Mycorrhiza (1996) 6:481–485 

ORIGINAL PAPER

**M. Schelkle · R. L. Peterson**

# Suppression of common root pathogens by helper bacteria and ectomycorrhizal fungi in vitro

Accepted: 23 October 1996

**Abstract** Root pathogens cause considerable loss of tree seedlings in nurseries and are generally difficult to control using conventional methods. Inoculation with ectomycorrhizal fungi may provide some suppression of pathogens. Bacteria (so-called mycorrhization helper bacteria) have been isolated that stimulate mycorrhiza formation on seedling roots and enhance seedling growth; however, their role in pathogen inhibition has not been explored. Four strains of helper bacteria were inoculated together with the ectomycorrhizal fungal species *Laccaria bicolor*, *L. proxima* and *Suillus granulatus* on culture plates to determine inhibition of the pathogens *Fusarium oxysporum* and *Cylindrocarpon* sp. Buffered medium was used to rule out acidification of the medium as a mechanism of inhibition. None of the ectomycorrhizal fungal species alone inhibited the growth of *Fusarium* but all showed slight inhibition of *Cylindrocarpon* growth. Helper bacterium strain MB3 (*Bacillus subtilis*) was effective in inhibiting both pathogens and, when inoculated with either *L. proxima* or *S. granulatus*, inhibition of *Fusarium* growth was enhanced over MB3 alone. With *Cylindrocarpon*, however, only *S. granulatus* inoculated along with MB3 showed enhanced inhibition over MB3 alone. The other three bacterial strains had little effect on the growth of *Fusarium* or *Cylindrocarpon*. More research is necessary to determine if these inhibitory effects are reproducible in situ.

**Key words** Helper bacteria · *Laccaria* · *Suillus* · *Fusarium* · *Cylindrocarpon* · Ectomycorrhiza

M. Schelkle · R. L. Peterson (✉)
Department of Botany, University of Guelph,
Guelph, Ontario, N1G 2W1, Canada
fax: +1-519-767-1991; e-mail: lpeterso§uoguelph.ca

## Introduction

Fungal root pathogens are a major cause of conifer seedling death in nurseries and most conventional practices are not effective at controlling the problem. Methods of biological control are, therefore, receiving increasing interest. Many researchers have documented suppression of root diseases in conifers by ectomycorrhizal (EM) fungi (Duchesne 1994), although field application of EM fungi has yielded somewhat erratic results (Sampangi et al. 1986; MacFall 1986).

Several mechanisms may be involved in disease suppression by EM fungi (see review by Duchesne 1994). These include the barrier effect provided by the mantle, competitive exclusion, plant-produced antimicrobials induced by EM colonization (Duchesne et al. 1987a, b), and antibiotics produced by EM fungi (Zak 1964; Marx 1972, 1973). The production of antibiotics by EM fungi has been studied using mostly in vitro tests; however, recent evidence suggests that inhibition of pathogenic fungi by EM fungi in vitro is due to acidification of the medium rather than to antibiotic production (Rasanayagam and Jeffries 1992).

Bacteria are also prolific producers of antibiotics, and the suppression of pathogenic fungi by bacteria has been demonstrated (Schroth and Hancock 1982; Malajczuk 1988). Several bacterial strains isolated from ectomycorrhizal tips and fungal sporocarps stimulate the formation of mycorrhizal tips on roots of several tree species when seedlings are inoculated with specific fungi (Garbaye et al. 1990; Duponnois and Garbaye 1991). Some of these bacteria, known as mycorrhization helper bacteria, stimulated the growth of the EM fungal species *Laccaria laccata* and *L. bicolor* while inhibiting the growth of other fungi in in vitro tests (Garbaye and Duponnois 1992). The same strains also proved their helper activity by promoting the formation of mycorrhizal tips on numerous tree species (*Picea abies* L. Karsten, *Pinus nigra* Arndd., *Pinus sylvestris* L., *Pseudotsuga menziesii* (Mirb.) Franco and *Quercus robur*

L.) that were inoculated with *L. laccata* in greenhouse experiments and nursery trials (Garbaye et al. 1992). The mechanisms involved in these processes are largely unknown. This study investigated the suppression of root pathogens as one of the possible mechanisms involved in enhanced growth of seedlings inoculated with helper bacteria.

## Materials and methods

Plugs (5 mm in diameter) of *L. bicolor* Maire Orton, *L. proxima* Boudier or *Suillus granulatus* (L. ex Fr.) O. Kuntze. were taken from the periphery of growing colonies maintained on modified Melin Norkrans (MMN) medium (Marx and Bryan 1975) and placed at the edge of a Petri plate containing 1.2% MMN or MMN buffered with 2-(N-morpholino)ethanesulfonic acid (MES) (Rasanayagam and Jeffries 1992). The plugs were allowed to grow in the dark at 25 °C (15 days for *S. granulatus*, 11 days for *L. proxima* and 9 days for *L. bicolor*) before they were inoculated either alone or along with helper bacteria to test effects on two fungal pathogens. Eight replicates of each treatment were prepared. Controls consisted of plates containing a plug of plain MMN with no EM fungi but inoculated with bacteria, and plates with plugs of EM fungi which were not inoculated with bacteria.

Four strains of patented helper bacteria were obtained from Jean Garbaye (INRA, Nancy, France): *Bacillus subtilis* (MB3), and *Bacillus* spp. (SHB1) both isolated from mycorrhizas of *L. laccata* (Scop. ex Fr.) Bk. & Br. S238 on Douglas fir, and *Pseudomonas fluorescens* (BBc6) and *Pseudomonas* spp. (SBc5), which were obtained from sporocarps of *L. laccata*.

The bacteria were inoculated into 50 ml of Tryptic Soy Broth and incubated overnight at room temperature on a rotary shaker (90 rpm). The cells were then collected by centrifugation (20 min at 4300 rpm) and washed once with 0.1 M $MgSO_4 \cdot 7H_2O$. The pellet was then resuspended in the same solution. Suspensions were serially diluted to $10^{-6}$ and plated in duplicate onto 1.2% Tryptic Soy Agar to determine the viable colony-forming units (cfu) per ml. The original suspensions (ca. $10^8$ cfu/ml) were then used to inoculate the EM fungi. A 0.1-ml aliquot of the bacteria was added aseptically to the growing colony, or to the control plug of plain MMN. For the second control, 0.1 ml of 0.1 M $MgSO_4 \cdot 7H_2O$ was administered to the growing EM colony.

The pathogens were inoculated at the same time as the bacteria. Plugs (5 mm in diameter) taken from the actively growing periphery of a fungal colony were placed directly opposite to the EM colony and against the edge of the Petri dish. *Fusarium oxysporum* Schlect. and *Cylindrocarpon* sp. previously isolated from diseased roots of *Pinus strobus* L. (white pine) seedlings grown in the St. Williams nursery in southern Ontario were the two pathogens used.

Once the plates had been inoculated with both the bacteria and the pathogens, the plates were sealed with Parafilm and incubated in the dark at 25 °C. Inhibition zones were measured after an 18-day incubation. The measurement was taken in a straight line between the two plugs using a ruler and a Zeiss dissecting microscope.

### Statistical analyses

A 2-way analysis of variance (ANOVA) was used to examine the effect of media and inoculation on the size of the inhibition zone. Linear contrasts were used to compare treatment means to controls. All analyses were performed using JMP (SAS Institute).

## Results

### *Fusarium inhibition*

Buffering of the culture medium reduced the size of the inhibition zone in all cases except with the fungus *S. granulatus* in combination with the bacterial strain MB3 (Table 1). All fungi alone produced an inhibition zone on MMN medium but not on buffered medium. Three of the bacterial strains (BBc6, MB3, SHB1) inoculated alone on MMN medium also resulted in inhibition zones when challenged with *Fusarium* but only MB3 showed an inhibition zone on buffered medium (Table 1). *L. bicolor* inoculated along with MB3 on both MMN and MES medium resulted in significantly wider inhibition zones than with the fungus alone (Table 1). This fungus, in combination with the bacteri-

**Table 1** Mean inhibition zones (cm ± standard error) on two different media measured between colonies of ectomycorrhizal fungus and *Fusarium* after inoculation with a suspension of helper bacteria. Values in the rows should be compared to the control with bacterium only, and column values should be compared to the control with fungus only (*MES* 2-(N-morpholino)ethanesulfonic acid, *MMN* Melin-Norkrans medium, *NA* not applicable)

| Bacterium | *Laccaria bicolor* | | *Laccaria proxima* | | *Suillus granulatus* | | Control (bacterium only) | |
|---|---|---|---|---|---|---|---|---|
| | MMN | MES | MMN | MES | MMN | MES | MMN | MES |
| BBc6 | $0.34^{+++}$ ±0.057 | 0.09 ±0.048 | $0.43^{***+}$ ±0.042 | 0.00 | $0.63^{**}$ ±0.076 | 0.00 | 0.60 ±0.065 | 0.00 |
| MB3 | $1.18^{***++}$ ±0.136 | $0.50^{***}$ ±0.053 | 0.91 ±0.142 | $0.79^{***+++}$ ±0.069 | $0.41^{++}$ ±0.063 | $0.66^{***+}$ ±0.159 | 0.84 ±0.115 | 0.34 ±0.072 |
| SHB1 | 0.30 ±0.087 | 0.00 | $0.87^{+}$ ±0.092 | 0.00 | 0.42 ±0.048 | 0.00 | 0.35 ±0.171 | 0.00 |
| SBc5 | 0.36 ±0.080 | 0.00 | $0.71^{***}$ ±0.077 | 0.00 | $0.34^{+++}$ ±0.084 | 0.00 | 0.00 | 0.00 |
| Control (fungus only) | 0.39 ±0.051 | 0.00 | 1.16 ±0.061 | 0.00 | 0.31 ±0.046 | 0.00 | NA | NA |

* $P<0.05$ control = EM fungus only
** $P<0.01$ control = EM fungus only
*** $P<0.001$ control = EM fungus only
$^{+}$ $P<0.05$ control = bacteria only
$^{++}$ $P<0.01$ control = EM fungus only
$^{+++}$ $P<0.001$ control = EM fungus only

al strain BBc6 on MMN medium, produced a significantly smaller inhibition zone than with the bacterium alone, but with MB3 the inhibition zone was significantly wider (Table 1). On MMN medium, *L. proxima* in combination with either BBc6 or SBc5 produced a smaller inhibition zone than that shown by the fungus alone. In combination with BBc6, the zone was smaller than with bacterium alone, while in combination with SHB1 the zone was wider than with the bacterium alone (Table 1). On MES medium, *L. proxima* in combination with MB3 resulted in an inhibition zone significantly larger than with either the fungus or bacterium alone (Table 1). *S. granulatus* in combination with MB3 showed a reduced inhibition zone on both media compared with bacterium alone but a wider inhibition zone on buffered medium compared with fungus alone (Table 1). This fungus in combination with BBc6 on unbuffered medium resulted in a larger inhibition zone than with fungus alone.

### *Cylindrocarpon* inhibition

The results for inhibition of *Cylindrocarpon* were very different to those for *Fusarium* (Table 2). Zones of inhibition were produced in all cases on both media; however, zones were generally smaller on MES, suggesting that inhibition was caused by antibiosis and not by excessive acidification of the medium. For this reason, only the results from MES will be discussed.

For inoculation with MB3, all three EM fungi showed significantly larger inhibition zones than the treatments with the fungi alone. Both *L. bicolor* and *L. proxima* also produced significantly larger zones when coinoculated with MB3 than with the bacterial treatment alone.

Both *L. bicolor* and *L. proxima* coinoculated with BBc6 produced significantly smaller inhibition zones than with the bacterium alone, although inhibition zones produced by the fungi alone were not significantly different from the inoculated treatments. *S. granulatus* inoculated with BBc6 had significantly larger zones than *S. granulatus* alone, although the zones were not significantly different from the bacterial treatment alone.

The results of inoculation with SHB1 and SBc5 were similar to BBc6. Both *L. bicolor* and *L. proxima* inoculations gave significantly smaller zones of inhibition than the treatment with bacteria alone. *L. proxima* inoculated with SHB1 showed a significantly larger inhibition zone compared with the fungus alone. *S. granulatus* exhibited no significant differences in the size of inhibition zones compared with either control when inoculated with SHB1 or SBc5.

## Discussion

Results from unbuffered media if taken alone could easily be misinterpreted as antibiosis (Rasanayagam and Jeffries 1992). The use of a buffered medium (MES) in this study showed that acid production was responsible for much of the suppression of pathogens by EM fungi and bacteria in vitro. True antibiosis was evident, however, on MES especially with bacterial strain MB3. Inoculation of the EM fungi together with MB3 in most cases enhanced suppression of both *Fusarium* and *Cylindrocarpon*. Strain MB3 was identified as *B. subtilis* by Garbaye and Duponnois (1992), and it is well-known for its ability to produce extracellular chitinase and laminarinase, which can degrade fungal cell walls (Mitchell and Alexander 1963). For this reason, it has been utilized as a biocontrol agent and has been effective in suppressing pathogens such as *F. solani* (Mart.) Sacc. (Sarhan 1989). None of the EM fungi used alone in this study were able to inhibit *Fusarium*

**Table 2** Mean inhibition zones (cm ± standard error) on two different media measured between colonies of ectomycorrhizal fungi and *Cylindrocarpon* after inoculation with a suspension of helper bacteria. Values in the rows should be compared to the control with bacterium only, and column values should be compared to the control with fungus only. Abbreviations as in Table 1

| Bacterium | *Laccaria bicolor* | | *Laccaria proxima* | | *Suillus granulatus* | | Control (bacterium only) | |
|---|---|---|---|---|---|---|---|---|
| | MMN | MES | MMN | MES | MMN | MES | MMN | MES |
| BBc6 | $0.41^{+++}$ ±0.074 | $0.15^{+++}$ ±0.063 | 2.44* ±0.174 | $1.06^{+++}$ ±0.139 | 2.73 ±0.152 | 1.99* ±0.059 | 2.71 ±0.137 | 1.84 ±0.143 |
| MB3 | $1.30^{*+++}$ ±0.241 | $1.61^{***+++}$ ±0.055 | $2.28^{++}$ ±0.053 | $2.69^{***+}$ ±0.088 | 2.83 ±0.141 | 2.53*** ±0.182 | 2.66 ±0.127 | 2.36 ±0.069 |
| SHB1 | $0.87^{+++}$ ±0.089 | $0.46^{+++}$ ±0.201 | $1.86^{+++}$ ±0.112 | $1.36^{*+++}$ ±0.31 | 2.35 ±0.093 | 1.90 ±0.139 | 2.63 ±0.137 | 1.92 ±0.125 |
| SBc5 | $0.84^{+++}$ ±0.123 | $0.35^{+++}$ ±0.145 | 1.95 ±0.144 | $0.58^{+++}$ ±0.175 | $2.32^{+}$ ±0.110 | 1.71 ±0.097 | 1.89 ±0.130 | 1.80 ±0.118 |
| Control (fungus only) | 0.73 ±0.126 | 0.10 ±0.100 | 2.03 ±0.077 | 0.91 ±0.100 | 2.44 ±0.107 | 1.58 ±0.111 | NA | NA |

* $P<0.05$ control = fungus only
** $P<0.01$ control = fungus only
*** $P<0.001$ control = fungus only
$^{+}$ $P<0.05$ control = bacteria only
$^{++}$ $P<0.01$ control = fungus only
$^{+++}$ $P<0.001$ control = fungus only

on the buffered media, suggesting that the bacteria were solely responsible for suppression of this pathogen. Interestingly, both *L. proxima* and *S. granulatus* had significantly larger inhibition zones when inoculated with MB3 than those produced by the bacteria alone, suggesting a synergistic effect by the bacteria and EM fungi together. MB3 also had a suppressive effect against *Cylindrocarpon* when inoculated onto the EM fungi. In this case, all three EM fungi showed some inhibition of *Cylindrocarpon* without bacterial inoculation, implying that this pathogen is more vulnerable than *Fusarium* to substances produced by the EM fungi (Sampangi and Perrin 1986; Duchesne 1994). Once inoculated, only *L. proxima* showed a significant increase in inhibition over the bacterial treatment alone, suggesting an additive or synergistic effect and the possibility that MB3 was not as effective in inhibiting *Cylindrocarpon* when inoculated alone.

Several factors, such as siderophore production, competition for nutrients and space in the rhizosphere, and antibiotic production may be involved in the suppression of pathogenic fungi by EM fungi or bacteria. Fluorescent pseudomonads synthesize a water-soluble pigment under iron deprivation which serves as a siderophore (iron chelator) and solubilizes ferric hydroxide, thus making soluble iron available for uptake by the bacteria (Schroth and Hancock 1982; O'Sullivan and O'Gara 1992). The siderophores are species specific and the iron chelates can only be absorbed by those species that produce them, thereby effectively partitioning the nutrients away from competing organisms such as pathogens (Neidhardt et al. 1990). *Pseudomonas* spp. also produce fluorescent siderophores which can effectivly inhibit fungal pathogens (Howell and Stipanovic 1979). Strains BBc6 and SHB1 used in this study are pseudomonads and although they were not successful in inhibiting *Fusarium* on MES, *Cylindrocarpon* was effectively suppressed. Inoculation of these bacteria onto the EM fungi produced less inhibition than the treatments with the bacteria alone, suggesting that the presence of the EM fungi somehow limited the fungistatic properties of the bacteria. Many EM fungi are also able to produce hydroxamate siderophores in pure culture (Szaniszlo et al. 1981; Watteau and Berthelin 1990) and may also contribute to the nutrition of the plant, or to effective partitioning of nutrients away from competing organisms such as the helper bacteria in this study.

Although antibiotic production is a prominent mechanism of antagonism in the rhizosphere (Rambelli 1973), the competitive ability of the microorganism may play a more important role. Strzelczyk (1966) found that resistance of flax and tobacco to *Fusarium* and other pathogens was due to large numbers of competitive bacteria, and not to the presence of antibiotic-producing strains. Fluorescent pseudomonads, bacilli and other organisms tend to compete strongly for nutrients in the rhizosphere because of their reliance on plant/fungal exudates for survival (Garbaye 1991). Katznelson (1965) showed that competition for thiamine and other nutrients by soil bacteria limited the development of pathogens on alfalfa roots. *Pseudomonas* spp. aggressively colonize root surfaces and are nutritionally versatile, allowing the bacteria to thrive while propagules of pathogenic fungi germinate (Schroth and Hancock 1982). It is possible that helper bacteria are able to out-compete pathogenic fungi for nutrients, but this needs to be tested using seedling roots.

Although there has often been a poor correlation between inhibition of pathogens by bacteria on agar and in the rhizosphere (Broadbent et al. 1971), these studies failed to take into account the possible accumulation of acids in the medium, causing a false perception of antibiosis. Many studies have shown suppression of pathogens by either EM fungi or by bacteria and, therefore, it is reasonable to assume, when these organisms are present together, that they increase resistance to pathogenic organisms. Inoculation with fungi and bacteria that exhibit biocontrol properties should be examined in combination with nursery practices conducive to forming healthy, disease-free root systems in order to produce better quality seedlings.

**Acknowledgements** We would like to thank the Natural Sciences and Engineering Research Council of Canada for funding this project, Dr. Jean Garbaye for providing the bacteria, Jane Gurney for her technical assistance, and Kevin Stevens for comments on the manuscript.

## References

Broadbent P, Baker KF, Waterworth Y (1971) Bacteria and actinomycetes antagonistic to fungal root pathogens in Australian soils. Austr J Biol Sci 24:925–944

Duchesne LC (1994) Role of ectomycorrhizal fungi in biocontrol. In: Pfleger FL, Linderman RG (eds) Mycorrhizae and plant health. APS Press, St. Paul, Minn, pp 27–45

Duchesne LC, Peterson RL, Ellis BE (1987a) The accumulation of plant-produced antimicrobial compounds in response to ectomycorrhizal fungi: a review. Phytoprotection 68:17–27

Duchesne LC, Peterson RL, Ellis BE (1987b) Interactions between the ectomycorrhizal fungus *Paxillus involutus* and *Pinus resinosa* induces resistance to *Fusarium oxysporum*. Can J Bot 66:558–562

Duponnois R, Garbaye J (1991) Mycorrhization helper bacteria associated with the Douglas fir-*Laccaria laccata* symbiosis: effects in aseptic and in glasshouse conditions. Ann Sci For 48:239–251

Garbaye J (1991) Biological interactions in the mycorrhizosphere. Experientia 47:370–375

Garbaye J, Duponnois R (1992) Specificity and function of mycorrhization helper bacteria (MHB) associated with the *Pseudotsuga menziesii-Laccaria laccata* symbiosis. Symbiosis 14:335–344

Garbaye J, Duponnois R, Wahl JL (1990) The bacteria associated with *Laccaria laccata* ectomycorrhizas or sporocarps: effect on symbiosis establishment on Douglas fir. Symbiosis 9:267–273

Garbaye J, Churin JL, Duponnois R (1992) Effects of substrate sterilization, fungicide treatment and mycorrhization helper bacteria on ectomycorrhizal formation of pedunculate oak (*Quercus robur*) inoculated with *Laccaria laccata* in two peat bare-root nurseries. Biol Fertil Soils 13:55–57

Howell CR, Stipanovic RD (1979) Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with antibiotics produced by the bacterium. Phytopathology 69:480–482

Katznelson H (1965) Nature and importance of the rhizosphere. In: Baker KF, Snyder WC (eds) Ecology of soil-borne plant pathogens. University of California Press, Berkeley, Calif, pp 187–209

MacFall JS (1986) Effect of two ectomycorrhizal fungi on growth and *Cylindrocladium* root rot susceptibility of red pine seedlings in a Wisconsin nursery. Phytopathology 76:1110

Malajczuk N (1988) Interaction between *Phytophthora cinnamomi* zoospores and micro-organisms on non-mycorrhizal and ectomycorrhizal roots of *Eucalyptus marginata*. Trans Br Mycol Soc 90:375–382

Marx DH (1972) Ectomycorrhizae as biological deterrents to pathogenic root infection. Annu Rev Phytopathol 10:429–454

Marx DH (1973) Mycorrhizae and feeder root diseases. In: Marks GL, Koslowski TT (eds) Ectomycorrhizae: their ecology and physiology. Academic Press, New York, pp 351–383

Marx DH, Bryan WC (1975) Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infected with the fungal symbiont *Pisolithus tinctorius*. For Sci 21:245–254

Mitchell R, Alexander M (1963) Lysis of soil fungi by bacteria. Can J Microbiol 9:169–177

Neidhardt FC, Ingraham JL, Schaechter M (eds) (1990) Physiology of the bacterial cell. Sinauer, Mass

O'Sullivan DJ, O'Gara F (1992) Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol Rev 56:662–676

Rambelli A (1973) The rhizosphere of mycorrhizae. In: Marks GL, Koslowski TT (eds) Ectomycorrhizae: their ecology and physiology. Academic Press, New York, pp 299–343

Rasanayagam S, Jeffries P (1992) Production of acid is responsible for antibiosis by some ectomycorrhizal fungi. Mycol Res 96:971–976

Sampangi R, Perrin R (1986) Attempts to elucidate the mechanism involved in the protective effect of *Laccaria laccata* against *Fusarium oxysporum*. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Proceedings of the 1st European Symposium on Mycorrhizae, Dijon, France, pp 807–810

Sampangi R, Perrin R, LeTacon F (1986) Disease suppression and growth promotion of Norway spruce and Douglas fir seedlings by the ectomycorrhizal fungus *Laccaria laccata* in forest nurseries. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Proceedings of the 1st European Symposium on Mycorrhizae, Dijon, France, pp 799–806

Sarhan ART (1989) Biological control of *Fusarium* root rot of broad bean. Acta Phytopathol Entomol Hung 24:271–275

Schroth MN, Hancock JG (1982) Disease-suppressive soil and root-colonizing bacteria. Science 216:1376–1381

Strzelczyk E (1966) Effect of associated growth of bacteria from rhizosphere and non-rhizosphere soil on the growth of fungi. Ann Inst Pasteur 111:314

Szaniszlo PJ, Powell PE, Reid CPP, Cline GR (1981) Production of hydroxamate siderophore iron chelators by ectomycorrhizal fungi. Mycologia 73:1158–1174

Watteau F, Berthelin J (1990) Iron solubilization by mycorrhizal fungi producing siderophores. Symbiosis 9:59–67

Zak B (1964) Role of mycorrhizae in root disease. Annu Rev Phytopathol 2:377–392