Symbiosis, 9 (1990) 267–273
Balaban Publishers, Philadelphia/Rehovot

The Bacteria Associated with *Laccaria Laccata* Ectomycorrhizas or Sporocarps: Effect on Symbiosis Establishment on Douglas Fir*

J. GARBAYE, R. DUPONNOIS and J.L. WAHL

INRA, Centre de recherches forestières de Nancy, Champenoux, F 54280 Seichamps

Abstract

A range of bacteria isolated form mycorrhizas and sporocarps of the ectomycorrhizal fungus *Laccaria laccata* were tested for their effect on ectomycorrhizal development of this fungus on Douglas fir seedlings, both in containers in the glasshouse and in a bare-root nursery. Some of them reduced infection, but some others were very stimulating. These results are discussed from the standpoint of both ecology of mycorrhizal symbioses and forestry practice.

Introduction

It has been shown on different plant – fungus couples that bacteria present in soil, rhizosphere and mycorrhizas strongly interact with the establishment of ectomycorrhizal symbiosis, with the frequent occurrence of a stimulating effect (Bowen and Theodorou, 1979; Garbaye and Bowen, 1987 and 1989; De Oliveira, 1988; De Oliveira et Garbaye, 1989). Some stimulating ("helper") isolates could be of practical interest for improving mycorrhizal inoculation techniques in forest nurseries.

Douglas fir is presently the dominant forest tree used for reforestation in France, and field experiments have shown that the ectomycorrhizal fungus *Laccaria laccata*, when inoculated to planting stocks in the nursery, stimulates the early growth of Douglas fir in plantations (Le Tacon et al., 1988). Moreover, *L. laccata* sporocarps always contain bacteria, suggesting that this fungus may be particularly dependent on some associated bacteria for completing its life cycle.

Therefore, it is worth exploring the possibilities of using helper bacteria in this system. In this paper, a range of bacterial isolates from *L. laccata* mycorrhizas and

*Reviewed

sporocarps have been tested for their effect on ectomycorrhizal development of Douglas fir with *L. laccata*. The experiments were carried out in the glasshouse and in a nursery.

Material and Methods

Plant

The seeds of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) were from provenance zone 421 of Washington State (USA). When used in nursery or glasshouse experiments, they were pretreated in moist sphagnum peat for 8 weeks at 4°C before sowing. In the case of axenic synthesis experiments, seeds were surface-sterilized in pure sulfuric acid for 1 min, then in 30% H_2O_2 for 5 min, washed 10 times in sterile water, and plated on water agar. Dishes were checked daily and contaminated seeds were discarded. The germinating ones were used when rootlets were 1–2 cm long.

Fungus

The fungus was the ectomycorrhizal basidiomycete *Laccaria laccata* (Scop. ex Fr.) Cke., isolate S-238 from USDA, Corvallis (Oregon). It was cultivated on Pachlewski agar medium (Pachlewski and Pachlewska, 1974) modified by increasing nitrogen content fivefold. Mycelial inoculum was grown for 4 weeks at 25°C in 1.6 litre glass jars containing 1.3 litre vermiculite-peat mixture (2:3–1:3, v:v) moistened with modified liquid Pachlewski medium.

Bacteria

Bacterial strains were isolated from sporocarps and surface-sterlized mycorrhizas of *L. laccata* associated with young plants of Douglas fir in France in a glasshouse pot experiment (S), in a nursery (Morvan (M)), and in two plantations (Bruyères (B) and Sainte-Hélène (SH)). Sporocarps were brushed clean and broken open. Pieces of tissue from inside the cap were blended in sterile water using an Ultraturax blender. Mycorrhizas were washed in running tap water, surface-sterilized in 1.5% NaClO for two min, rinsed 20 times in sterile water and blended in the same conditions as sporocarp tissues. The effectiveness of surface sterilization was checked by plating water from the last rinse on nutrient agar. Serial dilutions of the suspensions from sporocarps and mycorrhizas were plated on 0.3% TSA medium (Trypsic Soy Agar, DIFCO) and distinctive colonies were isolated and subcultured on the same medium. Isolates from mycorrhizas were called -Bx, and those from sporocarps were called -Bcx. Out of 110 isolates obtained, 46 were selected according to their effect on growth of *L. laccata*, using the *in vitro* confrontation test

described by Duponnois and Garbaye (1990): 30 stimulating, 6 neutral and 10 inhibiting isolates.

Glasshouse experiment

The three components of the system (bacterium, fungus, plant) were confronted in 95 ml polythene containers filled with non-disinfected peat-vermiculite (1:1, v:v), mixed with 1:10 (v:v) fungal inoculum. Five ml concentrated (more than 10^8 cells per ml) bacterial suspension in $MgSO_4$ 0.1 M were injected into each container with a syringe. A control treatment received the buffer solution alone. Three seeds were sown per cell; when at the cotyledon stage, plantlets were thinned to one per cell. Each treatment was represented by a block containing 40 cells. From 5 weeks and on, a nutrient solution (14,8 ppm N from nitrate and 2 ppm P) was applied twice a week. Ten plants per treatment were randomly sampled 8, 12 and 16 weeks after sowing. Mycorrhizal rate (mycorrhizal short roots: total short roots) was determined and transformed by arcsin(sqrt). The means of treatment *vs.* control were compared with Students "t" test at 0.05 probability level.

Nursery experiment

A nursery bed was filled with an acid brown humic soil from a forest nursery on granite in the center of France. It was fumigated with cold methyl bromide (75 g per m^2, soil covered with polythene film for 4 days) 3 weeks before inoculating and sowing. The bed was divided into 0.5 m^2 plots. All plots were inoculated with *L. laccata* (1 liter inoculum per plot), and there were 3 bacterial treatments (MB61, SBc6, BBc8, with 10^8 cells per plot in 1 liter 0.1 M $MgSO_4$), and a control with a buffer solution only. Treatments were randomly repeated in 3 blocks, and plots were separated from each other by 50 cm non inoculated and non sown zones. The nursery bed was shaded, watered and manually weeded during the experiment. No fungicide or herbicide were applied. Ten weeks, 15 weeks and 20 weeks after sowing the mean height of the seedlings in each plot was determined, and ten plants with heights equal to the mean value corresponding to the plot were sampled. Mycorhizal rate was determined and transformed as in the glasshouse experiment, and the results were treated with two-way analysis of variance (blocks and treatments). Means were compared with l.s.d. 5%.

Results and Discussion

Glasshouse experiment

On week 8, mycorrhizal rate in the control was 60%. Only three bacterial isolates out of 47 significantly increased mycorrhizal development: MB3 (89%),

SBc2 (88%) and SBc5 (88%). In contrast, 20 had a depressive effect, the resulting mycorrhizal rate ranging from 33% for MB21 to 14% for MB51.

On week 12, mycorrhizal rate in the control was 64%. Two isolates stimulated mycorrhizal infection: MB3 (90%) and MB69 (89%). Only 6 isolates were depressive: MB10 (37%), MB23 (33%), MB29 (39%), MB51 (31%), MB55 (24%) and BBc5 (32%).

On week 16, mycorrhizal rate in the control was 67%. A greater number of isolates (15) displayed a significant stimulating effect, the resulting mycorrhizal rate ranging from 83% for BBc6 to 97% for MB3. On the other hand, only 2 isolates were depressive: MB10 (46%) and MB51 (40%).

Thus, the number of "helper" isolates increases with time, while the number of antagonist ones decreases. Seven patterns of behaviour exist, as shown by examples in Figure 1:

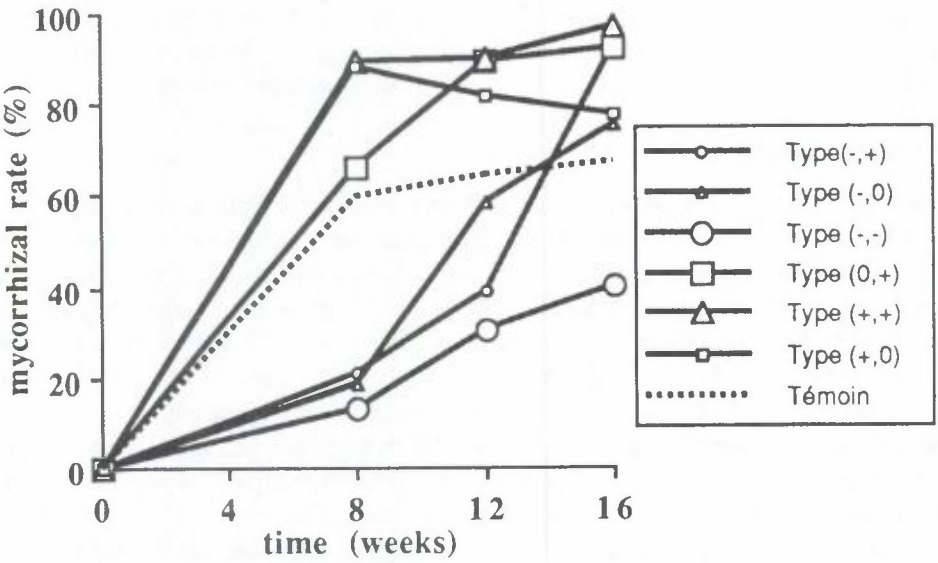

Figure 1. Glasshouse experiment: mycorrhizal rate of Douglas fir seedlings inoculated with *Laccaria laccata*, plotted against time, for the control and bacterial isolates chosen as examples of the six types of behavior. Type $(-,+)$:Mb29; type $(-,0)$:MBc4; type $(-,-)$:MB51; type $(0,+)$:MB69; type $(+,+)$:MB3; type $(+,0)$:SBc2. The signs $(+)$ or $(-)$ refer to significant differences from the control at 8 and 16 weeks, according to student "t" test ($P=0,05$).

type $(0,0)$: no significant effect during the whole season. The most important group numerically (16 isolates out of 46).

type $(-,0)$: early inhibition, then no significant effect. The second group for the numbers of isolates (13).

type (−, −): strong antagonist effect from the beginning to the end of the experiment (2 isolates).

type (0, +): late stimulation only (7 isolates).

type (+, +): significant stimulation during the whole observation period. Only those should be considered as effective "helpers". This group is only represented by 2 isolates MB3 and SBc5.

type (+, 0): early stimulation only (1 isolate).

type (−, +): an early inhibition is followed by a significant stimulation (5 isolates).

These observations suggest that several types of interactive mechanisms are involved. It can also be noticed that trends toward inhibition with time, such as (+, −) or (0, −), do not exist.

From another standpoint, no correlation was found between these results and characteristics of isolates such as taxonomic position, geographic origin, isolation place (mycorrhiza or sporocarp) and effect on fungal growth with *in vitro* confrontation tests.

Nursery experiment

On week 10, mycorrhizal rate in the control was 31%, and no bacterial treatment had any significant effect.

On week 15, the proportion of short roots mycorrhizal with *L. laccata* in the control had dropped to 18% and a large part of the root systems was infected by ectomycorrhizal fungi native to the nursery (mostly *Thelephora terrestris*, *Tuber albidum* and *Suillus sp.*). The bacterial isolate SBc6 significantly increased mycorrhizal rate due to *L. laccata* to 37%.

On week 20, *L. laccata* mycorrhizal rate in the control was 21% and the stimulation due to SBc6 was not significant any more.

The decline of the infection by *L. laccata* in the control indicates that the conditions in the nursery were limiting. Nevertheless, bacterial isolate SBc6 proved to be an efficient helper, precisely at the time when the decline took place. This isolate displayed an early depressive effect in the glasshouse, where it belonged to type (−, 0). This discrepancy shows that the studied interactions are very dependent on environmental factors, which differed widely in the two cultures for climate, mineral nutrition and uncontrolled microbial background. It also suggest that bacterium SBc6, which was isolated from a sporocarp, is not so closely, specifically and beneficially associated with *L. laccata* in it symbiotic state as it had been hypothesized in the Introduction. More results are needed to know if any such "quasi-symbiotic" bacteria exist.

Conclusion

These two experiments, carried out in conditions close to routine Douglas fir

planting stocks production (containerized seedlings in the glasshouse and bareroot nursery), clearly demonstrate that some helper bacteria, isolated from mycorrhizas or sporocarps, can effectively be used for improving the efficiency of ectomycorrhizal inoculation in spite of the complex and uncontrolled background rhizospheric microflora. Mycorrhizal development following inoculation with *Laccaria laccata* can be enhanced, near the end of the growing season, from 67 to 97% for containerized seedlings and from 18 to 37% in the bare-root nursery. Thus, new concepts and new techniques should be designed for ectomycorrhizas research and development, paying more attention to microorganisms closely associated with the fungal component of the symbiosis.

However, to date, the mechanisms involved in these interactions are poorly known (De Oliveira and Garbaye, 1989; Duponnois and Garbaye, 1990), and screening efficient helpers necessitates heavy and fastidious large-scale experiments for each specific nursery condition. That is why more basic research is needed in this field: a better understanding of the phenomenon is likely to cast a new light on ecology and physiology of mycorrhizal symbiosis and to be beneficial to forestry practice.

In future experiments, the evolution of introduced bacterial populations should be monitored in the rhizosphere by numeration at the end of the growth period.

REFERENCES

Bowen, G.D. and Theodorou, C. 1979. Interactions between bacteria and ectomycorrhizal fungi. *Soil Biol. Biochem.* **11:** 119–126.

De Oliveira, V.L. 1988. Interactions entre les microoganismes du sol et l'établissement de la symbiose ectomycorhizienne chez le Hêtre (*Fagus silvatica* L.) avec *Hebeloma crustuliniforme* (Bull. ex Saint-Amans) Quél. et *Paxillus involutus* Batsch. ex Fr. Thèse de doctorat de l'Université de Nancy I, 118 pp.

De Oliveira, V.L. and Garbaye, J. 1989. Les microorganismes auxiliaries de l'établissement des symbioses ectomycorhiziennes (revue bibliographique). *Eur. J. For. Path.* **19:** 54–64.

Duponnois, R. and Garbaye, J. 1990. some mechanisms involved in growth stimulation of ectomycorrhizal fungi by bacteria. *Can. J. Bot.*, in press.

Garbaye, J. and Bowen, G.D. 1987. Effect of different microflora on ectomycorrhizal inoculation of *Pinus radiata*. *Can. J. For. Res.* **17:** 941–943.

Garbaye, J. and Bowen, G.D. 1989. Stimulation of ectomycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas. *New Phytol.* **112:** 383–388.

Le Tacon, F., Garbaye, J., Bouchard, D., Chevalier, G., Olivier, J.M., Guimberteau, J., Poitou, N., and Frochot, H. 1988. Field results from ectomycorhizal inoculation in France. Proceedings of the Canadian Workshop

on mycorrhizae in Forestry, M. Lalonde and Y. Piché edit., Québec, Université Laval (Canada), 51–74.

Pachlewski, R. and Pachlewska, J. 1974. Studies on symbiotic properties of mycorrhizal fungi of pine (*Pinus sylvestris* L.) with the aid of the method of mycorrhizal synthesis in pure culture on agar. Forest Research Institute, Warsaw, p. 228.