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Specificity and Function of Mycorrhization Helper Bacteria (MHB) Associated with the *Pseudotsuga menziesii* — *Laccaria laccata* Symbiosis

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Abstract

Mycorrhization helper bacteria (MHB) associated with the Douglas fir – *Laccaria laccata* ectomycorrhizal symbiosis are found to be fungus-specific but not plant-specific. The most efficient strains of MHB were isolated from *L. laccata* sporocarps as well as from the mantle of its ectomycorrhizas. It is still unclear how MHB take benefit from living closely associated with the fungus, but a major mechanisms involved in mycorrhizal stimulation by MHB and in its fungus-specificity has been elucidated: direct stimulation of fungal growth by releasing volatile substances. Other hypotheses such as the production of growth regulators or pectinolytic enzymes are presently being investigated. The fact that MHB are fungus-specific may be of great significance in terms of competitive ability of different ectomycorrhizal fungi for colonizing a root.

Keywords: ectomycorrhizas, bacteria, helpers, *Pseudotsuga menziesii*, *Laccaria laccata*

1. Introduction

The occurrence of mycorrhization helper bacteria (MHB) associated with ectomycorrhizas of forest trees and stimulating the establishment of the symbiosis have been first demonstrated in the *Pinus radiata* D. Don.-*Rhizopogon*

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luteolus Fr. and Nordh symbiosis (Garbaye and Bowen, 1989) and more recently with Douglas fir and *Laccaria laccata* Scop. ex Fr. (Duponnois and Garbaye, 1991). This paper reports experimental results obtained with the latter symbiotic system, with two purposes: studying the specificity of MHB and understanding the mechanisms involved in the MHB effect.

2. Materials and Methods

Plant and microbial material

The seeds of Douglas fir (*Pseudotsuga menziesii* Mirb. Franco) were from the Pacific Northwest of USA. When used in aseptic *in vitro* experiments, they were surface-sterilized in hydrogen peroxide (Duponnois and Garbaye, 1991). Seeds of Norway spruce (*Picea abies* L. Karsten) and Scotch pine (*Pinus sylvestris* L.) were from the Vosges range in North-Eastern France, and those of Austrian pine (*Pinus nigra* Arnold, var. *austriaca*) from Austria.

The main ectomycorrhizal fungus used in all experiments was strain S238 of *Laccaria laccata* isolated by R. Molina from a sporocarp collected under *Tsuga mertensiana* in Oregon (USA). The other fungal strains listed in Tables 1 to 6 were from different geographical regions and from various host-plants, and are kept in the first author's laboratory. Fungal inoculum was prepared either as mycelial suspensions in sterile water when used in aseptic *in vitro* experiments, or by aseptically growing the mycelium in a vermiculite-peat mix moistened with a nutrient medium in the case of glasshouse and nursery experiments (Duponnois and Garbaye, 1991).

Four MHB strains were used: MB3 (*Bacillus subtilis*), SHB1 (*Bacillus* sp.), BBc6 (*Pseudomonas fluorescens*) and SBc5 (*Pseudomonas* sp.). They had been isolated from the Douglas fir-*Laccaria laccata* S238 symbiotic system in controlled inoculation experiments in France (Le Tacon et al., 1988), either from sporocarps (BBc6 and SBc5) or from the ectomycorrhizal mantel (MB3 and SHB1). They were grown in TSB medium (Tryptic Soy Broth, DIFCO), centrifuged, and resuspended in 0.1 M $MgSO_4$. This suspension was used as an inoculum.

Mycorrhizal synthesis experiments in the nursery

Douglas fir seeds were sown in spring, after soil fumigation with methyl bromide, in a forest nursery in the French Massif Central (sandy loam, pH 5.6, 8.9% organic matter, 0.52 g kg^{-1} available P determined by the Duchaufour and Bonneau method, 1969). The nursery bed was divided into 0.5 m^2 plots separated from each other by 50 cm unsown zones. One liter fungal inoculum

was mixed into the soil of each plot. Each plot received 2.5 L of bacterial suspension containing about 10^{12} cfu (or no bacteria in the control). Treatments were randomly distributed in 3 blocks. Ten plants per plot were lifted after 4 months; the root systems were washed, cut into pieces, mixed, and percent of ectomycorrhizal short roots was determined on a random sample of at least 100 short roots.

Glasshouse experiments

Douglas fir seedlings were grown in 95 ml containers filled with non-disinfected peat-vermiculite (1:1; v:v) mixed with 10% (v:v) fungal inoculum. Five ml bacterial suspension were injected in each container with a syringe immediately before sowing: the control received the solution of magnesium sulfate alone. The number of living bacterial cells per container ranged from 10^5 (MB3) to 10^9 (BBc6). One plant was grown per cell. After 5 weeks, a nutrient solution (14.8 mg l^{-1} N from nitrate and 2 mg l^{-1}P), which was previously experimentally determined as favourable to mycorrhizal development under these conditions, was applied in excess twice a week in addition to daily watering. Treatments were randomly repeated in 4 blocks, with plots of 20 seedlings as individual treatments. Four months after sowing, 10 plants were systematically sampled in each plot. The percent of mycorrhizal short roots was determined as above. For both types of experiments (nursery and glasshouse), data were analysed by two-way analysis of variance (block and treatment), and treatments were compared by lsd.

Experiments in aseptic conditions

Glass test tubes (3×15 cm) were filled to 2 cm from the top with a sphagnum peat-vermiculite mixture (1:1; v:v) moistened with a mineral nutrient solution: $MgSO_4$, $7H_2O$: 150 mg; $(NH_4)_2HPO_4$: 125 mg; $(NH_4)_2SO_4$: 125 mg; $CaCl_2$, $2H_2O$: 50 mg; KCl: 108 mg; Kanieltra (COFAZ, Paris): 0.1 ml; Distilled water: 1 l. They were covered with aluminium foil and autoclaved (120°C, 20 min). After cooling, the tubes were aseptically filled to the top with vermiculite-peat fungal inoculum prepared as above. One milliliter bacterial suspension in $MgSO_4$ 0.1 M solution was aseptically injected per tube, 2 cm-deep, with a syringe; tubes in the control treatment received 1 ml of the $MgSO_4$ 0.1 M solution without bacteria. Concentrations of the bacterial suspensions were 2×10^7 c.f.u. ml^{-1} for MB3, 2×10^8 for SHB1 and SBc5, 1×10^9 for BBc6. A hole was then made in the aluminium foil, and the rootlet (1 cm long) of an aseptically germinated Douglas fir seed was introduced. The sterility of

the root system was ensured by an autoclaved coachwork putty (Terosta 2, TEROSON S.A., F 92607 Asnières) which was stuck on the aluminium foil and around the root. The tubes (12 replicates for each fungus-bacterium treatment) were settled in a growth chamber (23°C day, 17°C night, 16 hr photoperiod with 240 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Mazda MAIH 400 lamps), 80% relative humidity). The determination of the percent of mycorrhizal short roots was done after 1 month (3 months for *Cenococcum geophilum*).

Effect of bacteria on fungal growth

Fungi were grown in Petri dishes on Pachlewski agar medium at 25°C for 2–3 weeks. Agar plugs (diameter: 6 mm; 4 mm thick) were taken from the margin of the colonies. Bacteria were grown in Petri dishes on 0.3% TSB agar medium at 25°C for 2 days. Five ml of sterile water were poured on the bacterial culture and mixed with a bend glass rod in order to obtain a homogenous bacterial suspension. A control solution was prepared the same way from a dish containing the same agar medium without bacteria. One set of experiments was done by direct liquid contact between the mycelium and the bacteria; fungal plugs were dipped for 1–2 min in the bacterial suspensions or in the control solution and transferred into empty Petri dishes (Duponnois and Garbaye, 1990). Another set of experiments was done with no liquid contact, using two-compartment dishes: one compartment contained the fungal plugs, laid on the dry bottom of the dish as before, while the other compartment contained TSA medium with the bacteria (or no bacteria in the control). The wall separating the two compartments did not touch the lid of the dish, permitting gas diffusion from one side to the other. In both experiment types, all the operations were done aseptically in a laminary flow cabinet, and the dishes were sealed with tape to prevent drying during incubation. Two dishes, each one with 5 mycelial plugs, were prepared for each treatment. Measurements were made through the lid with a stereomicroscope fitted with an ocular scale after 8 day incubation at 25°C. The mean radial growth in two perpendicular directions was calculated.

3. Results

The four bacterial isolates enhanced mycorrhiza formation by *Laccaria laccata* S238 with four different host-plants in a glasshouse experiment: Douglas fir, Scotch pine, Austrian pine and Norway spruce (Table 1).

Table 2 shows that, with Douglas fir in the nursery, the two bacterial isolates BBc6 and Sbc5 stimulated mycorrhiza formation by *Laccaria laccata* S238 and

Table 1. Effect of the four bacterial strains on mycorrhiza formation (per cent of mycorrhizal short roots) by *Laccaria laccata* S238 on four species of conifers. Values followed by a sign are significantly higher (+) or lower (–) than the one corresponding to the control treatment in the same line of the table (0.05 probability level); percents of mycorrhizal short roots were transformed by arcsin square root prior to test of significance.

Host plants	Bacterial isolates				
	None	MB3	SHBI	BBc6	SBc5
Pseudotsuga menziesii	61.1	86.9+	75.9+	90.7+	90.3+
Picea abies	61.9	77.7+	79.6+	85.1+	83.3+
Pinus nigra var. *austriaca*	40.7	81.4+	86.9	70.3+	90.0+
Pinus sylvestris	47.2	74.0+	70.3+	81.4+	70.3+

Table 2. Effect of two bacterial isolates on mycorrhiza formation on Douglas fir (per cent of mycorrhizal short roots) in a bare-root forest nursery. Values followed by a sign are significantly higher (+) or lower (–) than the one corresponding to the control treatment in the same line of the table (0.05 probability level); percents of mycorrhizal short roots were transformed by arcsin square root prior to test of significance.

Fungal isolates	Bacterial isolates		
	None	BBc6	SBc5
Laccaria laccata S238	75.2	93.6+	91.2+
Laccaria proxima	38.7	43.4	33.4
Laccaria bicolor D-101	55.9	89.8+	89.7+
Hebeloma cylindrosporum	89.6	57.8–	61.8–
Paxillus involutus	35.1	14.9	9.1

by the strain of *L. bicolor*; they had no effect on *L. proxima*, and reduced symbiosis establishment by the two fungal isolates belonging to the other genera.

The two glasshouse experiments (Table 3) gave similar results; the four MHB isolated from the Douglas fir-*Laccaria laccata* S238 system helped mycorrhiza formation by *Laccaria laccata* and *L. bicolor* but had a negative effect on *Thelephora terrestris*.

The experiments under axenic conditions (Table 4) confirmed the fungus-selectivity of the four MHB: their effect was positive with *Laccaria laccata* S238 and negative with three non-related fungal species. The reduction of mycorrhizal infection with two of the latter (*Hebeloma cylindrosporum* and *Paxillus involutus*) was linked with a reduction of the colonization of long root surface by the mycelium.

Table 5 shows that MHB also selectively interact with the mycelial growth

Table 3. Effect of the four bacterial isolates on mycorrhiza formation (per cent of mycorrhizal short roots) of Douglas fir in the glasshouse. Values followed by a sign are significantly higher (+) or lower (−) than the one corresponding to the control treatment in the same line of the table (0.05 probability level); percents of mycorrhizal short roots were transformed by arcsin square root prior to test of significance.

Fungal isolates	Bacterial isolates				
	None	MB3	SHB1	BBc6	SBc5
First experiment					
Laccaria laccata S-238	56.9	94.5+	93.0+	89.6+	86.2+
Laccaria bicolor 993	78.6	93.3+	92.8+	97.9+	93.9+
Laccaria bicolor S-3	5.2	30.4+	36.2+	19.6	20.8
Laccaria bicolor A4B3xA1B2	21.7	47.8+	31.4	45.7	47.5+
Second experiment					
Laccaria laccata S-238	32.4	67.5+	60.2+	62.5+	68.5+
Thelephora terrestris	18.0	5.2−	5.1−	4.2−	3.8−

Table 4. Effect of the four bacterial isolates on mycorrhiza formation (per cent of mycorrhizal short roots) and on the length of the tap root surface colonized by the mycelium (cm) of Douglas fir in aseptic conditions. Values followed by a sign are significantly higher (+) or lower (−) than the one corresponding to the control treatment in the same line of the table (0.05 probability level); percents of mycorrhizal short roots were transformed by arcsin square root prior to test of significance.

Measured parameter and fungal isolates	Bacterial isolates				
	None	MB3	SHB1	BBc6	SBc5
% mycorrhizal roots					
Laccaria laccata S-238	12.0	28.5+	37.8+	30.5+	24.0
Hebeloma cylindrosporum	96.9	66.4−	63.0	57.4−	79.5−
Paxillus involutus QBC	40.7	13.1−	6.6−	0.0−	0.0−
Cenococcum geophilum	44.3	2.8−	4.0−	5.4−	0.0−
Colonized root length					
Hebeloma cylindrosporum	6.24	2.9−	5.1−	4.1−	3.0−
Paxillus involutus QBC	4.5	2.9−	1.1−	0.0−	3.5−

Table 5. Effect of the four isolates on the mycelial growth of different fungal strains (mean radial growth of colonies in mm) with liquid contact. Values followed by a sign are significantly higher (+) or lower (−) than the one corresponding to the control treatment in the same line of the table (0.05 probability level).

Fungal isolates	Bacterial isolates				
	None	MB3	SHB1	BBc6	SBc5
Laccaria laccata S-238	0.50	0.65+	0.80+	0.67+	0.60+
Hebeloma cylindrosporum	3.05	1.57−	0.80−	1.52−	0.20−
Paxillus involutus NAU	4.45	0.00−	0.00−	0.00−	0.00−
Paxillus involutus QBC	4.00	1.10−	1.15−	0.57−	0.80−
Cenococcum geophilum	0.86	0.17−	0.06−	0.02−	0.13−
Thelephora terrestris	2.15	1.20−	1.35−	1.37−	1.25−

of fungal strains in pure culture: while *Laccaria laccata* S238 is stimulated, the growth of other species is systematically reduced in the presence of the bacteria. Moreover, these stimulative or depressive effects are reproduced and extended to a wider range of fungal strains when liquid contact between the fungus and the bacterium is prevented in the two-compartment Petri dish system (Table 6). Under these conditions, where gases only could diffuse from one organism to the other, volatile substances were responsible for the interaction.

A significant correlation (R=0.9 with 20 pairs of data pooled from five experiments in the nursery and in the glasshouse) was found between the volatile-mediated effect of a bacterium on a fungus and the effect of the same bacterium on mycorrhizal infection of Douglas fir by the same fungus (results expressed as per cent of the control treatment with no bacterium and transformed by arcsin square root).

4. Discussion

The four bacteria used in this work had been isolated from ectomycorrhizas and sporocarps of *Laccaria laccata* S238 (isolated in North America and transferred in France as a pure mycelial culture) which had established symbiosis with Douglas fir in French nursery and plantation soils. Therefore, these bacterial isolates did not evolve together with the American fungal strain, but were already present in French soils and were adapted to live in close association with *L. laccata* and *L. bicolor* which are common, non host-specific, ectomycorrhizal species in the French sites where bacterial isolations were done.

Indeed, the four bacterial isolates consistently acted as mycorrhization helpers (MHB) under a wide range of environmental and host-plant conditions, with *L. laccata* S238 as well as with other strains of *L. laccata* and *L. bicolor*,

Table 6. Effect of the four bacterial isolates on the mycelial growth (mean radial growth of colonies in mm) of different fungal strains with no liquid contact after 8 day incubation at 25°C. Values followed by a sign are significantly higher (+) or lower (–) than the one corresponding to the control treatment in the same line of the table (unpaired Student's "t" test with 10 replicates, 0.05 probability level).

Fungal isolates	Bacterial isolates				
	None	MB3	SHB1	BBc6	SBc5
Laccaria laccata S-238	1.15	1.75+	1.70+	1.95+	1.75+
Laccaria laccata S-1023	0.85	1.50+	1.95+	1.85+	1.40+
Laccaria laccata CHAM3	1.65	2.35+	2.35+	1.95+	
Laccaria laccata 003	0.70	1.55+	1.45+	1.70+	1.55+
Laccaria laccata 83222	1.15	1.85+	1.85+	1.37	1.46+
Laccaria laccata S-106	1.85	1.70	1.25	1.65	1.25
Laccaria laccata 152x123	1.62	2.90+	2.80+	2.45+	
Laccaria proxima	2.40	2.40	2.45	2.45	2.10–
Laccaria bicolor CRBF569	1.05	1.45+	1.60+	1.65+	1.50+
Laccaria bicolor CRBF591	4.05	7.05+	7.20+	6.40+	5.90+
Laccaria bicolor A4B3xA1B2	4.55	5.20	6.60+	6.60+	5.60+
Laccaria bicolor CRBF347	3.80	4.00	3.70	4.70+	4.40+
Laccaria bicolor CRBF348	2.40	2.80	2.60	2.70	2.45
Laccaria bicolor 83-216	0.60	1.85+	1.20+	1.40+	1.10+
Laccaria bicolor D-101	1.00	2.10+	1.90+	1.95+	1.90+
Laccaria bicolor 81-306	1.15	2.00+	1.95+	1.90+	1.75+
Laccaria bicolor 993	1.65	2.05+	2.00	2.50+	2.05+
Laccaria bicolor MUEL	2.75	3.05	3.30+	3.25+	3.00
Hebeloma cylindrosporum	4.50	4.10–	3.87–	3.75–	3.60–
Paxillus involutus QBC	1.75	0.95–	1.20–	1.50–	
Cenococcum geophilum	1.50	1.30	1.40–	1.10–	1.30–
Thelephora terrestris	3.70	2.65–	2.85–	2.55–	2.15–
Suillus bovinus	2.75	2.20–	1.35–	2.15–	2.20–
Pisolithus tinctorius	2.25	1.25–	1.70–	2.05	1.70–

while they have no effect on *L. proxima* and reduce mycorrhiza formation by other fungal genera. The specificity of MHB is thus demonstrated, at least within the Douglas fir – *Laccaria laccata/bicolor* system. In this respect, it is worth remarking that these two fungal species are taxonomically very closely related, and that isolate S238, initially called *laccata*, seems to be closer to *bicolor* according to recent investigations using rDNA polymorphism (Martin et al., 1991).

The ability of the bacteria to stimulate or inhibit mycelial growth by undetermined volatile substances is correlated to their promoting or reducing effect on mycorrhiza formation by the same fungi. Therefore, MHB mainly act by controlling the extention of the fungus in the rhizospheric soil and on the rhizoplan before mycorrhiza formation, and gas-mediated interactions are a major mechanism involved in the fungus-specificity of the phenomenon. But this does not preclude the possible occurrence of other mechanisms which are presently under investigation, such as interference with recognition or improvement of the receptivity of short roots to the fungal penetration (the bacterium producing growth regulators, pectinolytic enzymes, and modifying the pH of the rhizosphere).

The fungus-specificity means that MHB are an important factor controlling the fitness of different ectomycorrhizal fungi competing for infecting a root, forming together a three-partner association.

The results presented here also show that MHB are beneficial to the fungus, but whether or not the benefit is reciprocal remains to be found.

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