Plant and Soil **157**: 257–262, 1993.

PLSO 3127

The fungus-specificity of mycorrhization helper bacteria (MHBs) used as an alternative to soil fumigation for ectomycorrhizal inoculation of bare-root Douglas-fir planting stocks with *Laccaria laccata*

R. DUPONNOIS, J. GARBAYE[1], D. BOUCHARD and J.L. CHURIN
Centre de Recherches Forestières de Nancy, F-54280 Champenoux, France; [1]*Corresponding author*

Received 23 March 1993. Accepted in revised form 9 August 1993

Key words: bacteria, ectomycorrhizas, *Hebeloma cylindrosporum*, *Laccaria bicolor*, *Laccaria laccata*, nursery, *Paxillus involutus*, planting stocks, *Pseudomonas fluorescens*, *Pseudotsuga menziesii*, soil fumigation

Abstract

Mycorrhization helper bacteria (MHBs) isolated and selected from the Douglas fir-*Laccaria laccata* symbiotic system have previously been shown to be fungus-specific: they promote ectomycorrhizal establishment of *Laccaria laccata* but inhibit mycorrhiza formation by other fungi. In this paper, two experiments in a nursery producing two years-old bare-root Douglas-fir planting stocks confirm the specificity of MHBs under field conditions. They also show that, by selectively helping the introduced *L. laccata* against the resident symbionts, MHBs are an interesting alternative (safer and easier) to soil fumigation for the success of routine controlled mycorrhization of planting stocks in forest nurseries.

Introduction

Mycorrhization helper bacteria (MHBs) associated with mycorrhizas and sporocarps of the ectomycorrhizal basidiomycete *Laccaria laccata* have been shown to enhance the growth of this fungus and to promote its mycorrhiza formation with different host-plants in a wide range of conditions, from axenic in vitro experiments to large-scale nursery trials (Duponnois and Garbaye, 1991a, 1991b, 1992; Garbaye et al., 1992).

Another property of these MHBs is their fungus-specificity: they enhance mycorrhiza formation by *L. laccata* and some related *Laccaria* species but inhibit the symbiosis establishment by other fungi (Garbaye and Duponnois, 1992).

From a practical point of view, Le Tacon et al. (1988) demonstrated the growth-efficiency of *L. laccata* in conifer plantations. Therefore, the nursery production of Douglas-fir planting stocks mycorrhizal with *L. laccata* is an important challenge, and a commercial mycorrhizal inoculum is being developed in France.

The classical ectomycorrhizal inoculation techniques in bare-root forest nurseries rely on soil disinfection (usually with methyl bromide fumigation) in order to get rid of the resident symbiotic competitors before introducing the desirable fungal strain (Le Tacon and Bouchard, 1986; Marx, 1975).

Because of their selectivity, MHBs might be an interesting, cheaper and safer alternative to soil fumigation. The aim of the present work was to test the feasibility of this approach, focusing on three questions: (i) Is the fungus-specificity of MHBs strong enough to be expressed in complex nursery conditions? (ii) Does it operate in a non-disinfected soil? (iii) Can MHBs be as efficient as soil disinfection for optimizing the effect of the fungal inoculum? In order to answer these questions, two experiments were carried out with dual inoculation (fungus + bacterium) in

a large nursery representative of the French conditions for producing two years-old bare-root Douglas-fir planting stocks.

Material and methods

Seedlings

Seeds of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) from provenance zone 412 (Washington State, USA) were pretreated in moist sphagnum peat for 5 weeks at 4 °C before sowing (1200 seeds per m^2) and covered with fumigated (experiment 1) or non-fumigated soil (experiment 2).

Fungi

The ectomycorrhizal basidiomycetes *Laccaria laccata* (scop. ex Fr.) Cke., isolate S-238 from USDA, Corvallis (Oregon), *Laccaria bicolar* (Maire) Orton, isolate D 101, *Hebeloma cylindrosporum* (Romagnesi), isolate D15 and *Paxillus involutus* (Fr.) Fr., isolate QBC, were maintained on Pachlewski agar medium (Pachlewski and Pachlewska, 1974). The solid inoculum was prepared in 1.6 liter glass jars containing 1.3 liter vermiculite-peat mixture (4/1, v/v) moistened to water-holding capacity with liquid Pachlewski medium, inoculated with plugs from an agar culture and incubated for 6 weeks at 25 °C.

Bacteria

The nine strains of MHBs (mycorrhization helper bacteria) used for this work were isolated from the *Laccaria laccata*-Douglas fir symbiotic system and were described and selected by Duponnois and Garbaye (1991b). Four of them (MB3, SHB1, BBc6 and SBc5) are covered by the patent 521758, EP and deposited in the Collecton Nationale de Cultures de Microorganismes of the Institut Pasteur in Paris. All bacterial strains were grown in shaken glass flasks containing liquid TSB medium (3 g L^{-1} Trypsic Soy Broth, Difco) for 8 days at 25 °C. The concentrated bacterial suspensions (more than 10^{10} cells mL^{-1}) were centrifuged (2400*g*, 10 min), the supernatant was discarded, and the pellet was resuspended in 100 mL 0.1*M* $MgSO_4$.

Nursery

The two experiments were performed in the nursery of Peyrat-le-Château (Haute-Vienne, France) which produces bare-root planting stocks of conifers. The soil is brown podzolic, developed on granite. The upper horizon has been modified by 20-year cultivation of planting stocks; its present physicochemical characteristics are as follow: texture: sandy loam; organic matter: 8.9%; C/N: 12.5; pH (water): 5.6; exchangeable cations (meq per 100 g) Ca: 3.1, K: 0.67, Mg: 0.54; available P (Duchaufour and Bonneau, 1969): 0.52. In experiment 1, the soil was fumigated with cold methyl bromide (75 g m^{-2}, soil covered with clear polythene film for 4 days) three weeks before inoculation and sowing. In experiment 2, the soil was not fumigated.

Experimental design

The aim of this work was twofold: verify the fungus-selectivity of the MHBs in nursery conditions and test the effect of soil disinfection. Unfortunately, previous experience had shown that it was impossible to randomize the disinfection factor as small plots in a single experimental design because of the fast recontamination from one plot to another. Therefore, two experiments were set the same day in two different nursery beds, not adjacent but located in the same section of the nursery, and a completely randomized block design was used for each one. The experiment 1, in fumigated soil, had 15 treatments and 3 blocks; the treatments were the 15 combinations of a fungal factor with 5 level (no fungal inoculation, *Laccaria laccata*, *Laccaria bicolor*, *Hebeloma cylindrosporum*, *Paxillus involutus*) and of a bacterial factor with 3 levels (no bacterial inoculation, MHB SBc5, MHB BBc6). The experiment 2, in non-fumigated soil, had 11 treatments and 4 blocks; the treatments were a non-inoculated control and *Laccaria laccata* inoculated alone or together with one of 9 MHBs: MB3, BBc6, SBc5, SHB1, MB2, SBc4, SBc1, BBc7, BBc1 (Duponnois and Garbaye, 1991b). The one-meter wide nursery beds were divided into 0.5 m^2 plots separated from each other by 50 cm uninoculated and unsown zones. The different fungi were inoculated at the rate of 2 liter peat-vermiculite inoculum per m^2. Bac-

terial inoculum (about 10^{11} cfu m^{-2}) was applied as 2.5 liter suspension with a watering can on the surface of each plot after mixing the solid fungal inoculum into the top 10 cm of the soil. The solution for the non-inoculated control plots contained the same amount of $MgSO_4$ than for the bacterial treatments. Both fungal and bacterial inoculations took place immediately before and the same day as sowing. The plants were kept for two years and were not watered during the growth periods: the natural rainfall was enough to maintain soil moisture close to field capacity. In both experiments, no fertilization was applied either before sowing or during the two years.

Measurements and statistical analysis

Twenty seedlings per plot (i.e. 60 and 80 per treatment in experiments 1 and 2, respectively) were randomly sampled at the end of each growing season; their root-systems were pooled for each plot and cut into 2–3 cm pieces mixed together. Some of these root pieces were randomly picked and observed under a stereomicroscope; non-mycorrhizal short roots and four morphological types of ectomycorrhizas (*Laccaria*, *Paxillus*, *Hebeloma* and a contaminant white fungus forming mycorrhizas with a thick mantle and mycelial strands) were identified and counted until a total count of at least one hundred short roots. Mycorrhizal rates (mycorrhizal short roots/total short roots) were calculated for each mycorrhizal type and transformed by arcsin(sqrt) for statistical analysis (tests carried out on previous data of the same type had repeatedly shown that the variance was not homogenous). The shoot dry weights were also determined. The results were treated with a two-way analysis of variance (blocks and treatments) at a threshold of $\alpha = 5\%$. Means were compared with LSD at a threshold of $\alpha = 5\%$.

Results

The results of the experiment 1 (Table 1) show that, in the fumigated soil, the two MHB strains SBc5 and BBc6 markedly improved the efficiency of the inoculation by *L. laccata* and the very closely-related (Henrion et al., 1992) species *L. bicolor*, during both the first and the second year; this effect was more accentuated at the end of the second year, when the proportion of short roots mycorrhizal by the introduced fungi was enhanced by the bacteria from roughly 30 to 80%. In contrast, the same MHBs had a significant negative effect on mycorrhiza formation by *Hebeloma cylindrosporum* and the contaminant white fungus (only during the first year for the latter). *Paxillus involutus* was poorly infective and disappeared before the end of the second year; it was not significantly affected by the bacterial inoculation. However, this strain had previously proved to form abundant mycorrhizas with Douglas-fir in another section of the same nursery 10 years earlier (Le Tacon et al., 1988). As it was not reisolated from mycorrhizas or sporocarps since that time, it may have lost some of its aggressiveness.

In the non-fumigated soil (experiment 2, Table

Table 1. Effect of 2 MHBs (SBc5 and BBc6) on mycorrhizal establishment and stability of different fungi on Douglas-fir planting stocks in a methyl bromide-fumigated nursery soil (experiment 1)

Fungal and bacterial inoculation	Short roots mycorrhizal by the introduced fungus (%)	
	Year 1	Year 2
Contaminant white fungus (no fungal inoculation)	21	63
+ SBc5	0*	38
+ BBc6	2*	47
Laccaria laccata	75	35
+ SBc5	91*	89*
+ BBc6	94*	88*
Laccaria bicolor	56	25
+ SBc5	90*	84*
+ BBc6	90*	72*
Hebeloma cylindrosporum	90	66
+ SBc5	61*	19*
+ BBc6	58*	29*
Paxillus involutus	35	0
+ SBc5	10	0
+ BBc6	15	0

For each fungus, values followed by an asterisk are significantly different (at $\alpha = 0.05$) from the value corresponding to the treatment with no bacterial inoculation in the same column. Each value corresponds to 60 seedlings (see Material and Methods).

Table 2. Effect of 9 MHBs strains on mycorrhizal establishment and stability of *Laccaria laccata* on Douglas-fir planting stocks in a non-fumigated nursery soil, and on the shoot dry weight (experiment 2)

Fungal and bacterial inoculation	Short roots mycorrhizal by *Laccaria* sp. (%)		Mean dry weight of shoot at year 2 (g)
	Year 1	Year 2	
Non inoculated control	5 a	11 a	1.60 a
Laccaria laccata alone	7 a	22 ab	1.78 ab
L. laccata + MB3	23 b	45 bc	2.21 ab
L. laccata + BBc6	30 b	54 c	2.65 b
L. laccata + SBc5	28 b	48 bc	2.27 ab
L. laccata + SHB1	27 b	55 c	1.94 ab
L. laccata + MB2	21 b	47 bc	2.34 ab
L. laccata + SBc4	21 b	48 bc	2.21 ab
L. laccata + SBc1	22 b	52 c	2.50 b
L. laccata + BBc7	17 b	33 b	2.48 b
L. laccata + BBc1	23 b	44 bc	2.38 ab

Values in the same column followed by the same letter are not significantly different at $\alpha = 0.05$ probability level. Each value corresponds to 80 seedlings (see Materials and Methods).

2), the inoculation with *L. laccata* alone was unsuccessful: the percent of short roots mycorrhizal by *Laccaria* sp. was not significantly higher than in the non-inoculated control, where a low infection rate by a resident *Laccaria* was recorded. The addition of MHBs significantly improved mycorrhiza formation with the 9 strains tested the first year and with BBc6, SHB1 and SBc1 at the second year. Three bacterial strains (BBc6, SBc1 and BBc7) also led to a better seedling growth compared to the control with no fungal inoculation.

From the results concerning the bacterial strain BBc6 in both experiments, Table 3 compares the effects of fumigation with methyl bromide and of inoculation with the MHB BBc6 as soil treatments for ensuring the efficiency of *L. laccata* inoculation. Comparative statistics cannot be applied to two different experiments, each one with its own experimental design for the reasons discussed in Material and Methods; however, as they were set at the same time in the same section of the nursery with the same techniques and the same seeds, the comparison of raw data is of practical interest when differences are important (see Tables 1 and 2 for significant differences within each experiment). The best results were obtained with the combination of the two treatments, with very high mycorrhiza formation (88%) and seedling growth. When treatments were applied separately, fumigation provided the highest mycorrhizal colonization on the first year (75%); however, this colonization dropped down to 35% on the second year. In contrast, the MHB treatment gave only 30% at the first year (which was already markedly more than with the fungus alone, 7%), but this value increased to 54% on the second year, i.e. more than with the fumigation (35%). In both cases, the dry weight of the

Table 3. Comparison of effects (data from Tables 1 and 2) of soil fumigation with methyl bromide and of the MHB strain BBc6 on shoot dry weight and % of short roots mycorrhizal by *L. laccata* of two years-old Douglas fir planting stocks inoculated with *L. laccata* mycelium (experiments 1 and 20)

Soil treatment	Mycorrhization (%)		Shoot dry weight (g) Year 2
	Year 1	Year 2	
Experiment 2 (Table 2)			
Untreated control	5	11	1.6
L. laccata	7	22	1.8
L. laccata + MHB	30	54	2.7
Experiment 1 (Table 1)			
L. laccata + fumigation	75	35	3.5
L. laccata + MHB + fum.	94	88	3.2

Each value corresponds to 60 and 80 seedlings in experiments 1 and 2, respectively (see Material and Methods). Statistical comparison of mean values from the two experiments would not be valid; see Tables 1 and 2 for significance of differencies within each experiment.

seedlings was higher than for the untreated and uninoculated control.

Discussion

The various fungi used in experiment 1 had already been tested against the same MHB strains in previous in vitro and glasshouse experiments (Garbaye and Duponnois, 1992). In these former experiments, the two bacteria SBc5 and BBc6 had a positive effect on *Laccaria laccata* (from which they were isolated) and *L. bicolor*, and a negative effect on *Hebeloma cylindrosporum* and *Paxillus involutus*. This selectivity in favour of *L. laccata* and *L. bicolor* was also expressed here in real nursery conditions and extended to other bacterial isolates and to another fungus: the unidentified contaminant white symbiont. However, no significant effect was recorded with *P. involutus* because of its low infectivity in these experimental conditions: it had completely disappeared in all treatments by the end of the second year. These new experimental results confirm the strong fungus-specificity of *L. laccata*-associated MHBs even in natural conditions, which in turn explains the success of the MHBs in the non-fumigated soil (experiment 2): they acted by inhibiting the establishment of the white mycorrhizas and helping that of *L. laccata* ones. However, mycorrhiza types were only visually identified according to their morphology under the stereomicroscope. It was thus impossible to distinguish the introduced *Laccaria laccata* from the resident *Laccaria* sp.

From a practical point of view, the MHB strains BBc6, SHB1 and SBc1 applied in non-fumigated soil led to *L. laccata* mycorrhizal levels of about 50% at the end of the second year (when planting stocks are ready for lifting and outplanting). Previous results by Le Tacon et al. (1988) and Villeneuve et al. (1991) have shown that this mycorrhizal level of 50% was sufficient for significantly enhancing the growth of Douglas-fir plantations.

The MHB strain BBc6 (a *Pseudomonas fluorescens*) seems to be a good candidate as an alternative to soil fumigation with methyl bromide for optimizing the efficiency of *L. laccata* mycelial inoculum. In spite of a lower mycorrhizal level on the first year, it provides in non-disinfected soil two year-old planting stocks as big and as well mycorrhizal by *L. laccata* as those grown in fumigated soil. Thus, the feasibility of this new safe and non-polluting technique is demonstrated; it has now to be tried in other nurseries to determine its application range.

The fact that, during the two-year growth period, the mycorrhizal level decreases in the fumigation treatment and increases in the MHB treatment, suggests that the introduced bacteria survived and multiplied in the rhizosphere and had a long-lasting effect on helping *L. laccata* and/or inhibiting other fungal symbionts. This hypothesis is presently being tested by retrieving and specifically quantifying BBc6 with ribosomal DNA typing using PCR and RFLP techniques (Stenström et al., 1991).

This type of experiments under nursery conditions cannot provide any indication concerning which effect on the plant the bacteria may have on their own, because resident or contaminant fungi rapidly form mycorrhizas, the frequency of which is in turn modulated by the bacteria (as shown in Table 1). However, previous in vitro and glasshouse experiments (Duponnois and Garbaye, 1991b) have shown that the bacterial strains used here had no significant effect on plant growth in the absence of ectomycorrhizal symbiosis: these MHBs do not behave as PGPRs (Plant Growth Promoting Rhizobacteria) under any of our experimental conditions. On the other hand, the same in vitro experiments by Duponnois and Garbaye (1991b) have also shown that MHBs promoted mycorrhizal establishment by themselves and not by interfering with other microorganisms which would inhibit the symbiosis. In addition, the MHB effect does not depend on the tree species (Garbaye and Duponnois, 1992). These three series of observations suggest that MHBs are specifically adapted to living in close association with and provide a competitive advantage to the ectomycorrhizal fungus. Such properties are likely to open a wide field of applications whenever inoculating planting stocks with *Laccaria laccata* or *L. bicolor* is desirable. Whether or not the same situation may exist for other ectomycorrhizal fungus species remains an open question.

Acknowledgements

The nursery was made available for these experiments by the Centre d'Etude du Machinisme Agricole et des Eaux et Forêts, and the work was partly funded by the BIOCEM Company (LIMAGRAIN group).

References

Duchaufour P and Bonneau M 1969 Une méthode nouvelle de dosage du phosphore assimilable dans les sols forestiers. Bulletin de l'Association Française pour l'Etude du Sol 41, 193–198.

Duponnois R and Garbaye J 1991a Effect of dual inoculation of Douglas fir with the ectomycorrhizal fungus *Laccaria laccata* and mycorrhization helper bacteria (MHB) in two bare-root forest nurseries. Plant and Soil 138, 169–176.

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

Duponnois R and Garbaye J 1992 Application des BAM (bactéries auxiliaires de la mycorhization) à l'inoculation du Douglas par *Laccaria laccata* S238 en pépinière forestière. Rev. For. Fr. 6, 491–500.

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

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

Henrion B, Le Tacon F and Martin F 1992 Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal genes. New Phytol. 122, 289–298.

Le Tacon F and Bouchard D 1986 Effects of different ectomycorrhizal fungi on growth of Larch, Douglas fir, Scots pine and Norway spruce seedlings in fumigated nursery soil. Oecologia applicata 7, 389–402.

Le Tacon F, Garbaye J, Bouchard D, Chevalier G, Olivier JM, Guimberteau J, Poitou N and Frochot H 1988 Field results from ectomycorrhizal inoculation in France. *In* Proceedings of the Canadian Workshop on mycorrhizae in Forestry. Eds M Lalonde and Y Piché, Québec, Université Laval, Canada, pp 51–74.

Marx DH and Bryan WC 1975 Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. Forest Sci 21, 242–254.

Pachlewski R and Packlewska J 1974 Studies on symbiotic properties of mycorrhizal fungi of pine (*Pinus sylvestris*) with the aid of the method of mycorrhizal synthesis in pure culture on agar. Forest Research Institute, Warsaw, 139 p.

Stenström E, Henrion B, Martin F and Garbaye J 1991 Identification of Mycorrhization Helper Bacteria (MHB) by PCR Amplification of Nuclear Ribosomal DNA Genes. Mycorrhizas in Ecosystems—Structure and Function, 3rd European Symposium on Mycorrhizas, Sheffield.

Villeneuve N, Le Tacon F and Bouchard D 1991 Survival of inoculated *Laccaria bicolor* in competition with native ectomycorrhizal fungi and effect on the growth of outplanted Douglas fir seedlings. Plant and Soil 135, 97–107.

Section editors: G D Bowen and H Lambers