculations were performed inside cross-walled Petri dishes. Fifteen millilitres of MMN agar medium (Molina & Palmer, 1982) without a carbon source was added to one side of the cross-walled Petri dish, leaving the other side empty. Three 3-mm slots were melted into the cross-walls to ease the placement of seedlings. Four-week-old seedlings were placed inside the cross-walled Petri dishes, in a way that the roots were supported by the MMN medium whereas the needles and hypocotyls were positioned on the empty side. An overview of the inoculations performed is given in Table 2. For bacterial inoculation, 400 µl of AcH 505 suspension ($3 \times 10^5$ CFU $ml^{-1}$ in distilled water) was evenly spread on the agar medium. The MMN medium for control seedlings was supplied with 400 µl sterile distilled water. The seedlings were grown for 1 wk under standard conditions (Schrey *et al.*, 2005). After this, the spruce seedlings were inoculated with the fungal spore suspensions: *H. abietinum* 331 or *H. annosum* 005 were used for the root and *Botrytis cinerea* for needle inoculations.

To inoculate the roots with *Heterobasidion*, a conidiospore suspension ($3 \times 10^5$ spores $ml^{-1}$, based on Asiegbu *et al.*, 1993) was prepared by washing 2-wk-old mycelia with sterile water. From the *Heterobasidion* spore suspension, 400 µl were evenly spread on the agar surface. A moist filter paper was placed on the roots of the seedlings to prevent drying of the root system. After 1 wk in the growth chamber, seedlings were harvested for analysis. For all inoculations (water only, bacteria, fungus, bacteria and fungus), 14 replicates were made. Five of these were used for microscopic analysis. To obtain homogeneous material for enzyme and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analyses, the first 3 cm of roots and the needles of nine seedlings were separated, pooled as three fractions (three biological replicates) and powdered under liquid nitrogen.

Plant and bacterial influence on conidial germination and the growth of germ tubes of *H. abietinum* 331 were analysed *in vivo* using light microscopy. For this, bacterial and fungal treatments were implemented as explained above. Two-hundred fungal spores adjacent (first 100 µm) to the root surface were analysed for germination. At 48 h, when 60–70% of the spores were germinated, the length of the germ tubes of 100 randomly selected spores was measured.

The needles of Norway spruce were treated with the grey mould *Botrytis cinerea* 1 wk after bacterial inoculation. To obtain a fungal spore suspension, 2-wk-old heavily sporulating mycelia were washed with sterile water, the spores counted and diluted to a concentration of $1 \times 10^5$ spores $ml^{-1}$ water. The needles were gently abraded with carborundum before infection to increase the needle infection rate by the grey mould. Each seedling was sprayed with 1 ml spore suspension or with 1 ml water. After 4 d inoculation, the amount of diseased needles per seedling was estimated, dividing the symptoms of grey mould infection into four categories: (1) needle killed; (2) 50% or more of the needle infected; (3) < 50% of needle infected; (4) no visible symptoms. The needles of 10 seedlings from each treatment were examined, and the categories were used as values to obtain the means for the needle infection experiments.

## Tissue preparation for light and electron microscopy

For the light microscopy analysis, five Norway spruce roots from each treatment were examined. Starting from a distance of 2 cm from the root tip, two 5-mm sections were cut and fixed for a minimum of 1 h in 0.1 M sodium cacodylate buffer containing 2% glutaraldehyde. The samples were then washed with 0.1 M sodium cacodylate buffer, postfixed with 1% osmium for 1 h, washed with water and contrasted with

 

uranylacetate for 1 h. The samples were then washed again with water, dehydrated in acetone, infiltrated in an acetone–vinylcyclohexendioxide series and finally embedded in silicone. The incubation time of 24 h at 70°C was used for hardening. Semithin sections (0.75 μm) were made with a glass knife using an ultramicrotome (Ultracut; Reichert-Jung, Leica, Bensheim, Germany) and analysed with a light microscope. Ultrathin sections (70 nm) were cut from selected samples with a diamond knife (Drukker; W. Reichert Labtech, Munich, Germany), mounted onto copper grids (SCI, Science series GmbH, Munich, Germany), and coated with Formvar carbon film. Sections were contrasted with 6% lead-citrate for 13 min, and rinsed with distilled water. The ultrathin sections were examined with a Zeiss EM 109 transmission electron microscope at 80 kV.

### Chlorophyll fluorescence and water content measurements

Three needles of identical size from 10 seedlings per treatment were used for chlorophyll fluorescence measurements with a PAM fluorometer (Imaging PAM, Walz, Effeltrich, Germany) based on Nagy *et al.* (2004b). Dark-adapted seedlings (15 min darkness before the measurement) were used for the measurement of maximal photosystem II (PSII) efficiency ($F_v/F_m$) with a white saturating pulse (3000 μE $m^{-2}$ $s^{-1}$) of 0.7 s duration. The water content of the roots and shoots of 10 seedlings was calculated as a percentage of dry weight: (fresh weight-dry weight)/dry weight. Samples were dried for 3 d at 50°C.

### Peroxidase activity measurements and real-time PCR analysis of *PaSpi2* expression levels

Soluble proteins were extracted and peroxidase activity was measured according to Mensen *et al.* (1998) using guaiacol (Sigma-Aldrich, Taufkirchen, Germany) as substrate. The oxidation of guaiacol at 470 nm was followed for 2 min. First 3 cm of roots or needles were used for RNA extraction according to Nehls *et al.* (1998). RNA was quantified spectrophotometrically. The RNA samples were routinely checked for DNA contamination by PCR analyses and, if necessary, treated with DNAse I (Invitrogen, Groningen, Netherlands) according to the manufacturer's instructions. The expression of the Norway spruce *Spi*2 (spruce pathogenesis related protein 2) gene was assayed by real-time PCR analysis according to Hietala *et al.* (2004). The Norway spruce α–tubulin gene was used as an endogenous reference (Hietala *et al.*, 2004). Real-time PCR was performed using iScript One-Step RT-PCR Kit (containing Sybr green and fluorescein; Bio-Rad, Hercules, CA, USA). 1 μl RNA (adjusted to 8 ng), and 300 nM of each gene specific primer was used for the analysis in an iQ 5 Multicolour Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). In this reaction mixture, cDNA was synthesized with gene-specific primers. The PCR was always performed in triplicates together with a dilution series of the reference gene. Three biological replicates were used for the analysis. Primers used for analysis of *Spi*2 expression were: *Spi*2 sense, 5′-GGGCAGAAGGGACTCCAGAAG-3′; and Spi2 antisense, 5′-GCCGCCTGAGAGAACCAACC-3′. Reverse transcription was 10 min at 50°C and cycling conditions were 10 s at 95°C; 30 s at 55°C.

## Results

### Influence of the bacterium on fungal growth and colonization of wood disc

The influence on mycelial growth of 12 *Heterobasidion* isolates by *Streptomyces* sp. AcH 505 was evaluated by dual cultures on agar (Fig. 1a). The bacterium was antagonistic against the *Heterobasidion* isolates with one exception: *Heterobasidion abietinum* 331 was not affected by the treatment with AcH 505. Three of the isolates, *H. annosum* 005, *H. parviporum* 005 and *H. abietinum* 331, representing two isolates sensitive against and one tolerant to AcH 505, were subjected to the wood disc bilayer assay to estimate the relevance of the agar tests. As on agar, the mycelial growth of *H. abietinum* 331 was unaffected by the presence of the bacteria, but the growth of *H. annosum* 005 and *H. parviporum* 005 was severely retarded by AcH 505 (Fig. 1b). We have previously reported that AcH 505 produces the antibiotic WS-5995 B, and that fungi which are sensitive against this antibiotic are suppressed in dual culture with AcH 505 (Riedlinger *et al.*, 2006). Thus, we recorded the growth of the three *Heterobasidion* isolates on a culture medium supplemented with WS-5995 B. At 25 μM WS-5995 B concentration, mycelial extension of *H. annosum* 005 (56% radius in relation to control treatment) and *H. parviporum* 005 (52%), respectively, was inhibited, while that of *H. abietinum* 331 (98%) was not affected. We thus assume that the tolerance of *H. abietinum* 331 against AcH 505 is related to its tolerance against the antibiotic WS-5995 B.

### Primary root colonization by *H. abietinum* 331 and *H. annosum* 005 under bacterial influence

The antagonistic activity of AcH 505 against *H. annosum* 005 suggested that the bacterium may be capable of delaying the spread of the fungus and the invasion of the roots. By contrast, the resistance of *H. abietinum* 331 against the bacterial metabolites indicated that AcH 505 does not inhibit the colonization of the plant by the latter fungus. The presence of fungal mycelia on the root surface and the penetration of cortex cells at 25 mm distance from the root tip were used as the basis for assessing root health (Table 3; Fig. 2). The estimates were done 1 wk after fungal spore inoculation, at the time when the fungal hyphae covered the root surface in

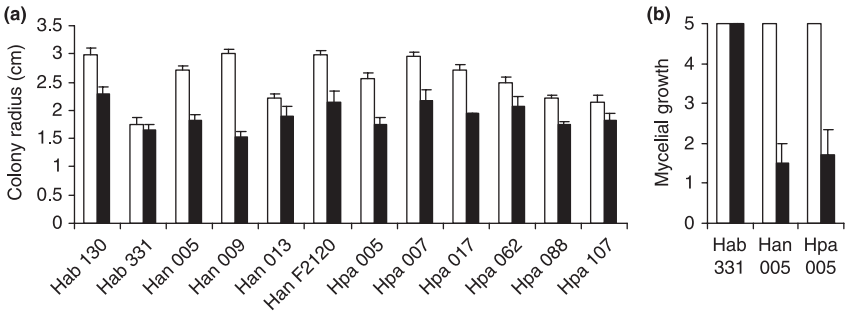


**Fig. 1** Growth inhibition of *Heterobasidion* spp. caused by *Streptomyces* sp. AcH 505 on agar and on wood discs from the bilayer assay. (a) Mycelial growth (colony radius) of 12 *Heterobasidion* isolates on agar medium. Open columns, radii of axenic mycelial colonies; closed columns, radii of dual cultures with AcH 505. The mycelial growth of the fungal isolate *Heterobasidion abietinum* 331 (Hab 331) is not significantly affected by the bacterial treatment. In all other isolates tested, the radius of mycelia is significantly smaller in dual culture ($P < 0.001$) according to student's *t*-test. (b) Mycelial growth of three *Heterobasidion* isolates on wood disc bilayer assay according to Schoeman *et al*. (1994). Open columns, growth of axenic mycelia; closed columns, growth of dual cultures with AcH 505. The growth of *Heterobasidion* isolates was assessed according to six categories: (0) no mycelial growth; (1) presence of a few dispersed mycelial bristles (short filaments) extending from the plug; (2) continuous growth of mycelial bristles around the plug periphery; (3) plug completely covered with mycelium but no growth onto the adjoining agar; (4) mycelial growth on the plug and extending linearly up to 1 cm onto the bilayer surface; (5) mycelial growth extending to more than 1 cm beyond the plug onto the bi-layer surface. The categories were used as values to obtain the means and standard deviations (error bars) for 10 replications each. The treatment with AcH 505 causes a statistically significant decrease ($P < 0.001$) in mycelial growth of *H. annosum* 005 and *H. parviporum* 005 according to student's *t*-test.

**Table 3** Influence of AcH 505 on the colonization of Norway spruce (*Picea abies*) roots by *Heterobasidion annosum* 005 and *Heterobasidion abietinum* 331

| Treatment | Fungal hyphae on root surface | Fungal hyphae in cortex cells |
|---|---|---|
| *H. annosum* 005 | 50/50 | nd |
| *H. annosum* 005 + AcH 505 | nd | nd |
| *H. abietinum* 331 | 50/50 | nd |
| *H. abietinum* 331 + AcH 505 | 50/50 | 50/50 |

nd, none detected.
Samples were examined 7 d after fungal inoculation. Ten cross-sections per seedling, from five seedlings, were analysed per treatment.

samples without bacterial treatment. The spread of *H. annosum* 005 was inhibited by AcH 505 and no fungal hyphae were observed on the root surface. By contrast, the preinoculation of roots with AcH 505 caused an extensive colonization of the epidermal and cortex tissues by *H. abietinum* 331. No colonization of the stele was observed at this stage (Table 3).

Light micrographs of the Norway spruce-AcH 505-*H. abietinum* 331 interaction suggested that fungal material penetrated the root cortex cells. Electron micrographs were taken to confirm the identity of the root colonizing microorganisms. The electron dense bacterial filaments (Fig. 3a,b) were easily distinguishable from the pale grey fungal hyphae (Fig. 3b). Only fungal hyphae were detected inside the root cells (Fig. 3c).

To investigate how far the known AcH 505 products contribute to the enhanced colonization rate of Norway spruce, we next tested whether auxofuran could increase spruce colonization by *H. abietinum* 331. We used 10 nM, 100 nM and 1 μM solutions, since this range covers the concentrations that have been recorded from AcH 505 culture media in single, bacterium–fungus, or tripartite cultivations (Riedlinger *et al*., 2006; J. Riedlinger unpublished). The colonization rate of Norway spruce roots was not increased after auxofuran or WS-5995 B applications, indicating that other factors (irrespective of fungal growth promotion) were responsible for the extensive colonization of spruce roots by *H. abietinum* 331 under the bacterial influence.

## Microbial interaction-related changes in plant physiology

Since AcH 505 promoted the colonization of seedling roots by a fungal strain that was not affected by the bacterium, we reasoned that the bacterium might affect the host physiology and/or defence response, resulting in increased fungal colonization. We thus measured the photosynthetic yield of spruce seedlings as a vitality marker (Fig. 4), in addition to root and needle water contents (Nagy *et al*., 2004b). AcH 505 inoculation led to a significantly (*t*-test, $P < 0.01$) increased $F_v/F_m$ value, representing an estimate of the maximum quantum efficiency of photosystem II in a dark adapted state (Maxwell & Johnson, 2000). The *H. abietinum* 331 treated seedlings showed a decreased value in root water content, and a drop in the $F_v/F_m$ value ($P < 0.01$). Dual (bacterium + fungus) infection caused a significantly decreased root water content ($P < 0.01$), but the $F_v/F_m$ values remained close to those in the controls.

 

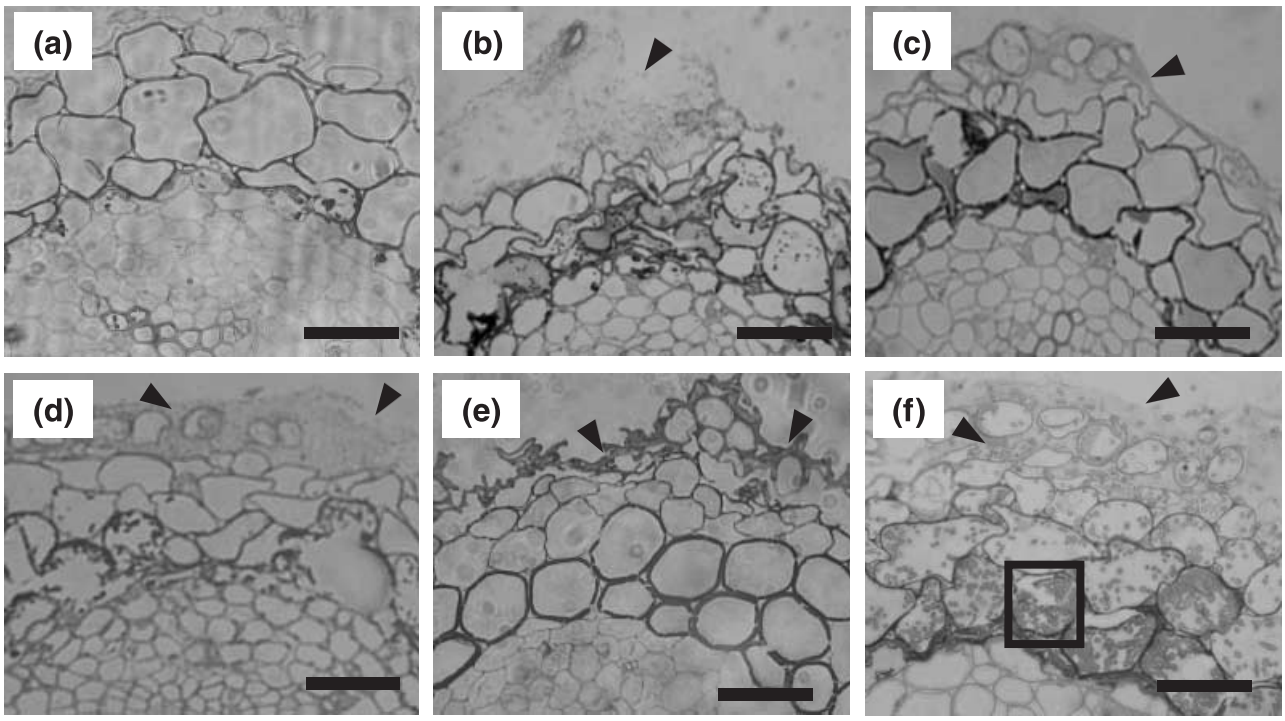


**Fig. 2** Light micrographs showing tripartite cultures of Norway spruce (*Picea abies*) roots together with AcH 505 and *Heterobasidion annosum* 005 or *Heterobasidion abietinum* 331. (a) No bacterial or fungal inoculation. (b) Inoculation with AcH 505. Note the bacterial slime on the root surface (arrow). (c) *H. annosum* 005. Fungal hyphae are present on the root surface (arrow). (d) *Heterobasidion annosum* 005 and AcH 505. Arrows depict the slime on the root surface. (e) *Heterobasidion abietinum* 331. Fungal hyphae are present on the root surface (arrow). (f) *Heterobasidion abietinum* 331 and AcH505. Note the presence of the bacterial slime (right arrow) and more densely stained fungal hyphae (left arrow) on the root surface, and the extensive colonization of the root cortex by the fungus (boxed). Bars, 0.5 mm.

### Quantitative changes in soluble peroxidase activities and in *PaSpi2* expression levels and the influence of Norway spruce–AcH 505–*H. abietinum* 331 interaction on the germination of fungal spores

Pathogen infection of Norway spruce roots leads to increased total peroxidase activity and increased levels of *PaSpi2* gene expression (Asiegbu *et al.*, 1993; Fossdal *et al.*, 2001; Nagy *et al.*, 2004b). We used these parameters to probe whether AcH 505 infection alters plant defence-related enzyme activity or gene expression (Fig. 5a,b). The results show that the inoculation with *H. abietinum* 331 leads to increased peroxidase activity and *PaSpi2* gene expression levels in the roots. Inoculation with AcH 505 reduced both activities, suggesting suppressed plant defence responses in roots. The negative effect of AcH 505 on root peroxidase activity was persistent after dual (bacterium + fungus) inoculations, but was overruled by the fungal influence as far as the *PaSpi2* expression was concerned.

In needles, peroxidase activity and gene expression levels were not significantly altered by bacterial pretreatment (Fig. 5c,d). Peroxidase activity increased slightly after fungal inoculation, and dual (bacterium + fungus) inoculation led to slightly increased *PaSpi2* expression levels.

To confirm that AcH 505 only affects host physiology or defence response and not the germination of fungal spores in the tripartite culture, we measured the germination rate and the growth of germ tubes of the fungal spores adjacent to (the first 100 μm) Norway spruce roots. The germination rate of *H. abietinum* 331 spores 1 d after inoculation was 1% with Norway spruce and 1.3% with Norway spruce and AcH 505. At 2 d after spore inoculation 65% of fungal spores with Norway spruce and 67% with Norway spruce and AcH 505 were germinated. At 2 d the length of the germ tubes from 100 randomly chosen spores was recorded. The average length of *H. abietinum* 331 germ tubes in culture with Norway spruce was 50.8 ± 29.0 μm, whereas in the cultures with Norway spruce and AcH 505 the length of germ tubes was 55.94 ± 27.0 μm. This indicates that the early interaction between the three organisms does not lead to significant changes in fungal spore germination or germ tube elongation.

### Needle infection after bacterial infection of the roots

According to the peroxidase experiments the response of Norway spruce to AcH 505 was nonsignificant in needles, and we reasoned that root inoculation by AcH 505 should not

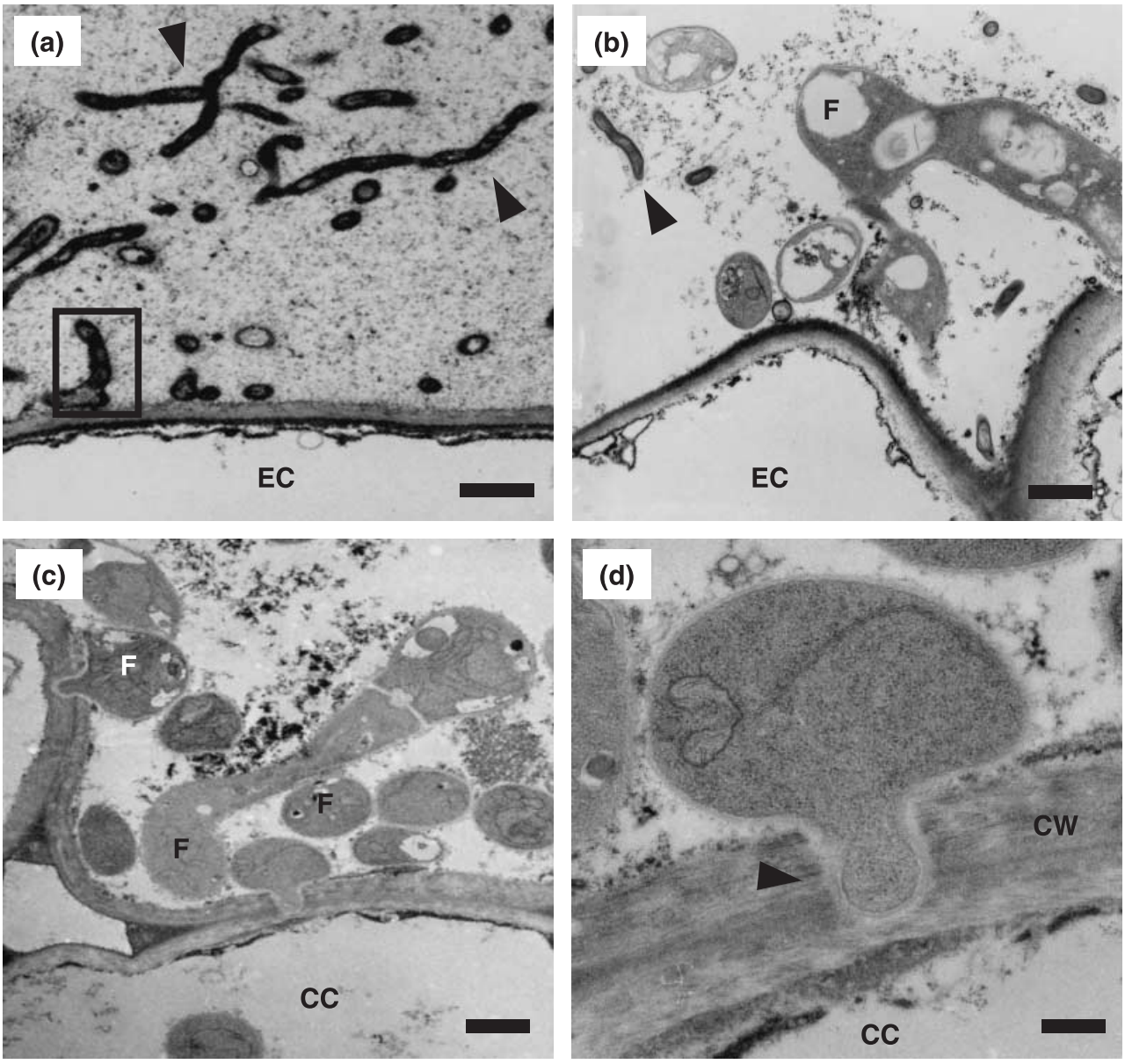


**Fig. 3** Electron micrographs from the interaction between Norway spruce (*Picea abies*), AcH 505 and *Heterobasidion abietinum* 331. (a) Inoculation with AcH 505. The densely stained bacterial filaments (arrow) are embedded in a slime. Occasionally, bacterial filaments attach to the surface (boxed) of the root epidermal cells (EC) but the bacterial filaments were never observed to penetrate the root cells. (b) Inoculation with AcH 505 and *Heterobasidion abietinum* 331. The larger fungal hyphae (F) can easily be distinguished from the electron-dense and thin bacterial filaments (arrow). (c) Inoculation with AcH 505 and *H. abietinum* 331. Fungal hyphae (F) grow inside the cortical cells (CC) of the root. (d) Magnification of (c). The cell wall (CW) of the host cortical cells (CC) is lysed during the penetration (arrow) of the fungal hyphae (F). The hyphal diameter is often reduced at the site of penetration. Bars: (a) 1 μm; (b,c) 2 μm; (d) 0.5 μm.

render the needles more susceptible to secondary infections. To test this, we used a spruce pathogenic strain of the grey mould, *Botrytis cinerea*, as a needle pathogen and recorded the outcome of AcH 505 inoculation of roots on needle infection by *B. cinerea* (Fig. 6). To our surprise, we found an increased number of healthy needles and those with mild disease symptoms after the pretreatment of roots with AcH 505. This indicates that root inoculation with the bacterium increases needle resistance against *B. cinerea*.

## Discussion

To our knowledge this is the first time that a mycorrhiza helper bacterium (MHB) has been shown to increase the

 

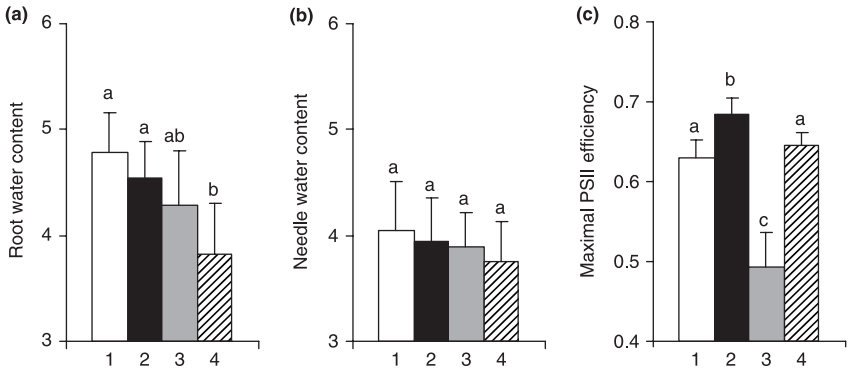


**Fig. 4** Physiological measurements in Norway spruce (*Picea abies*) after microbial treatments. Control values (open columns) were compared with those after treatments with AcH 505 (closed columns), *Heterobasidion abietinum* 331 (tinted columns) or dual (bacterium + fungus) inoculations (striped columns). (a, b) Root and needle water contents. Each data point represents the values from 10 seedlings. (c) Maximal photosystem II (PSII) efficiency ($F_v/F_m$) measured after 15 min in darkness on three needles from 10 seedlings each. Bars with different letters are significantly different according to student's *t*-test ($P < 0.01$).

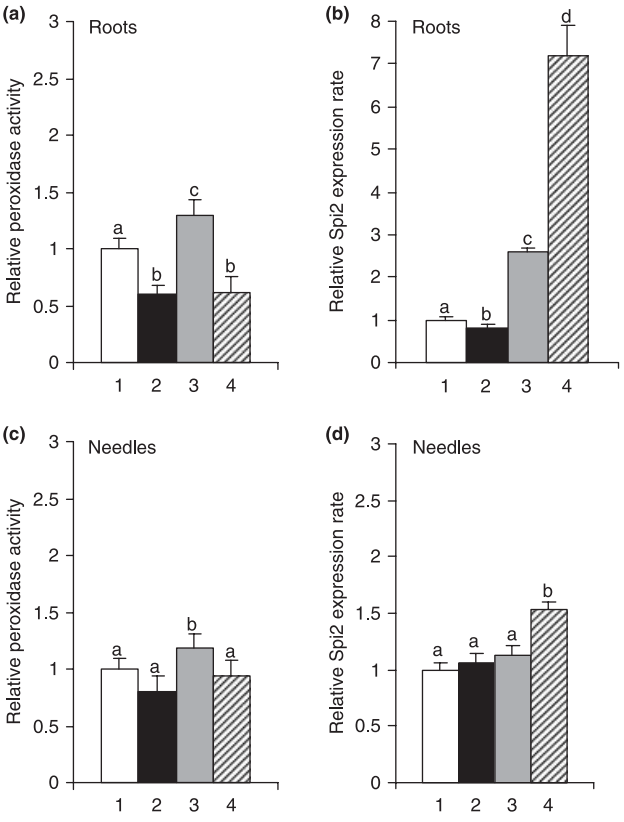


**Fig. 5** Peroxidase activity and *PaSpi2* gene expression responses in Norway spruce (*Picea abies*) roots after the interaction with AcH 505 and/or *Heterobasidion abietinum* 331. Control values (open columns) were compared with those after treatment with AcH 505 (closed columns), *H. abietinum* 331 (tinted columns) or dual (bacterium + fungus) inoculations (striped columns). Each data point represents three independent measurements from three seedlings. (a, c) Peroxidase activity in roots and needles of Norway spruce in relation to the control treatment without microbial inoculation. Data is represented as relative enzyme activity (μkatal $mg^{-1}$ total protein). (b, d) Expression profile of *PaSpi2* gene in roots and needles of Norway spruce as measured by real-time polymerase chain reaction using gene-specific primers. The signal intensities were calibrated according to a constitutively expressed Norway spruce α-tubulin gene (Hietala *et al*., 2004). Error bars represent standard deviations from three biological replicates that were used for the analysis. Bars with different letters are significantly different according to student's *t*-test ($P < 0.01$).

colonization of a plant root by a fungal pathogen. Our findings parallel those with previously tested mycorrhizal fungi *Amanita muscaria* and *Suillus bovinus* (Schrey *et al.*, 2005) with an important exception: whereas *A. muscaria* and *S. bovinus* were promoted by AcH 505 in cocultures, the mycelial growth rate of *H. abietinum* 331 was not affected by the bacterium. This suggests that the increased fungal colonization of Norway spruce roots with *H. abietinum* 331 could be based on a plant-related mechanism.

Garbaye (1994) suggested that a mycorrhiza helper bacterium could increase the rate of plant root colonization by positively affecting 'root receptivity'. Our data indicate that AcH 505 induces such a response by downregulating peroxidase activity and pathogenesis-related peroxidase gene (*Spi2*) expression. Both parameters have been linked to the defence response of Norway spruce towards *H. annosum* s.l. (Asiegbu *et al.*, 1993; Fossdal *et al.*, 2001; Karlsson, 2005) and other phytopathogens (Asiegbu *et al.*, 1999; Nagy *et al.*, 2004b). We therefore conclude that AcH 505 suppresses the defence response in this tree species, causing faster colonization of the roots by *H. abietinum* 331 after bacterial preinoculation. The question remains, which bacterial stimuli could be responsible for this effect? To answer this, we tested the dominant secondary metabolites identified from AcH 505, auxofuran and WS-5995 B. Neither substance showed any effect on plant colonization at concentrations up to 1 μM, indicating that AcH 505 produces other factors responsible for the enhanced susceptibility of Norway spruce against *Heterobasidion*. Bacteria can promote secondary infections in different ways. For example, Cui *et al.* (2005) observed that the primary *Pseudomonas syringae* infection of *Arabidopsis thaliana* rendered the plants more susceptible to secondary infections by the same bacterium, and this was caused by coronatine, a *P. syringae*-derived virulence factor that resembles the phytohormone jasmonic acid. It has also been suggested that the bacteria could release lipases or produce plant cell wall-degrading enzymes, making it easier for fungi to penetrate the cuticle of the leaves (Dewey *et al.*, 1999) or to separate the outer cells of the roots (Mosse, 1962). This indicates that based on their structure very different factors may lead to enhanced colonization of plant tissues.

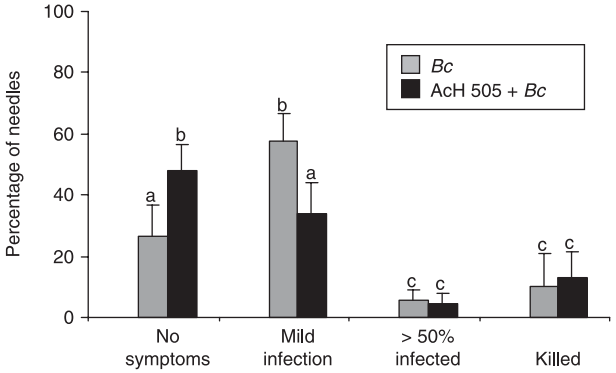


**Fig. 6** The analysis of needle infection of Norway spruce (*Picea abies*) seedlings after the interaction of seedling roots with AcH 505. The severity of needle infections by the grey mould *Botrytis cinerea* was investigated. Four-week-old seedlings were preinoculated with AcH 505 (closed columns) or water (tinted columns) for 7 d and postinoculated with *B. cinerea*. The severity of infection was analysed 1 wk postfungal inoculation. The needles of 10 seedlings per treatment were analysed. Note the increased number of nonsymptomatic needles in with AcH 505-treated plants. Bars with different letters are significantly different according to student's *t*-test ($P < 0.01$).

The missing effect of auxofuran on host root colonization by *H. abietinum* 331 contrasts with the significantly improved growth of this fungus in the presence of auxofuran at 10 nM to 1 μM concentrations (our unpublished data). It has still to be investigated by time series whether the fungus reaches the root surface earlier under the influence of auxofuran. In addition, WS-5995 B applications of up to 1 μM did not suppress spruce infection by *Heterobasidion* spp. in root inoculation tests. This indicates that the concentrations of the antifungal substances must be higher at the bacterium–fungus contact area to suppress fungal growth and infection. Our preliminary observations indicate that WS-5995 B concentrations of 15 μM are able to suppress the infection of spruce by *H. annosum* 005. Such concentrations should thus be expected in the bacterium–fungus contact area.

According to the data by Garbaye *et al.* (1992), a bacterial isolate may consistently promote mycorrhiza formation by a single mycorrhizal fungus with different host plants, including even conifer and angiosperm species. AcH 505 is capable of promoting mycorrhiza formation in Norway spruce and in Scots pine, indicating a similarly low specificity in this bacterium–plant interaction. We have thus suggested that fungal growth promotion is the major mechanism behind the MHB effect by AcH 505 (Schrey *et al.*, 2005). However, in the light of current results, the plant factor(s) should be analysed further, preferably with an AcH 505-fungus–plant model where the plant host is amenable to genetic studies.

The primary aim of this work was to confirm the results by Maier *et al.* (2004) and Schrey *et al.* (2005), indicating that AcH 505 could be used for applied purposes: the simultaneous suppression of plant pathogenic infections and promotion of controlled mycorrhization. Owing to growth suppression of 11 out of 12 *H. annosum* s.l. strains AcH 505 could play an important role in biocontrol. In comparison with fungal isolates (reviewed by Holdenrieder & Greig, 1998), including *Phlebiopsis gigantea*, which is commercially used as a biocontrol agent, only a few bacteria have been previously tested for antagonism against *H. annosum* s.l. From the bacteria that inhabit the rhizosphere of Norway spruce, Falk (1987) reported that out of 129 *Streptomyces* strains tested, 76% were inhibitory against *H. annosum* in *in vitro* assays, indicating the presence of potent suppressor strains in this environment. Very few assays on *in vitro* antagonism have been combined with tree inoculation assays (Holdenrieder & Greig, 1998), which would confirm the importance of the antibiosis in biocontrol (Whipps, 2001). Rose *et al.* (1980) showed correlation between the two approaches, as the inhibitory activity by *Streptomyces griseoalbus* was observed not only in culture medium but also on wood. In our report, AcH 505 showed antagonism against *H. annosum* 005 *in vitro*, on wood bilayer assays, and in root inoculation experiments. To confirm *in vitro* results under *in vivo* conditions, the organisms have to be competitive on a natural substrate (Whipps, 2001). According to our preliminary results, AcH 505 is able to grow on nonsterile wood surfaces and in gamma-sterilized organic litter cultures (M. Ecke & M. T. Tarkka, unpublished). The effect of AcH 505 against phytopathogens should be tested under these more natural culture conditions to determine the biocontrol efficiency of this bacterium.

The fact that the colonization of spruce roots by *H. abietinum* 331, an isolate tolerant against the antibiotics produced by AcH 505, was enhanced in the presence of AcH 505 indicates that the use of this bacterium for biocontrol may, however, be unsafe. It would now be relevant to divide the dikaryotic *H. abietinum* 331 into its homokaryotic components, to examine whether tolerance to AcH 505 antibiotics is also displayed by homokaryons, and whether tolerance against antibiotics is a dominant factor that is displayed by all heterokaryons containing one tolerant homokaryon. This would certainly limit the use of AcH 505 as a biocontrol agent. It would further be relevant to compare the populations of *H. annosum* s.l. from the area where AcH 505 was isolated (Haigerloch, Germany) with those where *H. abietinum* 331 was found (Klein Kotterbachtal, Austria) for their tolerance against antibiotics produced by AcH 505. In conclusion, the inoculation of AcH 505 (or other fungus specific MHB)-tolerant phytopathogens should be regarded as potentially risky, and should be restricted to the use in tree nurseries for simultaneously improved mycorrhization and biocontrol. In the most severe scenario, the application of a fungus-specific bacterium could lead to the selection of highly virulent phytopathogens at the cost of less virulent strains suppressed by the bacterium. Our results suggest that testing predominant native strains of phytopathogens for tolerance against the putative biocontrol

 

agent and for root colonization should precede their wider use for biological control.

Antibiosis seems to be the major mechanism behind the antagonism between AcH 505 and fungi. Our data show that *Heterobasidion* isolates that are suppressed by AcH 505 in dual culture (DC) are more sensitive to WS-5995 B than the AcH 505-tolerant strain *H. abietinum* 331. This observation could explain the 'fungus-specificity' (Garbaye, 1994) and is in line with our previous results. There (Riedlinger *et al.*, 2006) we showed that *Hebeloma cylindrosporum*, which is inhibited in coculture, is more sensitive to WS-5995 B than *Amanita muscaria*. The current results go along with data reported by Gregor *et al.* (2003) on root nodulation by *Bradyrhizobium japonicum*. These authors showed that wild type strains of *B. japonicum* were unable to form root nodule symbiosis with soybean following coinoculation with the antagonistic *Streptomyces kanamyceticus*, while *B. japonicum* mutants with increased antibiotic resistance formed significantly more root nodules in the presence of the streptomycetes than without them (Gregor *et al.*, 2003). This could perhaps indicate that the production of antibiotics by *S. kanamyceticus* masked the effect of 'nodule promotion factors'. Such factors may also be produced by *Streptomyces lydicus*, a bacterium that colonizes the outer layers of pea root nodules and promotes root nodulation. The root nodule colonization by this streptomycete leads to a significantly increased rate of nitrogen fixation (Tokala *et al.*, 2002), increasing the benefit of the symbiosis for the plant host. In addition, *S. lydicus* is a strong biocontrol agent against soil-borne plant pathogens (Yuan & Crawford, 1995). The interplay between antagonistic activities and compatibility with secondary infections seems to be a common phenomenon among streptomycetes.

Based on anatomical changes and systemic expression or activities of peroxidases and chitinases, the presence of both local and systemic signalling in Norway spruce has been suggested (Christiansen *et al.*, 1999; Fossdal *et al.*, 2001). In a similar experimental setup as used here, the infection of 8-wk-old Norway spruce seedlings by *Rhizoctonia* led to increased peroxidase activities in both roots and needles (Nagy *et al.*, 2004b), supporting the presence of local and systemic signalling during pathogen attack. In the present study, no such effect was found. The rate of needle infection with grey mould *Botrytis cinerea* was, however, slightly decreased in with AcH 505-infected plants, which was in sharp contrast to the increased colonization of the roots by *H. abietinum* 331 observed after the preinoculation with the bacterium. Since we observed increased photosynthetic yield of the needles as a result of AcH 505 colonization, but no significant changes in needle peroxidase activity or gene expression levels, we suggest that the needles endured *Botrytis cinerea* infection better because of factors unrelated to peroxidase-based defence response. In fact, the observation that needle infection with *B. cinerea* is less severe after pretreatment of seedling roots with AcH 505 is reminiscent of induced systemic resistance (ISR) (van Loon *et al.*, 1998), which allows plants to endure pathogen attacks that could otherwise be lethal. Induced systemic resistance has been described in angiosperms following root inoculation with plant growth-promoting rhizobacteria (PGPR) such as *Pseudomonas*, *Burkholderia* and *Bacillus* (Haas & Defago, 2005). With respect to gymnosperms, especially spruce, induction of systemic resistance via application of PGPR has, to our knowledge, not been reported. Unlike induced resistance through PGPR, infection of spruce stems with fungal pathogens, and to a lesser extent, mechanical wounding results in localized enhanced resistance to subsequent fungal infection (Christiansen *et al.*, 1999; Krokene *et al.*, 1999). To determine whether AcH 505 elicits a systemic resistance such as ISR we used the model plant *A. thaliana*. Roots of soil grown *A. thaliana* seedlings were inoculated with a bacterial suspension of AcH 505 and plant leaves were subsequently inoculated with a spore suspension of the pathogenic fungus *Alternaria brassicicola*. Evaluation of the infection sites formed on the leaf surfaces after germination of the fungal spores showed a reduction of fungal infection of about 30% in pretreated plants compared with control plants (S.D. Schrey & M.T. Tarkka, unpublished). Microarray and mutant line analyses of *A. thaliana* could now reveal the signalling pathways behind the resistance mediated by AcH 505.

Suppression of seedling death in nurseries is often not effective using conventional practices, and biological control combined with controlled mycorrhization could become an effective way to increase seedling fitness (Frey-Klett & Garbaye, 2005). In this report we showed that although AcH 505 initially appeared to be a good candidate for such applications, its ability to facilitate root penetration and the presence of a WS-5995 B-tolerant *H. abietinum* 331 strain should be regarded as potential hazards in the use of AcH 505 in silviculture. Future work will show which bacterial factors yield the plants susceptible to fungal infection, and what renders *H. abietinum* 331 tolerant against the antifungal substances produced by AcH 505.

## Acknowledgements

We thank Margret Ecke for excellent technical assistance in plant and microbial cultures, Julia Riedlinger for auxofuran and WS-5995 B preparations, Maren Babbick for introduction into real-time PCR, Magdalena Wagner-Eha for technical assistance in microscopy, and Matthias Reicherter for peroxidase activity measurements. We are also indebted to Uwe Nehls and Robin Sen for helpful discussions, and to U. Nehls for reading and commenting the manuscript. We thank Kari Korhonen and Franz Oberwinkler for *Heterobasidion* isolates, and Raija-Liisa Petäistö for the grey mould isolate. The German Research Foundation (D.F.G.; graduate school 'Infection Biology'; N.A.L. and S.D.S.) supported this study.

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