Location and Survival of Mycorrhiza Helper *Pseudomonas fluorescens* during Establishment of Ectomycorrhizal Symbiosis between *Laccaria bicolor* and Douglas Fir

PASCALE FREY-KLETT,^{1*} JEAN CLAUDE PIERRAT,² AND JEAN GARBAYE¹

Equipe Microbiologie Forestière, INRA, 54280 Champenoux,¹ and Equipe Dynamique des Systèmes Forestiers, INRA-ENGREF, 54042 Nancy,² France

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The mycorrhiza helper bacterium *Pseudomonas fluorescens* BBc6, isolated from a *Laccaria bicolor* sporocarp, consistently promotes *L. bicolor*-Douglas fir (*Pseudotsuga menziesii*) ectomycorrhizal formation, even with low doses of bacterial inoculum. In order to describe this phenomenon more accurately, we have looked at the location and survival of the introduced bacterial strain in the soil and in the rhizosphere during the establishment of mycorrhizal symbiosis in glasshouse and nursery experiments. Bacterial populations were quantified with a spontaneous, stable, rifampin-resistant mutant, BBc6R8, which phenotypically conformed to the parental strain. BBc6R8 populations declined rapidly, reaching the detection limit after 19 weeks, and did not increase either when *L. bicolor* sporocarps were forming in autumn or when Douglas fir roots resumed growing in spring. BBc6R8 was neither an endophyte nor a rhizobacterium. Furthermore, it was not particularly associated with either mycorrhizas of Douglas fir-*L. bicolor* or *L. bicolor* sporocarps. Surprisingly, a significant mycorrhiza helper effect was observed when the inoculated BBc6R8 population had dropped as low as 30 CFU g of dry matter⁻¹ in the soil. This study raises questions concerning the bacterial concentration in the soil which is effective for promotion of mycorrhizal establishment and the timing of the bacterial effect. It allows us to develop working hypotheses, which can be tested experimentally, to identify the mechanisms of the mycorrhiza helper effect.

The establishment of ectomycorrhizas on tree roots is affected by the microbial populations of the rhizosphere (11) and especially by some bacteria which can have either a positive or a negative effect on mycorrhiza formation (1, 12). The study of bacteria which have a positive effect has led to the concept of mycorrhiza helper bacteria (MHB) (6). Garbaye (10) defined MHB as telluric bacteria associating with some mycorrhizal fungi and promoting the establishment of mycorrhizal symbiosis.

Pseudomonas fluorescens BBc6, isolated from a sporocarp of *Laccaria bicolor* S238N (Maire P. D. Orton syn. *Laccaria lac-cata* S238N), consistently stimulates the growth of *L. bicolor* in vitro and the mycorrhizal establishment of this fungus with the Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] (6, 7, 13). When the Douglas fir is associated with *L. bicolor* S238N, its growth in plantations is consistently improved (18), and large-scale inoculation of seedlings in nurseries is now developing commercially.

To date, little is known about the ecology of MHB and the mechanisms involved in their effect on mycorrhizal symbiosis, and there is a lack of knowledge about the fate of introduced MHB in the soil and in the rhizosphere. The objective of this study was, therefore, to localize strain BBc6 and to describe its survival in the soil in relation to the stimulation of mycorrhiza formation in the Douglas fir-*L. bicolor* system. We monitored the bacterial population in glasshouse and nursery experiments using a spontaneous rifampin-resistant mutant, BBc6R8, that we had assessed for the stability of its resistance and its phenotypic conformity to the parental strain.

MATERIALS AND METHODS

Fungal strain and inoculum preparation. The ectomycorrhizal basidiomycete *L. bicolor* S238N (3) was maintained on Pachlewski agar medium (19). Fungal inoculum was prepared by growing the mycelium aseptically in a peat-vermiculite nutrient mixture (6).

Bacterial strain and inoculum preparation. *P. fluorescens* BBc6 was isolated from a sporocarp of *L. bicolor* S238N in a Douglas fir plantation (6). Its classification as *P. fluorescens* biovar 1 was ascertained by phenotypic and genotypic analysis (9).

Twenty-one rifampin-resistant (Rif^r) mutants of BBc6, named BBc6R1 to R21, were derived from a BBc6 culture by plating a cell suspension (10¹¹ CFU ml⁻¹) onto King's medium B (KB) (17) agar plates containing 100 mg of rifampin liter⁻¹. Developing colonies were selected after 5 days of incubation at 25°C. BBc6 and the Rif^r mutants were stored at -80°C in Luria-Bertani medium (21) with 20% glycerol added.

Bacterial inoculum was prepared by growing the bacteria on KB agar plates with (for the mutants) or without (for BBc6) 100 mg of rifampin liter⁻¹ at 25°C for 36 h. The bacteria were then suspended in 0.1 M MgSO₄ buffer, washed twice, and resuspended in the same buffer at the desired bacterial density. Bacterial density was measured as the absorbance of the suspension at 600 nm, with reference to a standard curve calibrated by plate enumeration.

Plant growth conditions and inoculation procedures. The seeds of Douglas fir trees from provenance zone 422 (Washington state) were pretreated in moist peat at 4°C for 1 month to break dormancy.

In glasshouse experiments, the seedlings were grown in 4-cm-diameter polyethylene containers (80 ml) filled with either a nonsterilized peat-vermiculite mix (1:1, vol/vol; pH 5.5) or a methyl bromide-fumigated soil from a sandy forest nursery (pH 5.4; 7.5% organic matter, 31 ppm P [extracted in 0.5 M NaHCO₃], 0.3% N). Soil disinfection before sowing is a common practice in forest nurseries when inoculating with ectomycorrhizal fungi in order to suppress competing resident symbionts. Before the containers were filled, the substrate was watered to field capacity and mixed with 2.5% (vol/vol) fungal inoculum, unless otherwise indicated. Five milliliters of a bacterial suspension (10⁹ CFU ml⁻¹) was poured on top of each container immediately after sowing; the controls received only sterile distilled water. One seedling was grown per container. The containers were arranged in trays containing 40 containers. The trays were watered daily with a mist system adjusted to maintain field water capacity. Beginning 7 weeks after sowing, a nutrient solution [KNO₃, 0.8 g; Ca(NO₃)₂ · 4H₂O, 1.9 g; NaH₂PO₄, 0.4 g; MgSO₄ · 7H₂O, 0.7 g; Kanieltra (a commercial micronutrient solution; COFAZ, Paris), 0.2 ml; distilled water, 1 liter; pH 6] was applied to the trays with peat-vermiculite mix twice a week with a watering can. The soil from the nursery was not fertilized. The experiments were performed during two

* Corresponding author. Mailing address: Equipe Microbiologie Forestière, INRA, 54280 Champenoux, France. Phone: 33 83 39 40 41. Fax: 33 83 39 40 69. E-mail: klett@nancy.inra.fr.

periods: in spring-summer (when the temperature in the glasshouse ranged from 15 to 28°C) and in autumn-winter (when the temperature ranged from 10 to 20°C). The photoperiod (16 h) was the same in both experiments (daylight was supplemented with artificial light).

In the nursery experiment, the soil (the same as in glasshouse experiments) was steam disinfected *in situ*. The nursery bench was divided into 0.5-m² plots. In each plot, *L. bicolor* was inoculated at 0.5 liters of peat-vermiculite inoculum per m². BBc6R8 (7×10^9 CFU m⁻²) was applied on the surface of each plot with a watering can after the fungal inoculum was mixed into the top 10 cm of soil. The Douglas fir seeds were then sown in rows. Nonlimiting water conditions were maintained by sprinkler irrigation.

Screening of the Rif^r mutants. The stability of rifampin resistance was tested by subculturing the 21 mutants on KB agar plates 15 times. After the 15th subculture, each mutant was grown on KB agar medium with or without 100 mg of rifampin liter⁻¹ and bacterial counts were compared. The effect of the Rif^r mutants on fungal growth *in vitro* was compared to that of BBc6 according to the test of Duponnois and Garbaye (5). Growth rates of BBc6 and the Rif^r mutants in liquid KB medium at 27°C were determined by measuring bacterial densities by the dilution plating method 0, 6, 10, and 25 h after inoculation of the cultures. The growth of mutants BBc6R7 and R8 in competition with BBc6 was quantified after coinoculating liquid KB medium with BBc6 and either of the mutants as described by Glandorf et al. (14). Two glasshouse experiments were performed to compare the mycorrhiza helper effects of the Rif^r mutants to that of BBc6. Each bacterial treatment comprised five blocks of 40 containers each. Ten seedlings per block were sampled 12 weeks after inoculation, and the mycorrhizal index (the proportion of short roots with mycorrhizas of *L. bicolor*) was determined by randomly examining 100 short roots per seedling with a stereomicroscope. Then, the mycorrhizal indices of the treatment with the mutants were compared to those of the treatment with or without BBc6.

Population dynamics of BBc6R8. Five glasshouse experiments and one nursery experiment were performed to monitor the population dynamics of BBc6R8.

In one of the glasshouse experiments, nursery soil was inoculated with BBc6R8 only, without *L. bicolor*, in autumn-winter. The four other experiments (two experiments with peat-vermiculite mix and two with nursery soil) involved inoculation with the fungus and BBc6R8. For each substrate tested, one experiment was done in spring-summer and the other was done in autumn-winter. Three of the four experiments were performed to monitor simultaneously bacterial and mycorrhizal kinetics, and one experiment was performed to monitor BBc6R8 kinetics only. Five containers were sampled periodically to monitor BBc6R8 survival. The entire root system was removed, gently shaken to remove the nonrhizospheric soil, and cut into small pieces, which were vigorously homogenized with 0.5 to 3 ml of TS buffer (Tris, 2.42 g; NaCl, 8 g; distilled water, 1 liter; pH 7.5), depending on the size of the root system, for 1 min. To monitor endophytic bacterial populations, entire root systems were vigorously washed with sterile water three times, dipped in a sodium hypochlorite solution (3.5%) for 30 s, and then washed with sterile distilled water four times. Complete surface disinfection was ascertained by imprinting root systems on KB agar. The surface-sterilized root systems were then crushed in 3 ml of TS buffer with a mortar and pestle. The whole substrate of each container was divided into three equal parts (bottom, medium, and top). Each part was homogenized and weighed. About 1 g of substrate per part was sampled, weighed, and suspended in 3 ml of TS buffer. The samples were then vigorously homogenized for 1 min.

The nursery experiment was performed in a bench divided into 10 plots. Each plot constituted one replication. One sample was periodically taken at random in each plot along the bench. Core samples were taken with a steel tube (12 cm long, 4 cm in diameter) centered on a seedling. During the experiment the root system was small enough to be almost entirely contained within the core sample. All the root pieces were removed from the core sample and processed as described above. The soil of the core sample was then homogenized, and about 1 g was subsampled, weighed, and processed as described above.

Quantification of BBc6R8 populations. Appropriate dilutions were plated for all samples in duplicate on KB medium containing 100 mg of rifampin liter⁻¹ and 100 mg of propiconazole (Tilt 125, a fungicide from Ciba-Geigy) liter⁻¹ with a Spiral system (Interscience, Saint Nom la Brtche, France). Total fluorescent pseudomonad populations were estimated on the selective medium of Simon and Ridge (23), which is KB medium amended with 13 mg of chloramphenicol and 60 mg of ampicillin liter⁻¹. For poorly colonized samples, crude suspensions also were directly plated, in triplicate, on the same media. Bacterial colonies were counted after incubation at 25°C for 48 h. When the bacterial population was below the level detectable by dilution plating, 0.5-g soil samples were enriched by shaking them in 3 ml of liquid KB-rifampin (100 mg liter⁻¹) medium at 27°C for 42 h. The presence of BBc6R8 in each sample was then checked by culturing 50 μ l of the enriched culture on KB-rifampin plates. The fluorescence of the colonies was confirmed under UV light.

All samples (substrates and roots) were then oven dried at 70°C for 1 week to determine dry weight. Weighing the root together with the rhizosphere soil led to an underestimation of bacterial densities in the rhizosphere by 0.1 log CFU g⁻¹. This error was considered negligible relative to the overall standard deviation of all data (0.5 to 1 log CFU g⁻¹). As discussed below, no bacteria were found within the surface-sterilized roots. Therefore, the root plus adhering soil compartment was considered the rhizosphere compartment.

Localization of BBc6R8. The distribution of BBc6R8 on the roots was observed periodically in two glasshouse experiments by imprinting five root systems on KB-rifampin agar medium; the nonadhering soil was removed by shaking before plating. The presence of BBc6R8 in the mycorrhizas was studied in the three experiments designed to monitor BBc6R8 kinetics. On each sampling date, one mycorrhiza per root system was sampled and crushed in 40 μ l of TS buffer. Then, the 40- μ l samples were cultured on KB-rifampin plates. The presence of BBc6R8 was also determined in the *L. bicolor* sporocarps which had occurred naturally in the three experiments designed to monitor BBc6R8 kinetics. All the sporocarps were sampled regardless of their stage of development. Each sporocarp was washed three times in 3 ml of sterile water, and 25 μ l of each was cultured on KB-rifampin plates. Each sporocarp was then cut into small pieces in a minimal volume of TS buffer. After 30 min, 25- μ l aliquots of the suspensions were cultured on KB-rifampin plates. All incubations were performed at 25°C for 48 h.

Assessment of mycorrhiza formation. Eight trays were inoculated with *L. bicolor* with or without BBc6R8 to measure mycorrhizal indices. The statistical design consisted of four complete blocks. In the experiments designed to monitor only bacterial kinetics, the mycorrhiza helper effect of BBc6R8 was checked 12 weeks after inoculation by randomly sampling five seedlings per block (i.e., 20 seedlings per microbial treatment). Then, samples were processed as described in the study of the mycorrhiza helper effect of the Rif^r mutants. In the experiments designed to monitor bacterial and mycorrhizal kinetics simultaneously, five seedlings per block (i.e., 20 seedlings per microbial treatment) were randomly sampled every 2 weeks beginning on week 6. The proportion of short roots forming mycorrhizas with *L. bicolor* was determined by examining the whole root systems.

Data analysis. All the statistical analyses were done at the probability threshold of 0.05. Bacterial populations were expressed as CFU per container or per gram of dry matter (gDM) and log transformed. For the experiments using the nursery soil, the detection limit was 10² CFU g DM⁻¹ in the root and soil compartments; for the experiments with the peat-vermiculite mix, it was 3 $\times$ 10² CFU DM g⁻¹. On the sampling dates when the bacterial populations were close to the detection limit, the densities of the samples in which no bacteria were detected were considered to be null and the zero values were used for calculating the mean. Pairs of bacterial kinetics values were compared in two statistical steps. First, the parallelism of the profiles was tested: for each sample, the differences of the bacterial densities at each sampling date were calculated and compared with a one-factor (time) ANOVA. Second, the equality of the profiles was tested: the mean of the differences was compared to zero with a *t* test.

Mycorrhizal indices (the proportions of short roots in mycorrhizas with *L. bicolor*) were transformed by arcsin (square root) for ANOVA analysis. The transformed mycorrhizal indices in all the experiments designed to monitor BBc6R8 populations were analyzed with a two-factor (experiment-microbial treatment) ANOVA with interaction. The transformed mycorrhizal kinetics in the presence and in the absence of BBc6R8 were compared with a two-factor (time-microbial treatment) ANOVA with interaction.

RESULTS

Screening of the Rif^r mutants. We screened 21 spontaneous Rif^r mutants, BBc6R1 to R21, for five phenotypic criteria: stability of rifampin resistance *in vitro*, effect on fungal growth *in vitro*, growth in liquid KB medium, competitiveness in liquid KB medium compared to BBc6, and mycorrhiza helper effect in glasshouse experiments. Many of the 21 mutants did not behave like parental strain BBc6. Nevertheless, we were able to select one mutant, BBc6R8, which was indistinguishable from BBc6. Its rifampin resistance was stable *in vitro* and in glasshouse experiments for at least five weeks (data not shown).

Survival of BBc6R8. The decline of the BBc6R8 population per container from the second week after inoculation (Fig. 1A) was observed in all glasshouse experiments, regardless of the substrate and the period of the year. The brief increase in population size that occurred during the first 2 weeks after inoculation in the experiment shown in Fig. 1A was not seen in three other experiments and probably occurred because of drainage of the bacterial inoculum, which reduced the initial bacterial density to 3 $\times$ 10⁶ CFU per container (Fig. 1A) compared to 10⁹ CFU per container in the other experiments. Such population increases are typical of bacterial recolonization of freshly disinfected soils when the inoculum density is lower than the initial bacterial capacity of the soil. Because the population dynamics of the fluorescent pseudomonads differed among experiments depending on the recolonization of the

FIG. 1. Population dynamics of *P. fluorescens* BBc6R8 in the presence of Douglas fir seedlings and *L. bicolor* in a glasshouse experiment performed with nursery soil in spring-summer. (A) Population sizes for BBc6R8 (closed circles) and fluorescent pseudomonads (open circles) per container; the bars are the standard deviations for five replications of one container each. (B) Population size of BBc6R8 per gDM in the soil (open squares) and in the rhizosphere (closed squares); each point represents the mean value of five replications, and the two curves are parallel (ANOVA) but not equal (*t* test) at $P = 0.05$.

substrate by contaminant or resident fluorescent pseudomonads (data not shown), differences in the rate of decline of fluorescent-pseudomonad and BBc6R8 populations were not always significant.

In all the glasshouse experiments, BBc6R8 significantly stimulated mycorrhizal establishment measured 12 weeks after inoculation, and the magnitude of that helper effect was dependent on the experiment (Table 1).

TABLE 1. Mycorrhiza helper effect of BBc6R8, 12 weeks after inoculation, in the four glasshouse experiments aimed at monitoring bacterial kinetics

Microbial inoculation treatment	Mycorrhizal index in expt no. ^a :			
	1	2	3	4
<i>L. bicolor</i>	48	71	47	69
<i>L. bicolor</i> plus BBc6R8	65	85	61	80

^a The mycorrhizal indices (proportions of short roots mycorrhizal with *L. bicolor*, expressed as percentages) transformed by arcsin (square root) were analyzed with a two-factor ANOVA (experiment-microbial treatment; $P = 0.05$) with interaction; a significant helper effect of BBc6R8 on mycorrhizal establishment, a significant effect of the experiment, and no interaction were found by the F test. Experiments 1 and 2 were performed with peat-vermiculite mix, and experiments 3 and 4 were performed with nursery soil. Experiments 1 and 4 were performed in autumn-winter, and experiments 2 and 3 were performed in spring-summer.

In the nursery, BBc6R8 populations in the soil and the rhizosphere decreased rapidly, reaching the detection limit after 2 weeks (Fig. 2). The bacterial density in the soil after the inoculation (2×10^3 CFU g DM⁻¹) was lower than that in the glasshouse (4×10^4 CFU g DM⁻¹) because the bacteria were diluted in a larger volume of soil. The use of the enrichment method after 2 weeks confirmed that BBc6R8 disappeared progressively and revealed that only 9% of the core samples contained BBc6R8 1 year after inoculation (data not shown).

Localization of BBc6R8. In the glasshouse experiments, the BBc6R8 density and kinetics in the three parts of the substrate (bottom, medium, and top) were not significantly different regardless of the substrate, although the bacterial suspension was poured on top of the substrate.

BBc6R8 was not detected within root tissues, and populations in the soil and in the rhizosphere declined similarly, reaching densities close to the detection limit 9 weeks after inoculation (Fig. 1B). The similarity of the bacterial decline in the soil and that in the rhizosphere was confirmed by the

FIG. 2. Population dynamics of BBc6R8 in the soil (open circles) and in the rhizosphere (closed circles) of Douglas fir seedling-*L. bicolor* mycorrhizas, in the nursery experiment.

FIG. 3. Population size of BBc6R8 per container in the presence (closed circles) or in the absence (open circles) of Douglas fir seedlings in a glasshouse experiment performed in autumn-winter with nursery soil, without *L. bicolor*. Each point represents the mean value of five replications, and the two curves are not significantly different on the basis of the two-factor (microbial treatment-time) ANOVA ($P = 0.05$).

one-factor (time) ANOVA performed on the difference (rhizosphere – soil). In the experiment shown, the rhizosphere and soil populations were significantly different according to the *t* test. However, considering the small difference between the two curves ($0.5 \log \text{CFU g DM}^{-1}$ on average), and considering the fact that no significant difference was ever found in the other experiments, we conclude that the rhizosphere population did not differ from that of the soil. In another experiment without *L. bicolor*, the decline of the BBc6R8 population per container was not significantly different in the presence or absence of Douglas fir seedlings (Fig. 3).

The imprints on KB-rifampin agar revealed a random distribution of BBc6R8 on the root systems, independently of tips, short roots, and mycorrhizas. BBc6R8 occurred in only 0 to 60% (depending on the experiment) of the first mycorrhizas, which were forming 8 weeks after fungal and bacterial inoculation. Then, the frequency of occurrence of BBc6R8 in the mycorrhizas progressively decreased.

BBc6R8 also was not systematically associated with the sporocarps of *L. bicolor*. Nineteen of the 57 sporocarps sampled in one experiment revealed the presence of BBc6R8, whereas none of the 40 sporocarps sampled in the two others experiments were colonized.

Kinetics of mycorrhizal establishment in the glasshouse. The presence of BBc6R8 in the vicinity of *L. bicolor*-inoculated seedlings did not shorten the interval between fungal inoculation and appearance of the first mycorrhizas (Fig. 4), which corresponds to the time at which the roots are receptive to *L. bicolor*, in this experiment. A significant improvement of the mycorrhizal index (i.e., the mycorrhiza helper effect) was ob-

FIG. 4. Kinetics of mycorrhizal establishment with (closed squares) or without (open squares) BBc6R8 in relation to the population size of BBc6R8 per container (closed circles) in the glasshouse experiment performed with nursery soil in spring-summer. Each point is the mean value of five replications. At each sampling time, an asterisk means that the mycorrhizal indices in the two treatments (*L. bicolor* and *L. bicolor* plus BBc6R8) are significantly different on the basis of the one-factor (microbial treatment) ANOVA ($P = 0.05$).

served 5 weeks after the beginning of mycorrhizal establishment, at a time when bacterial density had dropped as low as 2×10^3 CFU per container (i.e., 30 CFU g DM⁻¹ in soil). However, the divergence of the two mycorrhizal-index curves suggests that the helper effect began earlier. Moreover, as soon as a significant helper effect occurred, the two curves became parallel, as indicated by the absence of interaction time by microbial treatment revealed by the two-factor ANOVA. This suggests that the symbiosis-promoting effect of the bacterium had already stopped. The same results were also recorded in two other experiments aimed at simultaneously monitoring bacterial and mycorrhizal kinetics, both in the nursery soil and in the peat-vermiculite mix (data not shown).

DISCUSSION

The BBc6R8 population declined in all glasshouse experiments, regardless of the substrate and the period of the year, and in the nursery experiment as well, although the bacteria stimulated mycorrhizal establishment. We can thus conclude that BBc6R8 does not survive in the soil for a long period, at least in a culturable state. The decline of the BBc6R8 population led to the disappearance of the bacteria below the detection limit after 19 weeks, which corresponds to the annual growth period of Douglas fir seedlings in forest nurseries. Interestingly, the disappearance of BBc6R8 was independent of the physiological states of *L. bicolor* and Douglas fir trees, since the BBc6R8 population did not increase when the *L. bicolor* sporocarps were forming in autumn or when Douglas fir roots resumed growing in spring. In contrast, the populations of other fluorescent pseudomonad strains colonizing various crop plants increased in the rhizosphere (26) and in the soil (4) in spring, when the temperature increased and the vegetation resumed growing. The decline of the BBc6R8 population was consistent with previous results on *P. fluorescens* introduced into soils (2, 25). It cannot have been due to soil moisture since the cultures were kept under nonlimiting water conditions. van Elsas et al. (24) also noted a steady decline of the telluric population of a *P. fluorescens* strain after inoculation, a decline which was independent of soil moisture. The decline could be due to antibiosis of telluric bacteria or to predation by protozoa, which might have recolonized the disinfected soil. The nursery soil used had a low clay content, and it is now well demonstrated that clay confers on telluric bacteria partial protection against predation by protozoa (15, 16).

We conclude that BBc6R8 is not an endophyte since bacterial counts from surface-sterilized roots did not reveal bacteria within root tissues. Neither is BBc6R8 a rhizobacterium according to the common definition: free-living, plant-associated bacteria which have the ability to colonize roots aggressively (28). The BBc6R8 populations in the soil and in the rhizosphere declined at the same rate, and the rhizosphere population size did not differ from that of the soil. Furthermore, the presence of Douglas fir roots did not affect the population density of BBc6R8 per container. The population decline in the rhizosphere suggests that the bacterium is not well adapted to the utilization of root exudates and, as a result, is more vulnerable to the native microflora and fauna of the rhizosphere. Interestingly, BBc6R8 also was not particularly associated with either mycorrhizas of Douglas fir-*L. bicolor* or *L. bicolor* sporocarps, although strain BBc6 initially was isolated from an *L. bicolor* sporocarp. We may therefore wonder what BBc6's ecological niche is. We hypothesize that in nature it is associated with the mycelium of *L. bicolor* in the soil; this contention is supported by the results of Sen et al. (22), who showed that the wild type BBc6 attaches to the hyphal wall of

L. bicolor in in vitro experiments. This hypothesis also could explain why the bacteria are not systematically present in the sporocarps after inoculation: they would colonize sporocarps randomly when carried along the hyphae.

Selected beneficial bacterial strains frequently have been introduced into the soil for a variety of purposes (25), but their effectiveness often is reduced by the poor survival of the inoculum in the soil or in the rhizosphere (20, 27). However, Duponnois and Garbaye (8) showed that BBc6 was effective at a low inoculum dose (10 CFU g DM⁻¹) in a nursery experiment. In the same way, we observed a significant mycorrhiza helper effect when the bacterial population had already dropped as low as 30 CFU g DM⁻¹ in the soil. This raises the question of the threshold concentration of BBc6R8 which is needed to promote mycorrhizal establishment. Our results and those of Duponnois and Garbaye (8) suggest that it is not necessary to apply high levels of bacterial inoculum or to be concerned about the survival of the strain in the rhizosphere to have a helper effect. These results are novel in comparison to other systems in which a minimum initial bacterial inoculum density of 10^5 CFU g of root⁻¹ (20) is required for fluorescent pseudomonads to be effective as biocontrol agents.

Our study suggests three working hypotheses concerning the mechanisms of the helper effect. First, the bacteria could act when the mycorrhizas are already forming—for instance, by facilitating recognition mechanisms between the two symbionts. This hypothesis implies that the helper effect extends over time. However, our results suggest that it stops about 10 weeks after inoculation. Consequently, we consider this hypothesis to be of minor importance. Second, the bacterium could act within the first 5 weeks to improve the receptivity of the root to the fungus before the first mycorrhizas are formed. However, we consider this hypothesis also of minor importance because BBc6R8 is not a rhizobacterium and does not reduce the time required for mycorrhizal establishment. Finally, a third possible mechanism could be an early effect of the bacterium on the presymbiotic growth of the fungus. The bacterium could stimulate fungal growth, thus increasing the probability of a root-mycelium encounter. This hypothesis is supported by the fact that BBc6 attaches to the hyphal wall of *L. bicolor* (22) and stimulates mycelial growth of the fungus in vitro (reference 13 and this paper). Further, Garbaye (10) reported that the ability of bacteria to stimulate mycelial growth of *L. bicolor* in vitro was strongly correlated with their effect on mycorrhizal establishment of *L. bicolor* on Douglas fir trees. To test this hypothesis, we are presently measuring the biomass of *L. bicolor* in soil with and without BBc6R8.

In conclusion, this study shows that monitoring microbial populations is a powerful tool for focusing investigations on biological interactions in the rhizosphere: it points out possible mechanisms which can be set in order of importance and submitted to separate, unequivocal experimental analyses.

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