Root inoculation with a forest soil streptomycete leads to locally and systemically increased resistance against phytopathogens in Norway spruce

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Summary

• Soil streptomycetes are commonly antagonistic against plant pathogens. However, interactions involving increased defense responses in the host plant, leading to suppression of plant disease development, have not yet been detailed.

• Here, the mechanisms were studied of disease suppression by *Streptomyces* sp. GB 4-2 against *Heterobasidion* root and butt rot in Norway spruce (*Picea abies*) seedlings.

• GB 4-2 promoted mycelial growth of the phytopathogenic fungus, germination rate of fungal spores, extension of germ tubes and early colonization of outer cortical layers of the plant root. Reduced colonization of the inner cortical cell layers was accompanied by the induction of cell wall appositions, and increased xylem formation in the vascular cylinder emerged after bacterium–fungus coinoculation. Bacterial treatment led to decreased water content in roots and needles and increased photosynthetic yield (F_v/F_m) and peroxidase activities in needles. The infection of needles by *Botrytis cinerea* was reduced by bacterial pretreatment.

• Complex interactions of GB 4-2 with Norway spruce and *Heterobasidion abietinum* were discovered. The bacterium promoted the growth of the phytopathogenic fungus but induced plant defense responses. Host responses indicate that GB 4-2 induces both local and systemic defense responses in Norway spruce.

Key words: actinomycetes, biological control, *Botrytis*, *Heterobasidion*, induced resistance, Norway spruce (*Picea abies*), plant–microbe interactions, *Streptomyces*.

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Introduction

Plant-associated microorganisms influence soil fertility and plant health (Barea *et al.*, 2005; Morgan *et al.*, 2005). Filamentous soil bacteria from actinomycetes have been shown to be capable of antagonism against other microbial species, so influencing the microbial populations in the competitive rhizosphere surroundings (Huddleston *et al.*, 1997; Emmert & Handelsman 1999). Some of the actinomycete species are potent biocontrol agents of plant diseases, owing to their ability to exude a variety of antimicrobials and enzymes degrading fungal cell walls and insect exoskeletons (Crawford *et al.*, 1993; Weller *et al.*, 2002). These bacteria also serve other plant beneficial functions in the rhizosphere; for example, several strains are able to promote plant fungus symbiosis establishment (mycorrhiza, Ames 1989; Poole *et al.*, 2001; Schrey *et al.*, 2005; root nodules, Tokala *et al.*, 2002).

With regard to trees, conifers have both constitutive and inducible defense mechanisms against phytopathogens and insect attacks. Constitutive defenses include resin stored in ducts or blisters in the bark and sapwood and phenol-rich parenchyma cells in the phloem. Following attack by fungal

pathogens or insects, or after wounding, inducible defenses include a hypersensitive response, followed by accumulation of terpenes and phenolics in the cells surrounding the site of attack, and the triggering of defense-related gene expression (Franceschi *et al.*, 2005; Keeling & Bohlmann, 2006).

Local resistance against secondary infections may come about as a result of pathogen or insect attack; for example, preinoculation with sublethal doses of phytopathogenic fungi increases the resistance of Norway spruce to subsequent inoculation with phytopathogens (Christiansen *et al.*, 1999). In contrast to locally induced resistance, much less is known about systemic responses in spruce. Indications for a systemic response have come from studies with young spruce seedlings: *Rhizoctonia* infection of roots of Norway spruce seedlings resulted in enhanced peroxidase and chitinase activities in roots and needles (Nagy *et al.*, 2004), and root inoculation with *Streptomyces* sp. AcH 505 rendered the seedlings more resistant against needle infections by the gray mold *Botrytis cinerea* (Lehr *et al.*, 2007). This shows that Norway spruce seedlings can be used as a model system for the investigation of systemic responses towards phytopathogens.

Heterobasidion annosum sensu lato (s.l.) is a major forest pathogen that causes root and butt rot in conifers (Woodward *et al.*, 1998). In Europe, *H. annosum* s.l. forms a species complex, including *H. annosum* sensu stricto, *H. parviporum* Niemelä & Korhonen and *H. abietinum* Niemelä & Korhonen; these species show a preference but not specificity for pine, spruce and fir, respectively (Niemelä & Korhonen, 1998). The primary infection by *Heterobasidion* spp. occurs on fresh stump surfaces or wounds in roots or stems, and following stump or root colonization, the fungus is reported to grow via root-to-root contacts to the neighboring trees (Rishbeth, 1951). Although no gene-for-gene interactions have been observed for the *H. annosum* s.l.–conifer pathosystem, a number of plant defense responses that hinder the spread of the pathogenic fungus in the host tissues have been revealed. Diverse phenolic compounds are produced after fungal attack in the plant tissues, including phenylpropanoids and stilbenes (Asiegbu *et al.*, 2005). Structural barriers with distinct cell anatomies are formed (Asiegbu *et al.*, 1994, 1998). Pathogen-related proteins are accumulated in conifer tissues, including those targeted against the fungal cell walls, chitinases and glucanases, and peroxidases that have been implicated in cross-linking of phenolics into cell walls and in the production of toxic radicals (Karjalainen *et al.*, 1998). These responses to fungal colonization are backed up by defense-related plant gene expression patterns, including the up-regulation of genes related to the phenylpropanoid pathway and to antimicrobial activities (Hietala *et al.*, 2004; Adomas *et al.*, 2007).

Although actinomycetes are ubiquitous in the rhizosphere (Costa *et al.*, 2006; Fierer *et al.*, 2007) and show great potential as a group of biocontrol organisms (Emmert & Handelsman, 1999; Weller *et al.*, 2002), information on their influence on local and systemic plant resistance is sparse. Using Norway spruce as the plant host and spruce-colonizing fungi as secondary infection models, we have started to investigate the influence of mycorrhizosphere actinomycetes on plant disease and symbiosis (Schrey *et al.*, 2005; Riedlinger *et al.*, 2006; Lehr *et al.*, 2007).

The aim of the present study was to determine the response of spruce seedlings to inoculation with a streptomycete that strongly reduces the extent of *Heterobasidion* root rot. The streptomycete strain used in this analysis was isolated from spruce mycorrhizosphere and inhibits *Heterobasidion* root rot development but promotes vegetative growth and spore germination of the disease causing fungus. Evidence is presented that the treatment of spruce seedlings with *Streptomyces* sp. GB 4-2 leads to formation of infection barriers in the inner root cortex and vascular cylinder, and to an improved resistance of needles against infection by *B. cinerea*.

Materials and Methods

Bacterial and fungal strains, analysis of bacterial 16S rDNA

The actinomycete strain GB 4-2 was isolated from a Norway spruce stand in Schönbuch forest near Tübingen, Germany. Isolation was implemented from an organic litter layer rich in fungal hyphae around mycorrhizal roots of Norway spruce (the mycorrhizosphere). GB 4-2 was maintained on ISP2 agar medium (Shirling & Gottlieb, 1966). *Heterobasidion* strains *H. annosum* 005 from Kirkkonummi, Finland, *H. abietinum* 130 from Trentino, Italy, *H. abietinum* 331 from Klein Kotterbachtal, Austria, and *H. parviporum* 007 from Kirkkonummi, Finland, were obtained from K. Korhonen and maintained on 1.5% malt agar. All of the fungal strains were originally isolated from Norway spruce stumps. Spruce pathogenic gray 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.

For 16S rDNA analysis, genomic DNA was extracted from a loopful of bacterial spores by GenElute bacterial genomic DNA extraction kit (Sigma, Schnelldorf, Germany). The partial 16S rDNA sequence was amplified with the primers 27f and 765r as described in Coombs & Franco (2003). The DNA sequence was compared against the National Center for Biotechnology Information database by the BLASTn program using the nr database.

Fungus–bacterium interaction assays on agar, seedling preparation, bacterial and fungal inoculations

The strain *H. abietinum* 331 was used as a model fungus for spruce inoculation experiments to compare root rot development under the influence of GB 4-2 with that observed previously under the influence of *Streptomyces* AcH 505 (Lehr

et al., 2007). The conditions for bacterium-fungus dual cultures on ISP2 agar medium, and the screening of bacterial effects on fungal growth in the absence or presence of the host plant were implemented as described in Maier *et al.* (2004) and Lehr *et al.* (2007), respectively. To obtain spruce seedlings, 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). The treatments started with an inoculation of roots with GB 4-2 for 7 d. Following this initial pre-inoculation, roots were inoculated with *H. abietinum* 331 for a further 7–21 d, needles with *B. cinerea* for 7 d. Bacterial and fungal inoculations were performed inside cross-walled Petri dishes using MMN agar (Molina & Palmer, 1982) without an additional carbon source as the medium (Lehr *et al.*, 2007). For bacterial inoculation, 400 µl of GB 4-2 suspension (3×10^5 CFU ml^{-1} 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). Spore suspensions from *H. abietinum* 331 were inoculated to spruce roots as described in Lehr *et al.* (2007). For the root inoculations, 14 replicates were made. Five of these were used for microscopical analysis. To obtain homogenous material for enzyme and real-time RT-PCR analyses, the needles and the first 3 cm of roots of nine seedlings were separated, pooled as three fractions (three biological replicates) and powdered under liquid nitrogen. *Botrytis cinerea* was used as a needle pathogen (Petäistö *et al.*, 2004) to investigate the influence of GB 4-2 on needle infections. The needles were inoculated with fungal spores according to Lehr *et al.* (2007) 7 d after the treatment of roots with water or GB 4-2. The *in vitro* growth of *B. cinerea* was not influenced by GB 4-2. Because of this, and a later observation that GB 4-2 does not colonize spruce tissues, a coculture of GB 4-2 with *B. cinerea* on spruce needles was excluded from the analysis. The amount of diseased needles per seedling was estimated 7 d after the inoculation with *B. cinerea*. The severity of *B. cinerea* infection on needles was scored from 30 individual needles of 10 seedlings for each treatment. Disease severity was addressed by calculating the percentage of needles from each plant belonging to the following phenotypes; 0, no visible symptoms; 1, < 50% of needle infected; 2, 50% or more of the needle infected; 3, needle killed. The percentage values calculated from each plant were used as replicates for the statistical analysis with ANOVA and Tukey test.

Tissue preparation for light and electron microscopy

For the light microscopical analysis, five Norway spruce roots from each treatment were examined. Starting from a distance of 2 cm above the root tip, two 5 mm sections were cut. The samples were prepared for light and electron microscopy as described in Lehr *et al.* (2007). Briefly, samples were fixed in glutaraldehyde and osmium, and contrasted with uranylacetate. After a dehydration series, the samples were embedded in silicone. Semi-thin sections (0.75 µm) were made with a glass knife using an ultramicrotome and analyzed with a light microscope. Ultrathin sections (70 nm) were cut from selected samples with a diamond knife, mounted on to copper grids, and coated with a Fromvar carbon film. Sections were contrasted with lead-citrate. The ultrathin sections were examined with a Zeiss EM 109 transmission electron microscope at 80 kV.

Chlorophyll fluorescence, water content and peroxidase activity 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.* (2004). Dark-adapted seedlings (15 min darkness before the measurement) were used for the measurement of maximal PSII efficiency (F_v/F_m) with a saturating pulse (3000 µmol 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. Soluble proteins were extracted and peroxidase activity was measured according to Mensen *et al.* (1998) using guaiacol (Sigma-Aldrich, Taufkirchen, Germany) as a substrate. The oxidation of guaiacol at 470 nm was followed for 2 min.

Results

Inoculation with *Streptomyces* sp. GB 4-2 reduces *Heterobasidion* root rot symptoms in Norway spruce but has a positive influence on the growth of the fungus

After 5 wk of inoculation with *H. abietinum* 331, Norway spruce seedlings were dead. By contrast, the noninoculated control seedlings and those coinoculated with GB 4-2 and *H. abietinum* appeared healthy (Fig. 1). Strain GB 4-2 showed typical actinomycete morphology, with orange-pigmented substrate mycelium on ISP-2 agar. Partial 16S rDNA sequencing was used to investigate the homology of the 16S rDNA of GB 4-2 with those of other actinomycetes. The resolving power of this technique is sufficient down to the genus level, and for subgrouping purposes (Kämpfer, 2006). According to the 16S rDNA analyses, GB 4-2 is a member of the genus *Streptomyces*, and was accordingly classified as *Streptomyces* sp. GB 4-2. The 16S rDNA of GB 4-2 was not identical with any of the publicly available 16S rDNA sequences. From taxonomically characterized bacteria, the 16S rDNA of GB 4-2 showed highest identity, 98%, with that of *Streptomyces drozdowiczii*, a cellulolytic streptomycete isolated from forest soil in Mata Atlantica, Brazil (Semedo *et al.*, 2004).

Fig. 1 GB 4-2 inhibits the development of *Heterobasidion* root rot in Norway spruce (*Picea abies*). Untreated Norway spruce seedlings (a) were compared with seedlings treated with *Heterobasidion abietinum* 331 (b) and with seedlings pretreated with GB 4-2 before inoculation with *H. abietinum* 331 (c). Photographs were taken 5 wk after fungal inoculation.

To investigate if the bacterium had a direct negative effect on *H. abietinum*, mycelial extension in dual culture, spore germination and germ tube elongation in the presence of the bacterium and the host plant were analyzed. The bacterium promoted the growth of *H. abietinum* 331 in axenic culture (Table 1). The bacterium similarly promoted growth of other tested *Heterobasidion* isolates, *H. annosum* 005, *H. parviporum* 007, and *H. abietinum* 130, but no effect on mycelial extension of the phytopathogen *Fusarium oxysporum* was observed (data not shown). Owing to the similarity in response to GB 4-2

Table 1 Streptomyces sp. *GB 4-2* promotes the growth and spore germination of *Heterobasidion abietinum* 331

	Treatment with GB 4-2	
	No	Yes
Mycelial growth on agar (mm)	49.9	60.3+
Spore germination rate (%)	57.3	76.5+
Germ tube elongation (μm)	42.7	83.2+

The growth of fungal mycelium (colony diameter at day 7, mm) on pure and fungus–bacterium cultures is indicated ($n = 10$), as well as fungal spore germination rate ($n = 200$) and the average length of germ tubes ($n = 480$) in fungus–plant and fungus–plant bacterium cultures at 24 h after the inoculation of the spores. Values followed by + are significantly higher than those observed in control treatments using Student's t-test ($P < 0.001$).

among the *Heterobasidion* isolates, only a single fungal strain was used for the following experiments: the strain *H. abietinum* 331 that was previously investigated under the influence of *Streptomyces* AcH 505 and Norway spruce (Lehr *et al.*, 2007). When the fungus was coinoculated with spruce seedlings and bacteria, the germination rate of fungal spores and the elongation rate of germ tubes increased (Table 1). These results suggest that the disease-suppressing activity by GB 4-2 is not related to bacterium-fungus antagonism.

Fungal colonization of spruce root is at first promoted by the bacterium

Light and electron microscopy were used to investigate if bacterial inoculation rendered the plants more resistant to fungal colonization. Root anatomy in noninoculated seedlings and in those inoculated with *Streptomyces* GB 4-2 was identical at the time point for inoculation of fungal spores (Supplementary material, Fig. S1a). One week after fungal inoculation there was a moderate colonization of cortical cells in the roots from dual (bacterium + fungus)-inoculated plants; four to 10 dense structures per cortical cell were observed in the 0.75-μm-thick cross-sections of the roots (Fig. 2d). As a result of their appearance these structures were assumed to be cross-sections of *H. abietinum* hyphae. Transmission electron microscopy analyses of root cross-sections confirmed this assumption (Fig. 3). On the surface of the roots, bacterial filaments could be easily distinguished from the fungal hyphae (Fig. 3a). Inside the plant cells, only fungal hyphae were observed (Fig. 3b). Control roots (Fig. 2a) showed no colonization, whereas considerable amounts of bacterial slime were observed between the cells of the rhizodermis in the roots inoculated with GB 4-2 only (Fig. 2b). In plants inoculated with *H. abietinum* only (Fig. 2c), one to three layers of fungal mycelium appeared on the surface of plant roots. In conclusion, GB 4-2 promotes the fungal colonization of the root cortex,

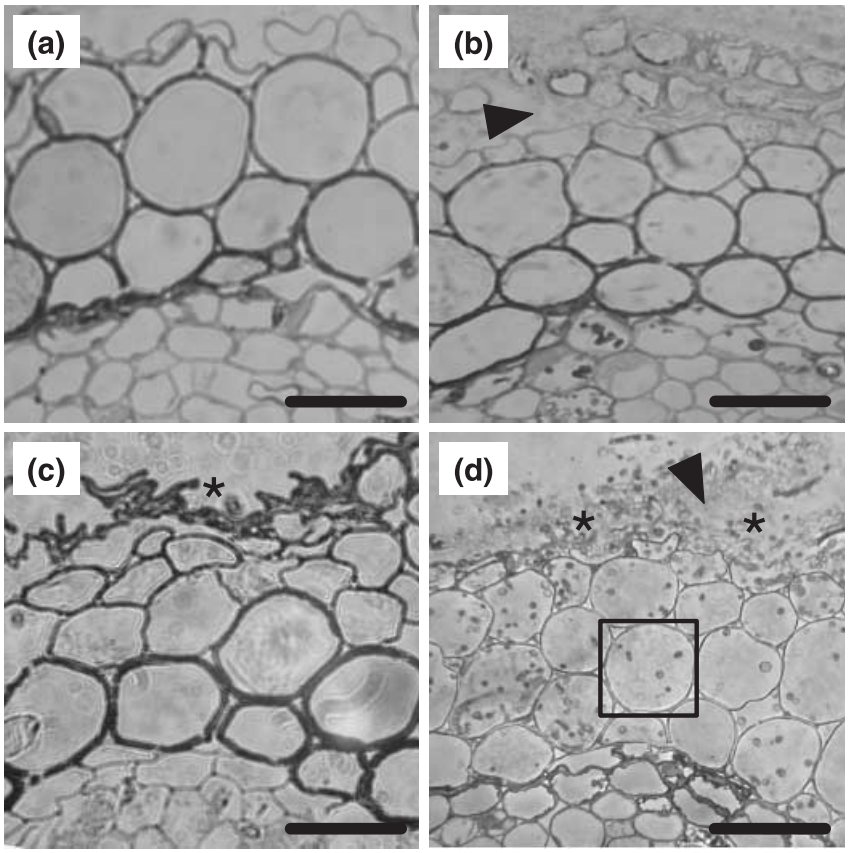

Fig. 2 *Streptomyces* sp. GB 4-2 promotes the colonization of root cortical cells by *Heterobasidion abietinum* 331. Light micrographs of Norway spruce (*Picea abies*) roots inoculated 2 wk with GB 4-2 and/or 1 wk with *H. abietinum* 331. (a) No bacterial or fungal inoculation. (b) Treatment with GB 4-2. The bacteria form slime on the surface of the spruce root (arrow). (c) Treatment with *H. abietinum* 331. Fungal hyphae are present on the root surface (asterisk). (d) Treatment with *H. abietinum* 331 and GB 4-2. The bacterial slime (arrow) and the fungal hyphae (round dots; asterisks) are visible on the root surface. The root cortical cells are infected with *H. abietinum* 331 (boxed), but no bacteria are visible. Bars, 0.5 mm.

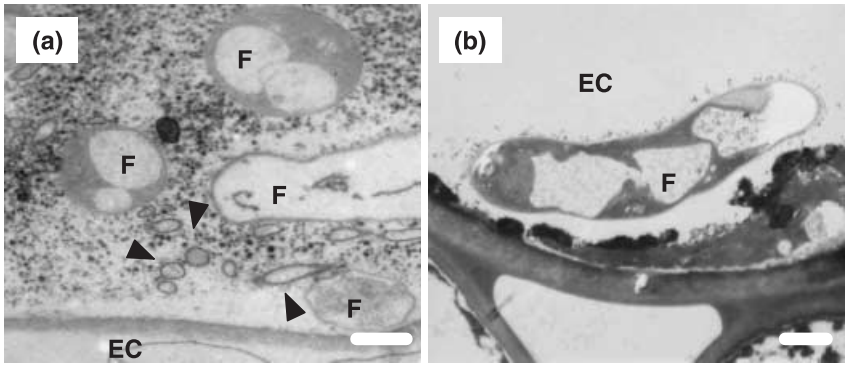

Fig. 3 Localization of *Streptomyces* sp. GB 4-2 on root surface. Electron micrographs of Norway spruce (*Picea abies*) roots inoculated with GB 4-2 and *Heterobasidion abietinum* 331 taken 1 wk after fungal inoculation. (a) GB 4-2 (arrows) and *H. abietinum* 331 (F) are imbedded in a matrix (dark granulous staining) on the surface of root epidermal cells (EC). (b) Only fungal hyphae (F) are present inside the root epidermal cells (EC). Bars, 2 μm (a); 1 μm (b).

and the inhibition of *Heterobasidion* disease development must consequently happen at later stages of infection.

Physiological state of inoculated seedlings

According to our earlier work with spruce seedlings (Lehr *et al.*, 2007), increased colonization by *Heterobasidion abietinum* 331 is associated with reduced seedling water content and photosynthetic yield, and increased soluble peroxidase activities. We used these parameters to investigate how GB 4-2 influences the physiology of spruce seedlings.

One week post-fungal inoculation (pfi), fungal treatment in root samples and bacterial and dual inoculations in shoot samples led to decreased water contents (Fig. 4a,b). The water contents of all inoculated seedlings were markedly reduced compared with control samples at 2 wk pfi. The presence of GB 4-2, however, improved photosynthetic yield, especially at 2 wk pfi (Fig. 4c). For the dual-inoculated plants, F_v/F_m values remained close to those of the controls. By contrast, a decrease in photosynthetic yield was already evident 1 wk after the inoculation with *H. abietinum* only.

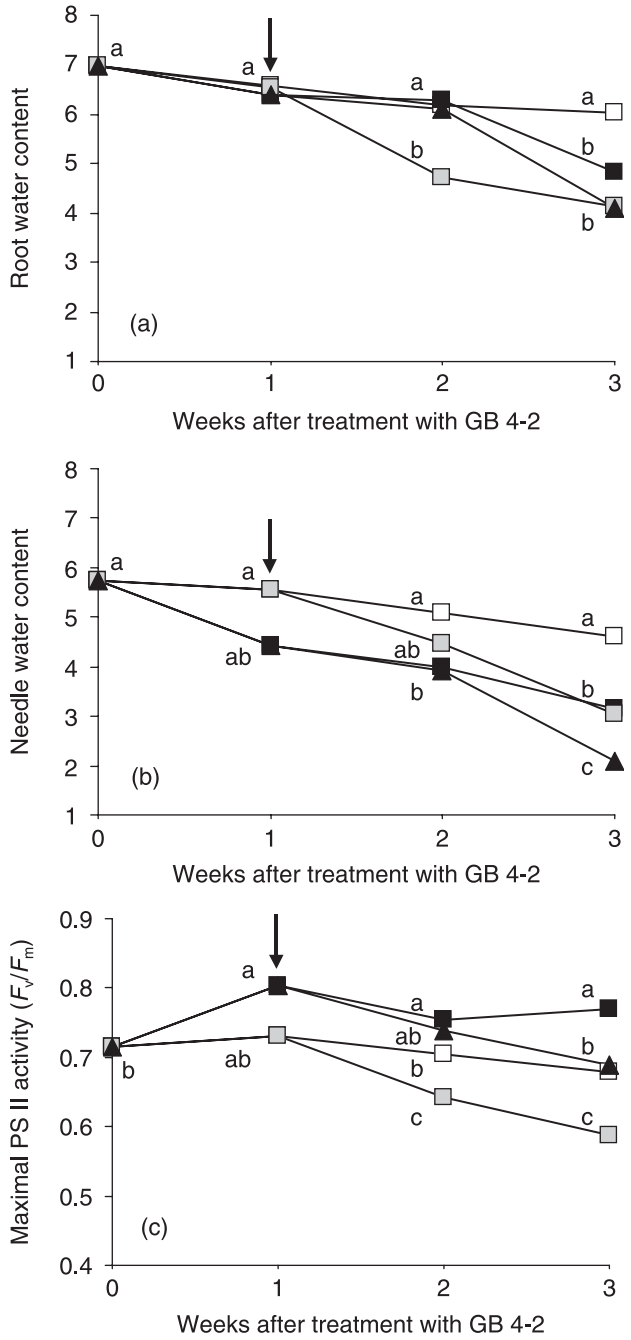

Fig. 4 Physiological responses in spruce seedlings inoculated with *Streptomyces* sp. GB 4-2 and/or *Heterobasidion abietinum* 331. Untreated Norway spruce (*Picea abies*) seedlings (open squares) were compared with seedlings treated with GB 4-2 (black squares) and *H. abietinum* 331 (gray squares), and with seedlings pretreated with GB 4-2 before *H. abietinum* 331 inoculation (triangles). Different letters next to the data points represent significant ($P < 0.05$) differences between means according to one-way ANOVA and Tukey test. The arrow indicates the time point of fungal inoculation. (a, b) Root and needle water contents from 10 seedlings each were measured. (c) Maximal PS II efficiency (F_v/F_m) of three needles from 10 seedlings each was measured after 15 min of dark adaptation. Values are means of 10 replicates.

Dual (bacterium + fungus) inoculation leads to the formation of cortical cells with encrustations and local cell wall thickenings, and to an increased lignification of the vascular cylinder

Next we analyzed if any alterations in root anatomy occur at later stages of the interaction between GB 4-2 and *H. abietinum*. For this, cross-sections of spruce roots were analyzed by light microscopy until 4 wk after fungal inoculation. Two weeks post-fungal inoculation, the first plant cells were colonized by *H. abietinum*, whereas in dual (bacterium + fungus)-inoculated plants the whole cortex was already colonized by fungal hyphae, and cells of the inner cortex showed dense staining in their outer cell walls (Fig. S1c). As the cells in the cortex of plants inoculated exclusively with the fungus were colonized by fungal hyphae 3 wk pfi, they became stained (Fig. S1d). Only in dual-inoculated roots did protrusions form on the inner surfaces of cell walls of cells in the inner cortex. Four weeks after fungal inoculation, the *H. abietinum*-infected seedlings showed the first fully necrotic needles, but dual-inoculated seedlings appeared healthy. The anatomy of the inner cortex and the vascular cylinder remained altered in the dual-inoculated roots (Fig. 5d,e; Fig. S1e). Parenchyma cells from the inner cortex showed dark staining indicative of polyphenolics and locally thickened cell walls, and a substantial number of evenly thick-walled cells were observed in the vascular cylinder. When compared with the situation in roots 1 wk after dual inoculation (Fig. 2d), few structures resembling colonizing *H. abietinum* hyphae were observed. Instead the cells contained dark encrustations (Fig. 5d). Little or no dark staining inside the cortical cells and no cell wall appositions were observed in controls or in GB 4-2 treated roots (Fig. 5a,b). By contrast, completely deteriorated cortex, endodermis and pericycle layers of *H. abietinum*-inoculated plants were detected (Fig. 5c). From these observations we conclude that GB 4-2 helps to prevent the colonization of the vascular cylinder by *H. abietinum*.

Extensive formation of thick-walled cells was apparent in the vascular cylinder of the dual-inoculated plants, and phloroglucin-hydrochlorid acid (PG) staining (Fig. 6d) revealed that the cell walls of the thick-walled cells were lignified. The intensity of PG staining of the thick-walled cells was comparable to that observed in roots treated with *H. abietinum* only (Fig. 6c). We did not observe enhanced lignification in other cell types. Both noninoculated and GB 4-2 treated roots showed a slight PG staining only (Fig. 6a,b). The analysis of a time series of cross-sections from seedlings roots (Fig. S2) revealed strong phloroglucin staining in dual- as well as GB 4-2-inoculated seedlings 2 wk after bacterial and 1 wk after fungal inoculation (Fig. S2b). By contrast, the seedlings that were exclusively inoculated with *H. abietinum* showed increased phloroglucin staining only a few days before the lysis of the nonlignified tissues (Fig. S2d,e).

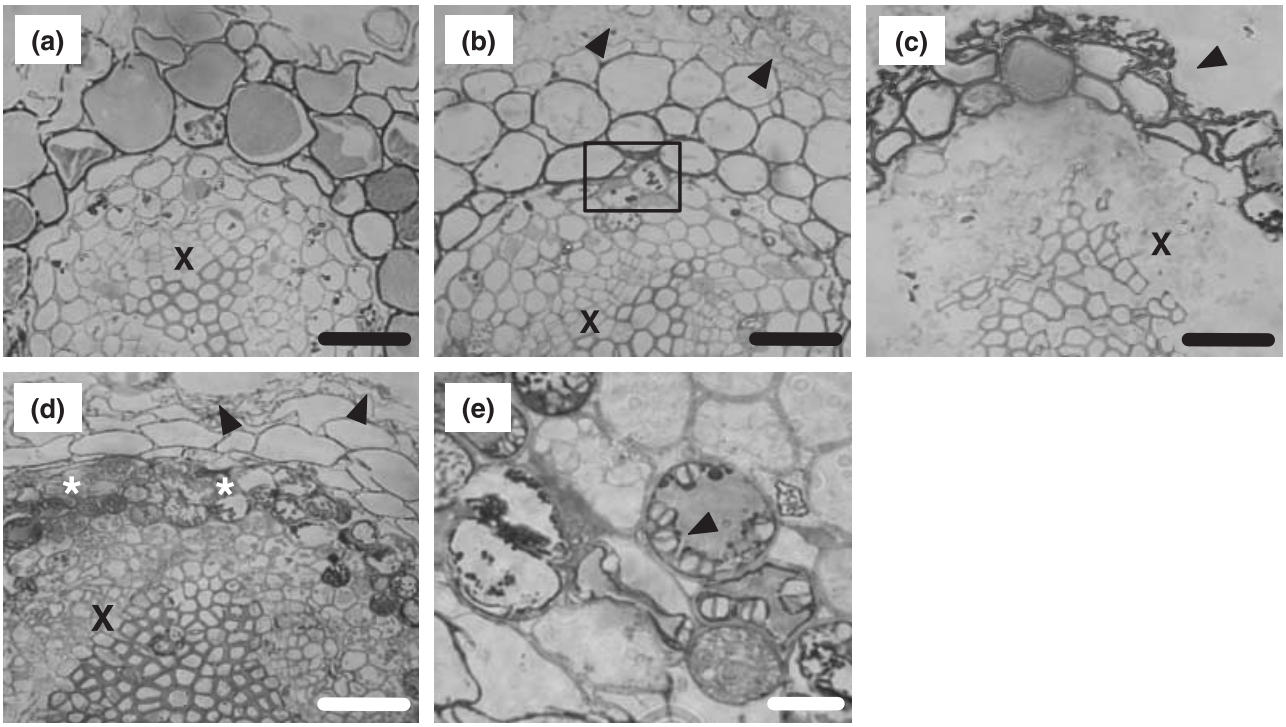

Fig. 5 Changes in cortical and xylem cells after dual inoculation of roots with *Streptomyces* sp. GB 4-2 and *Heterobasidion abietinum* 331. Light micrographs of Norway spruce (*Picea abies*) roots inoculated with GB 4-2 and/or *H. abietinum* 331 taken 3 wk after fungal inoculation. (a) No inoculation. Xylem (X) is visible in an intact vascular cylinder. (b) GB 4-2. Note the bacterial slime on the root surface (arrows). A few cortical cells show inclusions (boxed). (c) *H. abietinum* 331. Fungal hyphae (arrow) are present on the surface of the remaining cortical cell layer. Apart from the xylem (X), the vascular cylinder is completely degraded. (d) GB 4-2 and *H. abietinum* 331. Bacterial slime (arrow) is visible on the root surface. Fungal hyphae are only seldom visible in the cortex cells. Inner cortical cells show dark staining and appositions in the cell walls (asterisks). The vascular cylinder contains an increased number of thick-walled cells resembling tracheids (X). (e) Close-up of inner cortical cells showing locally thickened inner sides of the cell walls (arrow). Bars, 0.25 mm (a–d); 0.1 mm (e).

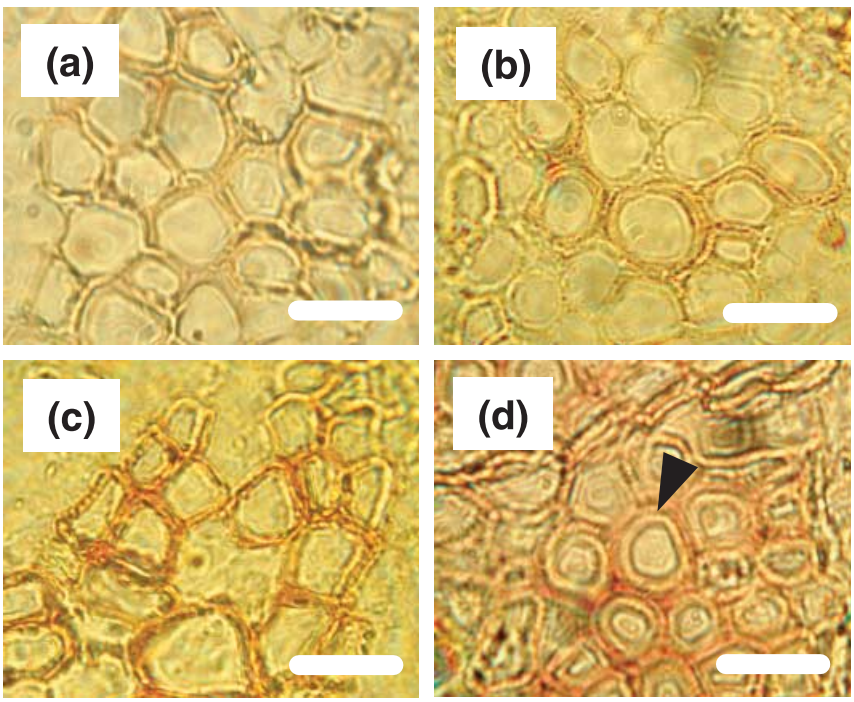

Fig. 6 Lignification in the central cylinder after microbial treatments. Lignin staining of Norway spruce (*Picea abies*) roots inoculated with *Streptomyces* sp. GB 4-2 and/or *Heterobasidion abietinum* 331. Figures were taken 3 wk after fungal inoculation from cross-sections that were stained with phloroglucin-hydrochloric acid. (a) No inoculation: (b) inoculation with GB 4-2; lignin staining is similar to that observed in control samples: (c) *H. abietinum* 331; note the increased lignin staining: (d) GB 4-2 and *H. abietinum* 331; lignin staining is comparable to that in (c), and tracheid-like cells have thick cell walls (arrow). Bar, 0.1 mm.

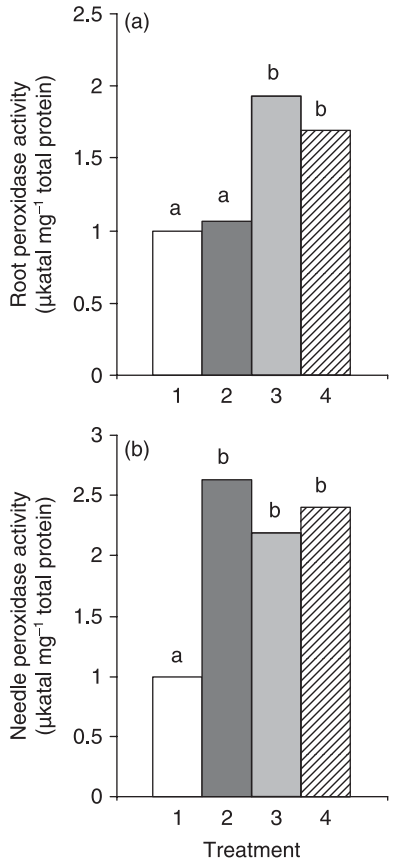

Fig. 7 Peroxidase activities in Norway spruce (*Picea abies*) roots and needles 2 wk after fungal inoculation. Data represent means of relative enzyme activities from roots (a) and needles (b) measured from nine seedlings each. Samples are marked as follows: 1, no treatment; 2, GB 4-2; 3, *Heterobasidion abietinum* 331; 4, GB 4-2 and *H. abietinum* 331. Bars with different letters represent significant differences between means according to one-way ANOVA and Tukey test ($P < 0.01$).

Fungal and dual inoculations lead to increased soluble peroxidase activity

Increased peroxidase activity is an important defense response mechanism in spruce against *Heterobasidion* (Asiegbu *et al.*, 2005), and also serves as a marker for drought stress (Nagy *et al.*, 2004). Here, peroxidase activities increased after fungal and dual inoculations in roots; this change was most significant 2 wk after fungal inoculation (Fig. 7a). In needles, peroxidase activities were comparable between the samples until 2 wk after fungal inoculation, when a strong increase in the enzyme activities was observed in all inoculated samples (Fig. 7b).

Systemic suppression of *B. cinerea* infection by GB 4-2

Increased peroxidase activities in needles suggested that GB 4-2 may have a suppressive effect on subsequent needle infections.

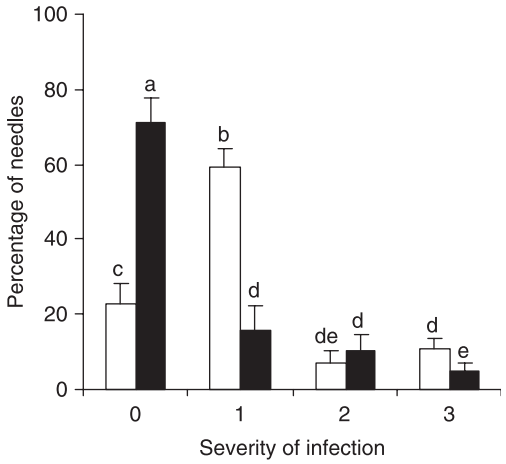

Fig. 8 Reduced needle infection rate by *Botrytis cinerea* after preinoculation of Norway spruce (*Picea abies*) roots with *Streptomyces* sp. GB 4-2 (closed bars) and without preinoculation (open bars). Severity of *B. cinerea* infection: 0, no symptoms; 1, < 50% infected; 2, ≥ 50%; 3, killed needle. Needles of 10 seedlings per treatment were analyzed. Note the strongly increased number of nonsymptomatic needles in GB 4-2-treated plants. Bars with different letters are significantly different according to one-way ANOVA and Tukey test ($P < 0.05$).

By the use of a spruce pathogenic strain of the gray mold *B. cinerea* (Petäistö *et al.*, 2004), we found an attenuated disease development in seedlings pretreated with GB 4-2 (Fig. 8).

Discussion

The results of the present work indicate that *Streptomyces* sp. GB 4-2 can protect spruce seedlings from pathogen infection in at least two ways: first, by evoking anatomical changes in root architecture (fortified cell walls); and secondly, by inducing systemic resistance. This report thus adds to previous evidence that *Streptomyces* spp. interact with plants and microbes in various ways, influencing plant physiology and the development of plant diseases and symbioses (Yuan *et al.*, 1995; Tokala *et al.*, 2002; Schrey *et al.*, 2005, 2007; Riedlinger *et al.*, 2006; Lehr *et al.*, 2007).

The positive influence of GB 4-2 on fungal growth and the early colonization of plant roots relates to previous work with bacteria that promote mycorrhiza formation (mycorrhization helper bacteria, MHB; see Garbaye 1994, Frey-Klett *et al.*, 2007 for reviews). Some of the MHB promote plant infection by enhancing fungal spore germination and mycelial extension (Poole *et al.*, 2001; Founoune *et al.*, 2002), whereas others may facilitate the fungal colonization of plants (Mosse, 1962). A reason for the latter mechanism could be a down-regulation of plant defenses towards colonization, which would also render plants more susceptible to phytopathogens. In our previous analysis, we showed that coculture with the MHB *Streptomyces* sp. AcH 505 has an inhibitory effect on plant peroxidase activity, and this may be the reason for facilitated colonization of Norway

spruce by *H. abietinum* 331 (Lehr *et al.*, 2007). However, no decrease in peroxidase activities was observed here after the treatment with GB 4-2. Thus we suggest that promotion of mycelial extension, and not a decline in plant defense, leads to enhanced early colonization of spruce roots preinoculated with GB 4-2.

While bacterial treatment facilitated the colonization of the cortical cells by *H. abietinum* 331, the same treatment later resulted in the attenuation of the colonization of the vascular cylinder (VC) by the fungus. This suggests that the defense of spruce against fungal colonization was in some way activated. Typical components of biochemical defense responses against *Heterobasidion* include oxidative and lytic enzymes and antifungal secondary metabolites (Asiegbu *et al.*, 2005). Our data show that the oxidative response (increased root peroxidase activity) taking place in dually inoculated spruce seedlings is comparable to that in plants treated with *Heterobasidion* only. However, only in plants that were exposed to both microorganisms was an altered anatomy in distinct regions of cortex and the vascular cylinder observed.

Defense-related anatomical changes are common in Norway spruce as a response to pathogenic fungi, insects, or wounding. These reactions may serve two functions: they aid the plant in resisting subsequent attacks, and inhibit mycelial extension in the already infected regions (Franceschi *et al.*, 2005). In the cortex of the roots infected with *H. annosum*, lignification and extensive formation of cell wall thickenings, papillae, has been observed (Asiegbu *et al.*, 1994; Heneen *et al.*, 1994). Together with lignification, papillae formation aims at restricting the spread of fungal mycelium into the VC. This is important with regard to pathogenesis, since the vascular cylinder is suggested to be more susceptible to *Heterobasidion* than the cortex (Asiegbu *et al.*, 1998). Here, treatment with *H. abietinum* 331 neither induced the formation of papillae nor the lignification in cortex cells, indicating that plant colonization by this fungal isolate did not activate the full spectrum of spruce defense responses. In dual (bacterium + fungus)-inoculated plants instead, cell wall thickenings resembling papillae formed in the inner cortex, and extensive formation of tracheid-like cells took place in the VC. This suggests that bacterial pretreatment rendered the plants more responsive to the fungus as it entered the inner cortical cell layers.

The pattern of fungal infections is commonly modulated by bacteria, in both plant and animal hosts (de Boer *et al.*, 2005; Wargo & Hogan, 2006). For example, the mycorrhization helper bacterium *Streptomyces* AcH 505 promotes the extension of fungal mycelium by the metabolite auxofuran and this leads to enhanced mycorrhiza formation (Riedlinger *et al.*, 2006). By contrast, infections by *Candida albicans* are inhibited by *Pseudomonas aeruginosa* and *Lactobacillus rhamnosus* through the production quorum sensing molecules and butyric acid, respectively; both molecules inhibit the extension of the pathogenic filamentous form of the fungus (Hogan & Kolter, 2004; Noverr & Huffnagle, 2004). The inhibition of root rot by *H. abietinum* observed here was, however, not associated with any obvious changes in the morphology of the fungal hyphae. Hence we suggest that the induction of the barrier anatomy in the root is the key behind root rot suppression by GB 4-2. Interestingly, *H. annosum* s.l. produces phytotoxins that may provoke a systemic response in the host plant (Basset *et al.*, 1967). D. Schulz & H.-P. Fiedler (unpublished) recently observed that the production rates of the *H. annosum* phytotoxins alter in response to GB 4-2. It remains to be investigated if GB 4-2-induces alterations in the fungal secondary metabolism in the plant as well.

In the stems of spruce trees, the presence of local acquired resistance is well established (Christiansen *et al.*, 1999; Krokene *et al.*, 1999) and some evidence exists concerning resistance signaling to adjacent regions (Krokene *et al.*, 2003). Experiments with young seedlings suggest that Norway spruce is also able to perform systemic signaling against pathogens (Nagy *et al.*, 2004; Lehr *et al.*, 2007; this study). Nagy *et al.* (2004) observed increased peroxidase activities in needles after the interaction between spruce roots and *Rhizoctonia* sp.; similar responses are reported here as a result of the presence of either GB 4-2 and/or *H. abietinum*. The expression and activity of peroxidases may confer plant resistance to pathogens by generating hydrogen peroxide for the oxidative burst (Bindschedler *et al.*, 2006). In conifers, peroxidases have also been suggested to confer defense responses (Fossdal *et al.*, 2001), including lignifying or rigidifying plant cell walls (Tarkka *et al.*, 2001; Marjamaa *et al.*, 2006). In this work, we confirmed that the increased peroxidase activity in needles after the inoculation with GB 4-2 is associated with increased resistance against *B. cinerea*.

The physiological responses of Norway spruce to the inoculation with GB 4-2 were similar to those following treatment with *H. abietinum*: both treatments led to lower water contents in roots and in shoots. In addition, 2 wk after all microbial treatments, the peroxidase activities in needles were increased. Nagy *et al.* (2004) observed that the influence of *Rhizoctonia* infection on these two physiological parameters may easily be confused with the influence of drought. Therefore the influence of GB 4-2 on peroxidase activities may partially be explained by decreased water contents of the seedlings. Neither in the *in vitro* culture system used here nor on organic litter cultures (M. Störk & M. Tarkka, unpublished) have we observed any obvious plant stress symptoms such as root or leaf necrosis in the seedlings treated with GB 4-2. However, the decreased water content of seedlings caused by GB 4-2 may be of functional significance in water-poor substrates.

According to Nagy *et al.* (2004), drought caused a significant decrease in the maximum PSII efficiency (F_v/F_m). Since GB 4-2 caused decreased water content in the seedlings, a drop in F_v/F_m was expected. To our surprise, we observed an increase in the F_v/F_m values after the treatment with GB 4-2, similar to that observed in our previous study with *Streptomyces* sp. AcH 505 (Lehr *et al.*, 2007). We can think of two reasons for

this: it could be that the drain of carbohydrates in spruce rhizosphere by the bacterium leads to a compensation of the lost photosynthates by the plant by increased CO_2 assimilation (as described for spruce mycorrhizas in Loewe *et al.*, 2000); or it could be a direct positive effect on photosynthetic yield by GB 4-2. We have recently obtained data supporting the second hypothesis, since the photosynthetic rate of spruce is induced even in cross-walled Petri dishes, where the plants and the bacteria are spatially separated from each other (S. Schrey & M. Tarkka, unpublished). This indicates that the bacterium produces volatiles whose perception leads to an increase in the F_v/F_m values. In any case, our results add to previous data (Nagy *et al.*, 2004; Lehr *et al.*, 2007) indicating that the determination of photosynthetic yield of the host plant is a valuable tool to screen for beneficial or detrimental organisms infecting the plant.

The use of spruce seedling health as a marker for successful disease suppression enabled us to detect GB 4-2 in our screen for bacteria that inhibit the establishment of *Heterobasidion* root and butt rot. Some idea as to how useful GB 4-2 could be for biotechnological applications has come from our studies with *Arabidopsis thaliana*. GB 4-2 inoculation of *Arabidopsis* roots leads to an increase in photosynthetic yield and a sharp decrease in *Alternaria brassicicola* disease susceptibility in leaves. The outcome of these interactions has been confirmed not only in sterile Petri dish cultures but also in nonsterile agricultural soil (S. Schrey, unpublished). Two important conclusions may be drawn from this: GB 4-2 is able to perform its functions on nonsterile soils; and it can be used to increase systemic resistance in different plant species. This makes GB 4-2 a promising candidate for biocontrol approaches.

Bacterial strains influence the establishment of infectious diseases and symbioses in plants (Morgan *et al.*, 2005). How this is achieved is most thoroughly investigated in fluorescent pseudomonads (Ton *et al.*, 2001; Cui *et al.*, 2005; Haas & Defago, 2005; Frey-Klett *et al.*, 2007). The role of actinomycetes has gained increased interest, and it has been observed that the functions first characterized for fluorescent pseudomonads may be served by the actinomycetes as well (Yuan *et al.*, 1995; Poole *et al.*, 2001; Tokala *et al.*, 2002; Riedlinger *et al.*, 2006; Lehr *et al.*, 2007). Our report adds novel, encouraging data showing that actinomycetes may be able to restrict plant colonization by fungal strains that are able to escape the biological warfare exuded by the bacteria. That these bacteria are also able to elicit a defense response in the host plant warrants further investigations in the future.

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Time course of the interaction between Norway spruce seedlings, *Streptomyces* GB 4-2 and *Heterobasidion abietinum* 331.

Fig. S2 Increased lignification during the interaction between Norway spruce seedlings, *Streptomyces* GB 4-2 and *Heterobasidion abietinum* 331.

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