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Bacteria Associated with *Suillus grevillei* Sporocarps and Ectomycorrhizae and their Effects on *In Vitro* Growth of the Mycobiont

GIOVANNA CRISTINA VARESE[1], SABRINA PORTINARO[2], ANTONIO TROTTA[1], SILVANO SCANNERINI[1], ANNA MARIA LUPPI-MOSCA[1], and MARIA GIOVANNA MARTINOTTI[2*]
[1]*Department of Plant Biology, I Faculty of Sciences; and* [2]*Department of Sciences and Advanced Technologies, II Faculty of Sciences, University of Turin, Corso Borsalino 54, Alessandria 15100, Italy. Tel. +39-131-283725, Fax. +39-131-254410, E-mail. martinot@venere.unial.it*

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Abstract

Twenty seven bacterial species were isolated from both the sporocarps of *Suillus grevillei* and the ectomycorrhizae of *Suillus grevillei-Larix decidua*. The genera *Pseudomonas*, *Bacillus* and *Streptomyces* were predominant. Several species were common to both the sporocarps and the ectomycorrhizae. Dual culture trials between Gram-positive, Gram-negative, *Streptomyces* and five different isolates of *S. grevillei* showed several behavior patterns depending on the bacterial group, the fungal isolate and the time. Gram-positive bacteria seldom stimulated fungal growth. Among Gram-negative bacteria, *Pseudomonas fluorescens* strain 70 and *Pseudomonas putida* strain 42 showed the greatest enhancement of growth. *Streptomyces* always caused significant inhibition of the fungus. Bacterial supernatants never significantly stimulated fungal growth; volatile metabolites frequently enhanced fungal growth but seldom significantly. Most of the bacterial isolates produced siderophores. The results obtained suggest for some bacterial strains a very high fungus selectivity at the intraspecific level.

Keywords: Rhizobacteria, *Suillus grevillei*, *Larix decidua*, ectomycorrhizae

*The author to whom correspondence should be sent.

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1. Introduction

Sustained plant growth in natural ecosystems occurs because of the balance that has been developed over evolutionary time between host plants, their microbial associates and the environment. Disturbance of this balance decreases any chance for sustainability but the equilibrium could be restored by incorporating the necessary microflora. The re-establishment should begin with the establishment of appropriate populations of mycorrhizal fungi and as many of their beneficial associates as possible. Understanding of the factors governing microbial interactions in the mycorrhizosphere is needed t 129 to promote plant growth more efficiently.

Several genera of bacteria have been isolated from the fruiting bodies of ectomycorrhizal fungi (Li and Castellano, 1987; Garbaye et al., 1990; Danell et al., 1993). Bacteria associated with the hyphae of the mantle in *Arbutus unedo* L. have been found (Filippi et al., 1995). Germination of spores and propagules, and growth of mycorrhizal fungi mycelia (both AM and ECM) are mainly influenced by the genera *Pseudomonas, Corynebacterium, Bacillus* and *Streptomyces*. This influence may be positive or negative, depending on the bacterial isolate and on volatile or non-volatile metabolites able to act at a distance (Bowen and Theodorou, 1979; Bowen, 1980; De Oliveira and Garbaye, 1989; Paulitz and Linderman, 1991; Azcon-Aguilar and Barea, 1992; Garbaye and Duponnois, 1992; Garbaye, 1994).

Mycorrhizal infection is often enhanced by mycorrhization helper bacteria (MHB) commonly found in the rhizosphere, in different soils and plant-fungus associations. MHB can also be fungus-specific in that they are beneficial to the fungus from which mycorrhizae they were isolated, but inhibit other species (Garbaye and Duponnois, 1992; Garbaye et al., 1992; Duponnois et al., 1993). The mechanisms underlying this interaction are varied and not well understood (Duponnois and Garbaye, 1990).

The aim of the present research was to identify bacteria isolated both from the sporocarps and the ectomycorrhizae of *Suillus grevillei* (Klotz) Sing.-*Larix decidua* Miller and to examine in an *in vitro* system the effects of these bacteria on the growth of the mycobiont. *S. grevillei* is a highly specific ectomycorrhizal symbiont for *Larix* spp., therefore, bacterial populations associated to the fungus may be particularly important in the establishment of the symbiosis.

2. Materials and Methods

Fungal isolates

The five isolates of *Suillus grevillei* (Klot.) Sing. were aseptically obtained from

five different fruiting bodies collected under five different trees at different sites of a pure *Larix decidua* wood in Valle di Susa (Piedmont, Italy). They were maintained on Modified Melin-Norkrans medium (MMN) (malt extract-Difco 3 g; glucose 10 g; $(NH_4)_2HPO_4$ 1.9 mM; HK_2PO_4 3.7 mM; $MgSO_4 \times 7\ H_2O$ 0.61 mM; $CaCl_2 \times 2\ H_2O$ 0.45 mM; FeEDTA 0.05 mM; NaCl 0.43 mM; thiamine HCl 100 µg; bacteriological agar-Difco 20 g per liter) at 22°C. The isolates were subcultured every three or four weeks. Standard fungal explants were taken as plugs with a sterile 5 mm cork borer from the edge of an actively growing colony.

Isolation of fungus-associated bacteria

Bacterial strains were isolated from the very same sporocarps from which the five fungal isolates were obtained and from the mycorrhizae of *S. grevillei* collected under the sporocarps. Pieces of tissue from inside the sporocarps were blended in sterile water and serially diluted. Mycorrhizae were washed in running tap water, surface-sterilized in 1.5% NaClO for 2 min, and rinsed 15 times in sterile water. The effectiveness of surface sterilization was checked by plating water from the last rinse on Nutrient agar. Mycorrhized roots as a whole and serial dilutions of the suspensions from sporocarps were inoculated at 28°C on Trypticase Soy Agar (TSA) (Difco), Mac Conkey agar (Difco), Pseudomonas F agar (Difco) to isolate Gram-positive and Gram-negative bacteria, and at 22°C on Starch-Casein agar (starch 10 g; casein without vitamins 0.3 g; $NaNO_3$ 2 g; NaCl 2 g; $KHPO_4$ 2 g; $MgSO_4 \times 7\ H_2O$ 0.05 g; $CaCO_3$ 0.02 g; $FeSO_4 \times 7\ H_2O$ 0.01 g; bacteriological agar 15g per liter) to isolate *Streptomyces*.

Identification of bacteria

The most commonly occurring bacterial colonies were purified for identification and biotyped with the biochemical systems API 20 NE, API 20 E, API 50 *Bacillus* (Oxoid) with the additional tests of oxidase and motility. For the oxidase test, paper disks impregnated with dimethyl-p-phenilenediamine oxalate (Oxoid) were placed over a 24 hr confluent monolayer of bacteria. Oxidase positive colonies turned the disks black within a few minutes. For the motility test, bacteria were grown overnight on motility test medium (Beef extract -Difco 3 g; Peptone -Difco 10 g; NaCl 5 g; bacteriological agar-Difco 4 g per liter; final pH 7.3). The motility was interpreted by making a macroscopic examination of the medium for a diffuse zone of growth flaring out from the line of inoculation.

Effects of bacteria on in vitro growth of S. grevillei

All Gram-positive and Gram-negative bacteria were cultivated on TSA at 28°C for 24 hours, except for *Pseudomonas* species, which were cultivated on Pseudomonas F agar, and *Streptomyces* isolates which were cultivated on Starch-Casein agar at 22°C for 7 days. One mycelium disk, 5 mm in diameter and 4 mm thick was placed at the center of a 9 cm Petri dish containing MMN agar. This medium was chosen because it was the only one, among the various we checked, that could support the growth of both the fungus and the bacteria and is one of the most used for the *in vitro* studies of interaction between bacteria and mycorrhizal fungi. Four inocula obtained from an overnight monolayer of one bacterial species were streaked 1.5 cm from the fungal disk in four perpendicular directions. *Streptomyces* isolates were preinoculated on MMN agar 7 days earlier than the fungus. Controls were fungal disks without bacteria, and bacterial inocula paired with disks of MMN agar. Three replicates of each fungal isolate were inoculated in dual cultures with each bacterial strain. Petri dishes were incubated at 22°C. The mean radial growth of the mycelium in two perpendicular directions was measured every 7 days for three weeks. The data were compared by ANOVA, Tukey's test ($p< 0.01$; $p< 0.05$). When *Streptomyces* isolates were used, at the end of the third week, the mycelial disk was transferred to a new Petri dish containing MMN agar to evaluate the fungistatic or fungicidal effect of *Streptomyces*.

Effects of bacterial supernatants on in vitro growth of S. grevillei

Supernatants were obtained by culturing bacteria at 28°C for 48 hours in Tripticase soy broth (TSB) in a continuous shaker at 300 rpm to a density of 10^{11} CFU/ml. After centrifuging at 4,500 g for 25 min, supernatants were filtered through a 0.2 μm Millipore and concentrated ten times with a rotovapor. Controls were obtained from TSB without bacteria. Eighty microliters of each supernatant were mixed with 3 ml of molten MMN agar at 50°C, and poured on a Petri dish containing 20 ml of solidified MMN agar. A mycelial disk was then placed in the center of the Petri dish. Each experiment was done in triplicate. The mean radial growth of the mycelium in two perpendicular directions was measured after 7 days. The data were treated as described above.

Effect of bacterial volatile metabolites on in vitro growth of S. grevillei

Two 6 cm Petri dishes each containing 10 ml of MMN agar were faced. A disk of the isolate of the fungal symbiont was transferred to the center of the lower dish, while in the upper dish four perpendicular bacterial inocula were streaked

at a distance of 1.5 cm from the center. *Streptomyces* isolates were preinoculated seven days before the inoculum of the fungal symbiont. Controls were the upper dish without bacteria, and the lower with the mycelial disk. The two faced dishes were sealed with tape and incubated for 1 week at 22°C. Mycelial growth was measured as previously described after 7 days and the data were treated as described above.

Production of siderophores

Bacteria were inoculated on Chrome azurol S (CAS) agar plates and incubated at 28°C for 5 days (Schwyn and Neilands, 1987). The diameter of the orange halo was measured and regarded as evidence of production of siderophores.

All the experiments were repeated twice under perfectly similar conditions. Results are given from one experiment only but are representative of both.

3. Results

Isolation and identification of bacteria from sporocarps and ectomycorrhizae of S. grevillei

As shown in Table 1, 16 bacterial species and 9 *Streptomyces* isolates were isolated from the sporocarps, and 19 bacterial species and 2 *Streptomyces* isolates from the ectomycorrhizae: 8 bacterial species (with asterisks) were common to the sporocarp and the ectomycorrhizae. Many *Bacillus* and *Pseudomonas* species were isolated from sporocarps, whereas there was a wider variety of genera in the ectomycorrhizae. *Streptomyces* were classified as different isolates, but not distinguished to species, on the basis of their macroscopic and microscopic characteristics.

Effects of Gram-positive and Gram-negative bacteria on in vitro growth of fungal isolates

The effects of Gram-positive and Gram-negative species from the sporocarp and the ectomycorrhizal mantle on growth of five *S. grevillei* isolates at 7, 14 and 21 days are illustrated in Tables 2 and 3. The effect of *Bacillus* species varied considerably and was seldom significant in the first week. Moreover, different isolates of the same species could significantly inhibit one fungal isolate and stimulate another (*B. megaterium* 8 inhibited isolate AB7 and stimulated AB8). In the second week, inhibition, particularly of isolates AB1 and AB7, became more

Table 1. Bacteria isolated from both the sporocarps and the ectomycorrhizae of *S. grevillei.*

Sporocarps	Ectomycorrhizae
Bacillus brevis	*Acinetobacter calcoaceticus*
Bacillus cereus *	*Aeromonas hydrophila*
Bacillus megaterium	*Agrobacterium radiobacter*
Bacillus mycoides *	*Bacillus cereus* *
Bacillus polymyxa	*Bacillus mycoides* *
Bacillus pumilus	*Enterobacter agglomerans 3*
Bacillus subtilis	*Enterobacter agglomerans 4* *
Enterobacter agglomerans 4 *	*Enterobacter sakazakii*
Flavobacterium oryzihabitans	*Klebsiella ozaenae*
Micrococcus luteus *	*Klebsiella pneumoniae*
Pseudomonas aureofaciens	*Micrococcus luteus* *
Pseudomonas cepacia *	*Pseudomonas aeruginosa*
Pseudomonas chlororaphis *	*Pseudomonas cepacia* *
Pseudomonas fluorescens *	*Pseudomonas chlororaphis* *
Pseudomonas putida *	*Pseudomonas fluorescens* *
Serratia fonticola	*Pseudomonas putida* *
Streptomyces spp. (9)	*Serratia liquefaciens*
	Serratia marcescens
	Serratia rubidaea
	Streptomyces spp. (2)

*Species common to the sporocarps and the ectomycorrhizae.

significant and continued in the third week. In six cases (*B. cereus* 6, *B. megaterium* 8, *B. megaterium* B6B, *B. polymixa* versus AB7 and *B. pumilus* versus AB1 and AB8), inhibition was significant throughout the experiment. Stimulation of growth was less frequent and time-dependent (*B. subtilis* and *B. cereus* versus AB7), except for *B. megaterium* 8 versus AB8. *M. luteus* significantly inhibited all five isolates (AB1, AB3 and AB7 from the first week, AB6 and AB8 from the second week).

E. agglomerans 4 49, *K. pneumoniae*, and *S. fonticola* mostly resulted in significant inhibition throughout the experiment, and *A. calcoaceticus* and *E. agglomerans 4* 92 inhibited all isolates tested. Inhibition of mycelial growth by *F. oryzihabitans* was rare and significant stimulation of isolates AB1 and AB8 was observed in the first week. The effects of *Pseudomonas* species varied from one

Table 2. Growth of five *S. grevillei* isolates (% inhibition or -% stimulation ± s.d.) when paired with Gram-positive bacteria.

Bacteria	Isolate AB1			Isolate AB3			Isolate AB6		
	Days 7	14	21	7	14	21	7	14	21
B. brevis	–15±8	6±3	–15±4*	9±4	–3±2	–18±4**	–22±9*	–13±9	–3±2
B. cereus 6	6±4	15±5**	17±3**	–3±1	1±0	–2±1	–22±9*	3±2	–6±3
B. cereus 7	4±3	6±4	1±1	–2±1	–7±3	–5±5	6±4	2±1	2±1
B. cereus 8	2±2	12±6*	3±4	–2±2	–5±2	–4±5	–2±1	–11±5*	–11±6*
B. megaterium 8	0±0	15±5*	13±3*	4±3	–3±4	–15±5*	–6±3	7±4	1±1
B. megater. B6B	–9±7	10±6	1±1	11±6	11±3*	1±1	0±0	–22±10**	–11±8
B. mycoides	5±2	13±6*	5±4	–9±4	–7±4	–2±1	–28±13*	–5±4	–7±4
B. polymyxa	5±3	29±8*	16±14	22±9	28±6**	22±11	–13±7	–6±4	–3±2
B. pumilus	21±8*	26±7**	18±4**	2±1	3±2	4±3	–14±6	2±1	0±0
B. subtilis	8±5	18±3**	14±5**	–3±2	4±1	7±5	6±5	–4±3	–3±2
B. luteus	29±11*	56±3**	53±13**	10±2**	71±3**	79±3**	0±0	42±7**	35±12**

Table 2. Continued.

Bacteria	Isolate AB7			Isolate AB8		
	Days 7	14	21	7	14	21
B. brevis	18±11	25±11*	32±10*	–23±8	4±3	9±4
B. cereus 6	23±3**	26±4**	27±3**	–20±12	–9±6	–5±2
B. cereus 7	0±1	–8±6*	–5±3	1±1	4±3	–4±2
B. cereus 8	–2±1	6±4	–8±2*	1±0	1±2	–9±4*
B. megaterium 8	31±5**	37±5**	38±7**	–26±13**	–25±5**	–30±4**
B. megat. B6B	29±11*	30±13*	38±19**	–11±9	24±10*	39±14**
B. mycoides	6±5	3±2	14±2**	4±4	2±1	–3±1
B. polymyxa	19±9*	17±9*	27±12*	3±2	–1±1	–2±1
B. pumilus	3±2	–4±3	1±1	16±12	16±2**	20±5**
B. subtilis	–11±6	–18±5**	–17±5**	–2±1	4±2	7±4
B. luteus	56±6**	66±11**	64±11**	8±6	32±7**	34±7**

Values followed by * or ** are significantly different from the controls at a probability level of 0.05 or 0.01, respectively (ANOVA, Tukey's test).

Table 3. Growth of five *S. grevillei* isolates (% inhibition or -% stimulation ± s.d.) when paired with Gram-negative bacteria.

Bacteria	Isolate AB1			Isolate AB3			Isolate AB6		
	Days 7	14	21	7	14	21	7	14	21
A. calcoaceticus	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
E. agglom. 4 49	25±0**	66±5**	62±5**	15±4	22±8*	26±5**	4±7	16±4**	10±4
E. agglom. 4 '92	25±0**	83±1**	77±0**	52±3	77±0**	83±0**	56±2**	7±1**	78±0**
F. oryzihabitans	–18±11*	6±6	7±0	–2±4	–1±1	–7±2	–5±3	–9±5	–1±1
K. pneumoniae	1±1	36±16	51±26*	2±5	12±3**	30±3**	0±0	32±4**	35±5**
P. putida 42	–18±15	–1±0	3±2	–19±8*	–25±4**	–26±5**	1±1	0±0	–3±3
P. aeruginosa	–12±12	9±5	18±3**	4±7	15±3**	28±3**	3±2	18±5*	22±5**
P. aureofaciens	–19±13	0±0	–6±6	25±14*	28±12*	26±12*	–13±9	9±6	2±8
P. cepacia 89	6±9	44±3**	47±1**	–9±2	–19±4**	–13±3*	–29±6	–20±6	–14±6
P. cepacia '92	–7±6	3±3	0±3	13±9	13±6*	11±7	–15±10	18±7*	23±13*
P. chloror. 162A	–10±3	1±4	15±4*	1±1	–2±4	5±4	–25±10	–13±6	–6±4
P. chloror. '92	–13±8	–5±3	–17±9*	7±4	10±6	0±0	–2±0	–5±4	3±4
P. fluores. 70	–22±18	–13±7	–6±2*	–8±1	–29±5**	–21±2**	–42±21*	–57±7**	–51±6**
P. fluores. '92	–2±1	15±10	18±2*	0±0	–6±3	5±0	–10±6	8±5	8±3
P. putida 167	–20±17	13±6*	22±2**	–8±2	0±0	19±2	10±5	25±8*	25±3**
S. fonticola	–10±3	23±4**	20±6**	47±10**	50±14**	40±14**	14±6	8±8	0±0

isolate to another. In the first week, significant stimulation was confined to isolate AB8. From the second week, inhibition dominated especially against AB7 and stimulation of AB8 decreased or even changed to inhibition (*P. cepacia* 89 and *P. chlororaphis* 162A, *P. fluorescens* 70). Some stimulatory effects not significant during the first week became significant in the second and third weeks (*P. fluorescens* 70 and *P. cepacia* 89 versus AB3). *P. cepacia* 89 significantly reduced the growth of AB1 and enhanced that of AB3. Moreover, different isolates of this bacterial species could significantly inhibit (*P. cepacia* 89) or stimulate (*P. cepacia* 92) the same fungal isolate (AB8).

Effects of Streptomyces isolates on in vitro growth of fungal isolates

The *Streptomyces* isolates were frequently responsible for 100% inhibition (Table 4). With the exception of isolate 01 which was always fungistatic, and

Table 3. Continued.

Bacteria	Isolate AB7			Isolate AB8		
	Days 7	14	21	7	14	21
A. calcoaceticus	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
E. agglom. 4 49	24±4**	35±1**	38±7**	–20±16	–11±5*	–5±4
E. agglom. 4 '92	66±0**	83±0**	88±0**	37±5**	72±1**	75±1**
F. oryzihabitans	–3±1	–4±2	–5±2*	–25±11**	–10±6*	–4±3
K. pneumoniae	–2±1	37±4**	30±9*	–32±12*	8±4	28±15*
P. putida 42	7±5	15±5*	19±6*	–51±17**	–42±5**	–35±4**
P. aeruginosa	15±11	41±5**	36±5**	–24±1*	5±3	10±2*
P. aureofaciens	13±8	20±14*	37±13**	–42±33	–14±9	–10±9
P. cepacia 89	–9±6	11±2*	–14±2*	–26±7*	17±4**	30±5**
P. cepacia '92	12±7	13±9	23±17	–28±16	–17±13	–21±12*
P. chloror. 162A	8±5	40±1*	36±2**	–18±10*	9±1*	14±3*
P. chloror. '92	8±6	22±13*	37±12**	–35±25	–12±8	–1±1
P. fluores. 70	–2±2	23±3**	9±3	–36±13*	2±2	10±2*
P. fluores.'92	1±3	31±2**	28±2**	–32±12*	–15±5**	–5±3
P. putida 167	13±10	36±2**	28±2**	–20±9*	6±4	19±6**
S. fonticola	12±3**	35±9**	46±15**	–2±1	24±10**	31±7**

Values followed by * or ** are significantly different from the controls at a probability level of 0.05 or 0.01, respectively (ANOVA, Tukey's test).

isolate F2, which was only fungicidal when paired with isolates AB6 and AB8, they inhibited the fungi tested.

Effects of bacterial supernatants on in vitro growth of fungal isolates

The effects of bacterial supernatants at 7 days are illustrated in Table 5. Stimulation of fungal growth by supernatants was never significant. Bacterial supernatants which showed significant inhibition confirmed results observed in dual culture with the exception of *B. mycoides* with AB3.

Table 4. Growth of five *S. grevillei* isolates (% inhibition or -% stimulation ± s.d.) when paired with *Streptomyces* isolates.

Strepto-myces	Isolate AB1 Days 7	14	21	Isolate AB3 7	14	21	Isolate AB6 7	14	21
F1	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
F1a	10±9	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
F1a1	100±0**	100±0**	100±0**	45±11**	70±5**	76±5**	100±0**	100±0**	100±0**
F2	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
F4	100±0**	100±0**	100±0**	41±8**	62±11**	62±11**	100±0**	100±0**	100±0**
G1	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
M1	100±0**	100±0**	100±0**	44±7**	63±15**	63±15**	100±0**	100±0**	100±0**
01	100±0**	4±2	15±5	2±2	32±6**	51±4**	27±12*	31±2**	45±3**
9	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**

Table 4. Continued.

Strepto-myces	Isolate AB7 Days 7	14	21	Isolate AB8 7	14	21
F1	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
F1a	43±5**	66±3**	73±2**	100±0**	62±6**	78±4**
F1a1	40±6**	63±5**	70±3**	100±0**	62±3**	78±14**
F2	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
F4	40±6**	60±2**	68±3**	100±0**	58±10**	77±3*
G1	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**
M1	40±6**	64±5**	71±4**	21±21	57±9**	77±3**
01	26±8**	39±8**	39±6**	60±4**	42±2**	44±4**
9	100±0**	100±0**	100±0**	100±0**	100±0**	100±0**

Values followed by * or ** are significantly different from the controls at a probability level of 0.05 or 0.01, respectively (ANOVA, Tukey's test).

Table 5. Effects of bacterial supernatants on *in vitro* growth of five S. *grevillei* isolates (% inhibition or -% stimulation ± s.d.) at seven days.

Bacteria	Isolates				
	AB1	AB3	AB6	AB7	AB8
A. calcoaceticus	2 ± 1	11 ± 8	12 ± 8	1 ± 0	8 ± 5
B. brevis	0 ± 0	0 ± 0	–14 ± 9	14 ± 7	25 ± 0**
B. cereus 6	12 ± 7	10 ± 4	10 ± 7	20 ± 5*	13 ± 7
B. cereus 8	–8 ± 4	1 ± 0	–12 ± 9	14 ± 9	–6 ± 4
B. megaterium 8	–2 ± 1	3 ± 2	3 ± 2	21 ± 6*	–9 ± 6
B. mycoides	15 ± 10	20 ± 5 *	10 ± 7	15 ± 1*	0 ± 0
E. agglomerans 492	10 ± 6	14 ± 3 *	10 ± 7	17 ± 4 *	2 ± 1
F. oryzihabitans	–2 ± 1	–7 ± 5	–12 ± 10	8 ± 6	–9 ± 8
M. luteus	15 ± 10	1 ± 1	17 ± 6 *	15 ± 1 *	1 ± 1
P. fluorescens '92	–4 ± 2	11 ± 6	7 ± 5	1 ± 1	–8 ± 5
P. fluorescens 70	0 ± 0	0 ± 0	–4 ± 3	6 ± 5	–8 ± 4
P. putida 42	0 ± 0	–3 ± 2	–12 ± 10	2 ± 1	–2 ± 2
Streptomyces O1	3 ± 1	–7 ± 5	7 ± 5	7 ± 5	0 ± 0
Streptomyces F1	10 ± 8	6 ± 3	23 ± 7 *	9 ± 6	10 ± 8

Values followed by * or ** are significantly different from the controls at a probability level of 0.05 or 0.01, respectively (ANOVA, Tukey's test).

Effects of bacterial volatile metabolites on in vitro growth of fungal isolates

The effects of volatile metabolites at 7 days are illustrated in Table 6. Growth enhancement dominated, but was seldom significant. Mycelial growth of isolate AB1 was significantly enhanced by *Streptomyces* 01 only, while that of isolate AB3 by 50% of the bacteria. Isolates AB6 and AB8 were never significantly stimulated, AB7 was significantly stimulated by *B. cereus* 8, *B. megaterium* 8 and *P. putida* 42 and *P. fluorescens* 70. *P. putida* 42 significantly stimulated AB3 as in the dual culture, whereas in three cases, significant stimulation replaced the significant inhibition observed in dual culture: *B. megaterium* 8 versus AB7; *Streptomyces* 01 versus AB1 and AB3. Moreover, by contrast with the dual culture, *Streptomyces* F1 and 01 were always stimulating. Only *A. calcoaceticus* significantly inhibited all five isolates.

Table 6. Effects of bacterial volatile metabolites on *in vitro* growth of five S. *grevillei* isolates (% inhibition or -% stimulation ± s.d.) at seven days.

Bacteria	Isolates				
	AB1	AB3	AB6	AB7	AB8
A. calcoaceticus	25 ± 4 *	100 ± 0 **	100 ± 0 **	100 ± 0 **	100 ± 0 **
B. brevis	–1 ± 0	8 ± 5	6 ± 3	–6 ± 4	–11 ± 8
B. cereus 6	–23 ± 13	–42 ± 33 *	–6 ± 4	–5 ± 3	–12 ± 9
B. cereus 8	0 ± 0	–4 ± 3	–1 ± 0	–34 ± 13 **	4 ± 3
B. megaterium 8	–2 ± 1	–40 ± 37 *	7 ± 3	–33 ± 14 **	0 ± 0
B. mycoides	–27 ± 9	–20 ± 1 *	–11 ± 9	–14 ± 5	–4 ± 3
E. agglomerans 492	–2 ± 2	–8 ± 5	–11 ± 8	–12 ± 5	0 ± 0
F. oryzihabitans	–23 ± 13	–30 ± 14 *	0 ± 0	–18 ± 5	–12 ± 10
M. luteus	4 ± 3	–15 ± 9	3 ± 0	7 ± 5	3 ± 1
P. fluorescens '92	–16 ± 10	4 ± 3	–6 ± 5	–21 ± 8 *	0 ± 0
P. fluorescens 70	–23 ± 13	–30 ± 14 *	0 ± 0	–14 ± 5	1 ± 0
P. putida 42	–33 ± 10	–36 ± 14 *	–25 ± 19	–32 ± 12 **	–16 ± 11
Streptomyces O1	–12 ± 8	–2 ± 1	–6 ± 5	–12 ± 11	–13 ± 11
Streptomyces F1	–52 ± 19 **	–22 ± 8 **	–11 ± 7	–9 ± 7	–16 ± 9

Values followed by * or ** are significantly different from the controls at a probability level of 0.05 or 0.01, respectively (ANOVA, Tukey's test).

Siderophore production by bacteria

Most bacteria produced siderophores on CAS medium with the exception of *M. luteus, S. fonticola, Streptomyces* G1 (Table 7). *A. calcoaceticus* and *P. fluorescens* 92 were the best producers.

4. Discussion

Twenty seven bacterial species were isolated from both the sporocarps of *S. grevillei* and the ectomycorrhizae of *S. grevillei x L. decidua* (Table 1). As in other ectomycorrhizal associations, the genera *Pseudomonas, Bacillus* and *Streptomyces* were predominant (Bowen and Theodorou, 1979; Ali and Jackson, 1989; Duponnois and Garbaye, 1990, 1991; Danell et al., 1993). Some species were

Table 7. Siderophores production by bacteria on CAS medium.

Bacteria	Siderophores*
Acinetobacter calcoaceticus	+++
Bacillus brevis	+
Bacillus cereus	+
Bacillus megaterium	++
Bacillus mycoides	+
Bacillus polymyxa	+
Bacillus pumilus	+
Bacillus subtilis	++
Enterobacter agglomerans 4	+
Flavobacterium oryzihabitans	++
Klebsiella pneumoniae	++
Micrococcus luteus	–
Pseudomonas aeruginosa	++
Pseudomonas aureofaciens	++
Pseudomonas cepacia 89	++
Pseudomonas chlororaphis 162A	++
Pseudomonas fluorescens 70	++
Pseudomonas fluorescens '92	+++
Pseudomonas putida 42	++
Serratia fonticola	–
Streptomyces 9	++
Streptomyces F1	++
Streptomyces F1a	+
Streptomyces F1a1	++
Streptomyces F2	++
Streptomyces F4	+
Streptomyces G1	–
Streptomyces M1	++
Streptomyces O1	++

* +++: width of the halo of discolouration > 20 mm; ++: width of the halo of discolouration between 10 mm and 20 mm; +: width of the halo of discolouration < 10 mm; – : no halo.

common to both the sporocarp and the mycorrhizae as already reported for *Laccaria laccata-Pseudotsuga menziesii* symbiosis (Garbaye and Duponnois, 1992).

S. grevillei is a highly specific ectomycorrhizal symbiont for *Larix* spp. It has been shown to form *in vitro* mycorrhizae with *Larix kaempferi*, *Pinus silvestris* and *Pseudotsuga menziesii* although ultrastructural changes in the host-fungus interface of associations with the latter two hosts suggest that in both cases the symbionts were not entirely compatible (Duddridge, 1986). Due to this narrow host range, bacterial populations associated with the *S. grevillei-L. decidua* model may be particularly important in the establishment of the symbiosis.

In this study, the effects on the growth of five mycelial isolates ranged from highly significant growth enhancement to highly significant inhibition depending on the bacterial groups (Gram-positive, Gram-negative, *Streptomyces*) and the fungal isolate. A similar variability was observed (Richter et al., 1989) in the effects of *Streptomyces* strains isolated from *Pinus resinosa* mycorrhizae on the *in vitro* growth of *L. laccata, L. bicolor* and *Thelephora terrestris* , and for several bacteria (mainly *Pseudomonas*) from the sporocarps of *Cantharellus cibarius* on its growth *in vitro.* (Danell, 1993). It has also been reported that bacteria isolated from the sporocarps and ectomycorrhizae of *L. laccata* have very different and temporally variable effects on the establishment of the *L. laccata-P. menziesii* symbiosis (Garbaye et al., 1990).

Several behavior patterns emerged from the present study. Gram-positive bacteria seldom stimulated growth (Table 2). Their highly significant inhibition, particularly after the first 7 days, was probably due to the formation of secondary metabolites during sporulation. Microbial stain during the experiment confirmed that all the *Bacillus* species were sporulated after the first week. *Bacillus* spp., in fact, produce a great number of polypeptides with antibiotic activity when grown in a rich medium.

The effects of Gram-negative bacteria varied depending on the genera. *Pseudomonas* behavior was quite irregular but *P. fluorescens* 70 and *P. putida* 42 have some claim to be regarded as stimulators of the growth of some fungal isolates (Table 3). With the exception of *F. oryzihabitans*, the other Gram-negative bacteria inhibited fungal growth. All nine *Streptomyces* inhibited each of the five isolates (Table 4).

The effects of the bacterial supernatants generally mirrored those observed in the dual cultures, whereas those of the volatile metabolites were usually the opposite, this being particularly evident in the switch to general (and occasionally significant) stimulation induced by *Streptomyces* F1 and *Streptomyces* 01 metabolites (Table 6). Stimulation of AM fungi by *Streptomyces* volatile metabolites, and of *L. laccata* by those produced by *Bacillus* and *Pseudomonas* has been reported (Mugnier and Mosse, 1987; Garbaye and Duponnois, 1992). It is well known that volatile and non-volatile metabolites produced by soil microrganisms could have opposite effects on the growth of fungi (Paulitz and

Linderman, 1991). In addition, non-volatile metabolites (antibiotics, siderophores) generally have stronger activity.

Some of the bacterial species we have identified, have been shown, in other systems, to stimulate the growth of an ectomycorrhizal fungus by both producing amino acids, vitamins, growth factors and organic acids (Azcon-Aguilar and Barea, 1992; Duponnois and Garbaye, 1990, 1991; Garbaye, 1994) or by removing fungal catabolites such as polyphenols (Duponnois and Garbaye, 1990), and to inhibit fungal growth with production of toxic metabolites or competition for nutrients. The harmful effects of bacterial metabolites on the germination of propagules, mycelial growth and establishment of a mycorrhiza, in fact, have been observed by many researchers (Bowen and Theodorou, 1979; Garbaye and Bowen, 1987; Paulitz and Linderman, 1991). Competition for nutrients seems an improbable explanation in the present study, since the relatively rich MMN medium was employed. Production of antibiotics and toxins is likely to explain the inhibiting effects of some bacterial species. It is interesting to note, that siderophores were produced by 26 out of 29 strains (Table 7). These molecules are known to be both bacterio- and fungistatic, and they are currently regarded as responsible for the biocontrol of many phytopathogens by plant-growth promoting rhizobacteria (PGPR) (Neilands and Leong, 1986; Schippers et al., 1987; Schwyn and Neilands, 1987; Défago et al., 1990; Jayaswal et al., 1990; Hamdan et al., 1991; Lookwood, 1992; Lemanceau et al., 1993;). They may also stimulate the host plant directly (Klepper et al., 1980; De Weger et al., 1988) or by promoting absorption of iron by the mycorrhizal fungus. Since the nutritional balance of the host plant is an essential factor in the establishment of a mycorrhiza (Nylund, 1988; Wallander and Nylund, 1991), bacteria associated with it, through mobilization of mineral ions or competition for their absorption, can play a major role in this regulation. Therefore, bacteria expressing this character *in vitro* could have some chance to be the right species to be used *in vivo* also.

The use of mycorrhizae to improve the production of forest trees and other plants is the subject of many ongoing research programs worldwide. Many data are available about the positive influence on plants by nitrogen-fixing bacteria (*Rhizobium* and *Azospirillum*), phosphate-solubilizing bacteria or PGPR (Azcon-Aguilar and Barea, 1992) nevertheless, most of them refer to herbaceous plants and interactions with AM fungi. Investigation, in the present study, on the bacteria associated with the sporocarps and ectomycorrhizae of *S. grevillei* and their effect on the fungus offers a better understanding of the part played by individual species in the highly specific association it forms with *L. decidua*. Identification of bacteria that greatly stimulate the *in vitro* growth of *S. grevillei* isolates also open the way to the selection of both bacterial and fungal strains to promote the establishment of this association. Helper bacteria, in fact, display

both plant growth response specificity (Chanway et al., 1991) and fungal specificity (Garbaye and Duponnois, 1992). The fact that some bacterial strains (*P. cepacia* 89) can have opposite significant effects on different strains of the same fungal species suggests a very high fungus selectivity at the infraspecific level. This specificity may be adaptive as the outcome of a previous natural coexistence or the result of metabolic compatibility not dependent on such coexistence (Chanway et al., 1988; Chanway et al., 1989).

The simplified and artificial conditions in this study, of course, are very different from those in the natural rhizosphere, where the roots provide a great variety of complex substrates and soil colloids absorb some of the substances released by bacteria and fungi. The test used for assessing the effect of bacteria on the fungal growth prevents direct contact of the bacteria on the hyphae, therefore, diffusible or volatile substances were only analyzed. Garbaye and Duponnois, (1992) did not find different results by using direct liquid contact or no liquid contact for the *in vitro* study of the effects of MHB, isolated from sporocarps or mycorrhizae of *Laccaria laccata* associated with Douglas fir, on the mycelial growth of different fungal strains. On the contrary, Sen et al. (1994) have hypothesized that efficient attachment and subsequent colonization of the fungal surfaces of some ectomycorrhizae by MHB would be an important step for exertion of either positive or negative effect on mycorrhization.

The conclusion to be drawn from these results, when extending the experiments *in vivo,* is that close attention should be directed not only to the assessment of beneficial rhizobacteria for the *S. grevillei-L. laccata* symbiosis but also to the fungal isolate that selectively respond to stimulation by the selected bacteria.

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