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Receptiveness of forest soils to ectomycorrhizal association:

I. Concept and method as applied to the symbiosis between *Laccaria bicolor* (Maire) Orton and *Pinus pinaster* Art or *Pseudotsuga menziesii* (Mirb.) Franco

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Abstract Soil receptiveness to a mycorrhizal association can be estimated by standard bioassay from a dose-response relationship. The method was developed using the association *Pinus pinaster* or *Pseudotsuga menziesii* with *Laccaria bicolor* as a model and was successfully used to characterize the receptiveness of two forest soils. From a physical and chemical point of view, both soils were receptive to the *Laccaria bicolor* association. Our results show that microbial factors are very important in the receptiveness of soil to ectomycorrhizal association. Ectomycorrhizal development on seedlings at outplanting sites is discussed in relation to soil receptiveness and the ecological competence of selected strains.

Key words Soil receptiveness · Ectomycorrhizal association · *Laccaria bicolor* · Bioassay

Introduction

Mycorrhizas improve seedling survival and growth by enhancing uptake of nutrients and water, lengthening root life, protecting the root system against soil-borne pathogens and increasing tolerance to adverse conditions (Harley and Smith 1983; Strullu et al. 1991). The formation of ectomycorrhizas depends on the availability of inocula but is also influenced by environmental factors and soil properties such as moisture, fertility, temperature, pH and organic matter (Slankis 1974). Soil fumigation, fertilization and other nursery practices can affect population levels of mycorrhizal fungi and select for strains adapted to nursery conditions. Mycorrhizas are sometimes deficient in modern bare-root or container nurseries. A few dominant aggressive fungi, well adapted to the nursery, commonly and spontaneously colonize seedlings before outplanting. Aggressive symbionts such as *Thelephora terrestris* may quickly dominate root systems, reducing the ectomycorrhizal diversity within the first years of seedling development (Perry et al. 1987; Gagnon et al. 1987). However, differences in inoculum source and cultural practices result in variation in the spectrum of mycorrhizal fungi (Chu-Chou and Grace 1990; Pera 1992; Perrin unpublished data). Mycorrhizal fungi on nursery seedlings often disappear after outplanting into forest sites (Stenstrom et al. 1990; Chu-Chou and Grace 1990; Dahlberg and Stenstrom 1991) and are replaced by the indigenous mycorrhizal flora.

Little is known about the persistence of ectomycorrhizal soil infectivity in the absence of living hosts. The ability of ectomycorrhizal fungi to persist or reinvade soil after clearcutting varies with geographical location and is affected by the history and preparation of sites (Stenstrom et al. 1990). Dramatic decreases in or total elimination of active mycorrhizal fungi sometimes persist for years after clearcutting (Harvey et al. 1980; Parke et al. 1984; Perry et al. 1987; Dahlberg and Stenstrom 1991). Low levels of ectomycorrhizal infectivity were measured in forest soil that had not previously supported ectomycorrhizal plants (McAfee and Fortin 1986; Perrin et al. 1988; Parlade 1992; Pera 1992). Failure in field plantation or poor field performance of seedlings is likely related to the lack or low level of native mycorrhizal fungi and the ineffectiveness of mycorrhizas developed at the nursery. For these reasons, positive effects can be expected on outplanted seedling performance after inoculation with selected mycorrhizal fungi (Marx et al. 1977; Marx and Artman 1979; Ruehle 1982; McAfee and Fortin 1986; Le Tacon and Bouchard 1986; Valdes 1986; Amaranthus and Perry 1987; Stenstrom and Ek 1990). However, neutral or negative effects of mycorrhizal inoculation on seedling growth and

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survival have also been observed (Trappe 1977; Bledsoe et al. 1982; Stenstrom et al. 1990). These conflicting results may be related to the ability of the selected mycobiont to persist on the root system after outplanting (Perry et al. 1987; Chu-Chou and Grace 1990). Using a rapid field method, McAfee and Fortin (1986) showed that different species of mycorrhizal fungi vary greatly in their ability to compete with the native mycota. This was also observed by Chu-Chou and Grace (1990) in New Zealand.

Few studies have addressed the ecological competence of efficient ectomycorrhizal isolates, i.e. its ability to develop a mycorrhizal association and to express a beneficial influence under a range of environmental soil conditions. Perrin et al. (1988), Plenchette et al. (1989) and Duvert et al. (1990) extended to mycorrhizas the concepts of soil infectivity and soil receptiveness to inoculation widely used for soil-borne pathogens. Soil infectivity, which is the ability of a soil containing mycorrhizal fungi to induce the development of mycorrhizal associations on host plants, corresponds to the expression of the native inoculum potential. Soil receptiveness to inoculation is defined as the ability of a soil to allow the development of mycorrhizas on host plants from an introduced fungus. The authors have developed accurate and sensitive models based on mathematical relationships to quantify the infectivity and the receptiveness of soils in controlled conditions.

The purpose of this present study was to examine the relevance of the receptiveness concept for the study of the ecological competence of ectomycorrhizal isolates and to propose a standard biological method for its determination. Soil receptiveness to an ectomycorrhizal fungus can be estimated by standard bioassay from dose-response relationships, according to the biological assay principle (Finney 1971). The method proposed here involves a population of seedlings produced with various initial rates of *Laccaria bicolor* mycorrhizal colonization. The seedlings were transferred to a range of concentrations of two forest soils achieved by dilution with the same autoclaved soil, in order to obtain various levels of competition from the native mycorrhizal fungi. Plant response was measured as the final percentage of *L. bicolor* mycorrhizal short roots formed during the time of the bioassay.

Materials and methods

Production of ectomycorrhizal seedlings

Seeds of *Pinus pinaster* (pine, provenance Aquitaine France Cemagref lot 81245) and *Pseudotsuga menziesii* (Douglas fir, provenance Oregon USA origin 261 lot 313) were washed under running tap water for 12 h, surface-sterilized by soaking in 30% H_2O_2 for 30 min, and then carefully rinsed with sterilized water. Seeds were stratified in Petri dishes at 4 °C for 30–40 days. Five days after germination, seeds were sown in 150-ml plastic pots filled with autoclaved perlite. Pots were placed in a greenhouse (18–27 °C, 16-h day, with a minimum of 200 μmol $s^{-1}m^{-2}$, 50% RH) for 30–40 days, and watered three times weekly with deionized sterile water. Plants were carefully removed from the perlite, gently washed with running tap water and transplanted into plastic containers (Sherwood-type Rootrainers, Spencer-Lemaire Ind. Edmonton, Alberta, Canada) filled with a 175-ml mixture of autoclaved peat-vermiculite (1:1, v:v) and fungal inoculum in various proportions (Table 2), and grown for 6 weeks in the conditions previously described.

Mycelial inoculum of *L. bicolor* (isolate S 238 from USDA, Corvallis, Ore) was produced in a peat-vermiculite mixture (1:10, v:v) moistened with MNM liquid medium (1210:700, v:v) in 2-l glass bottles. The fungus was grown for 2 months at 25 °C.

Bioassay

Forest soils were collected from the A1 horizon in the Province of Girona, Catalonia, Spain at various times between November 1990 and April 1991. Details of soil locations and chemical properties are summarized in Table 1. The soils, designated as Douglas and Pine, were sieved through a 5-mm screen to remove roots, stones and wood pieces. Part of each soil sample was autoclaved in a plastic bag at 120 °C for 1 h to eliminate indigenous mycorrhizal fungi, then left in containers for at least 1 week before use. Dilutions were made of each soil type by thoroughly mixing the original soil in various quantities with the autoclaved soil, in order to provide various levels of competition pressure from indigenous fungi. Ten-week-old mycorrhizal pine and Douglas fir seedlings, previously examined for initial percentage colonization by *L. bicolor*, were transplanted into Spencer-Lemaire containers filled with 175 ml of each soil or soil dilution and grown as described above.

Experimental design

Ten single *L. bicolor* mycorrhizal plant replicates were used for each treatment in various combinations of inoculum rate × soil × soil dilutions in four successive experiments (randomized block design). The first experiment was performed with seedlings of both species preinoculated at rates of 0, 2.5, 5.5 and 12.5% ino-

Table 1 Physical and chemical characteristics of the forest soils Douglas and Pine

Soil	Stand	Texture	pH (H_2O)	N μg g^{-1}	P μg g^{-1}	K meq 100 g^{-1}
Montseny (Douglas)	Douglas fir 4 years old	Sand loam	4.93	3.0	41.7	0.04
Bisbal (Pine)	*Pinus pinaster* 25 years old	Sandy clay loam	5.64	1.4	1.1	0.33

culum/substrate (v/v) in both soils (at 3, 30, 100% nonautoclaved soil). The second experiment was performed with pine seedlings preinoculated at rates of 0, 2.5, 5.5 and 12.5% inoculum/substrate (v/v) on Pine soil (at 1, 3, 10% nonautoclaved soil). The third experiment was performed with pine seedlings preinoculated at rates of 0, 1, 3 and 9% inoculum/substrate (v/v) on Pine soil (at 0, 1, 3, 10% non autoclaved soil). The fourth experiment was performed with seedlings of both species preinoculated at rates of 0, 1, 3 and 9% inoculum/substrate (v/v) on both soils (at 0, 10, 30% nonautoclaved soil).

Two months after transplanting, the plants were carefully removed and the root systems were gently washed under running tap water and observed under a dissecting microscope for mycorrhizal assessment. Mycorrhizal types were identified on the basis of colour and morphology (Agerer 1987–1995). Final percentages of mycorrhizal colonization (number of mycorrhizal short roots: total number of short roots) were determined on the part of the root system developed after transplantation. The amount of short roots produced after planting, in bioassay conditions, was determined following a previously determined pattern of root development (number and length of long roots and number of shorts roots) with time (Perrin unpublished data).

Statistical analyses

Data expressed as percentages were arcsin-transformed prior to analysis. The transformed data were subjected to analysis of variance (ANOVA) and the treatment means were compared by LSD ($P<0.05$). All analyses were performed with the STATISTICA program for the Windows™ operating system 1994 (Statsoft Inc., Tulsa, Okla., USA).

Results

The variation in initial percentage colonization by *L. bicolor* at transplanting was not as high as expected. More than 60% of the short roots were mycorrhizal with *L. bicolor* at the lowest rate of inoculum (2.5%) for the two first experiments, and this level increased to more than 95% at the highest rate (12.5%). The variation was lower (86.4–96.9% mycorrhizal short roots) in the third and fourth experiments, despite the reduced levels of inoculum used.

Tables 2, 3 and 4 give the total mycorrhizal infection ratings (all fungi) of all treatments for experiments 1, 2, 3 and 4. After 2 months, control nonmycorrhizal seedlings transplanted in Pine soil had developed many more native mycorrhizas than in Douglas soil. Indeed, mycorrhizal infection of pine seedlings by indigenous mycobionts in Pine soil was higher than 94%, irrespective of the percentage of nonsterilized soil (Tables 2, 3). In contrast, mycorrhizal infection levels reached less than 4% in non diluted Douglas soil (Tables 2, 4).

The principal indigenous mycorrhiza observed on seedlings in Pine soil was a brownish-orange *Lactarius*-like type with a smooth, shiny mantle surface. In Douglas soil, the main type was a white, smooth, finely grainy, opaque type resembling mycorrhizas formed by *Lyophyllum decastes* on *P. pinaster* as described by Pera (1992). The other less frequently observed native mycorrhizas were a white *Amanita*–like type, one closely resembling a *Laccaria* type on Pine soil, a white *Hebeloma*–like, and a pink-white *Rhizopogon*–like type on Douglas soil.

The mycorrhizas of *L. bicolor* declined on pine and Douglas fir seedlings grown in Pine soil with decreasing levels of inoculum (Fig. 1A,D) and with increasing proportions of nonautoclaved soil (Fig. 2A,B). The influence of soil dilution was generally more pronounced than that of inoculum rate, especially with freshly collected soil samples (Fig. 2A,B), where values for the initial percentage mycorrhizal colonization of the introduced mycobiont were less variable. The highest *L. bicolor* mycorrhizal percentage was reached on the plants grown in the autoclaved soil (Fig. 2A,B).

The decrease in mycorrhizal infection with soil dilution was much more drastic in experiments 3 and 4, with freshly collected soils (Fig. 2A,B), than in experiments 1 and 2, carried out with soil samples stored 2–3 months before use (Fig. 1A,D). This difference may be related to changes in indigenous microflora during soil storage. A low percentage of freshly collected nonautoclaved Pine soil (3–10% in experiments 3 and 4) was

Table 2 Experiment 1. Overall mean percentage mycorrhizal development (all fungi) after 2 months growth of test seedlings with varying soil dilution and rate of inoculation. Soil was collected in November 1990 and seedlings transplanted in January 1991

Soil dilution (% non-autoclaved soil)	*Laccaria bicolor* inoculum rate (% v/v)	Pine seedlings on Pine soil	Pine seedlings on Douglas soil	Douglas seedlings on Douglas soil
3	0	94.2	2.5	0
	2.5	96.8	83.9	92.2
	5.5	95.9	72.4	96.2
	12.5	94.4	91.3	92.5
30	0	98.7	1.0	0
	2.5	96.6	91.6	96.3
	5.5	92.3	85.1	94.9
	12.5	98.5	94.9	95.4
100	0	98.6	3.6	0
	2.5	96.7	93.6	98.3
	5.5	99.4	90.9	97.3
	12.5	99.0	88.6	95.3

Table 3 Experiments 2 and 3. Overall mean percentage mycorrhizal development (all fungi) after 2 months growth of test seedlings with varying soil dilution and rate of inoculation. Soil was collected in November 1990 for experiment 2 and in February 1991 for experiment 3. Seedlings were transplanted in February 1991 (experiment 2) and March 1991 (experiment 3)

Solution (% nonautovlaved soil)	Experiment 2		Experiment 3	
	Laccaria bicolor inoculum rate (% v/v)	Pine seedlings on Pine soil	*Laccaria bicolor* inoculum rate (% v/v)	Pine seedlings on Pine soil
0			0	9.0
			1	93.9
			3	91.6
			9	95.1
1	0	94.4	0	98.9
	2.5	93.7	1	98.2
	5.5	92.3	3	94.2
	12.5	92.2	9	97.4
3	0	99.8	0	99.3
	2.5	98.5	1	97.2
	5.5	95.2	3	99.1
	12.5	96.1	9	93.7
10	0	99.0	0	99.1
	2.5	99.1	1	97.5
	5.5	96.4	3	97.7
	12.5	93.9	9	97.1

Table 4 Experiment 4. Overall mean percentage mycorrhizal development (all fungi) after 2 months growth of test seedlings with varying soil dilution and rate of inoculation. Soil was collected in March 1991 and seedlings transplanted in April 1991

Soil dilution (% non-autoclaved soil)	*Laccaria bicolor* inoculum rate (% v/v)	Pine seedlings on Pine soil	Pine seedlings on Douglas soil	Douglas seedlings on Pine soil
0	0	4.0	0.05	0.43
	1	38.4	52.9	72.3
	3	60.6	76.4	82.3
	9	70.6	–	28.0
10	0	96.8	1.06	1.33
	1	95.4	86.4	69.7
	3	99.2	92.6	68.4
	9	98.6	94.9	55.6
30	0	99.0	–	2.0
	1	98.5	88.2	45.8
	3	98.7	97.2	53.5
	9	99.4	90.1	61.4

enough to suppress the formation of *L. bicolor* mycorrhizas in most cases (Fig. 2A,B).

In the first experiment (Fig. 1B,C), there were no significant differences in *L. bicolor* mycorrhizal colonization on pine or Douglas fir seedlings grown in Douglas soil, regardless of the percentage of nonautoclaved soil or the rate of inoculation. In the fourth experiment, *L. bicolor* development on pine seedlings was reduced by addition of nonautoclaved soil (Fig. 2C), whilst the opposite was true for Douglas fir seedlings (Fig. 2D). Plants grown in 100% autoclaved Douglas soil in the 4th experiment showed root necrosis and the root system was poorly developed. This is likely due to an accidental contamination in the greenhouse by *Fusarium oxysporum*, which was frequently isolated from the necrotic part of the root system. The reduced level of *L. bicolor* mycorrhizal infection and the difference from previous experiments were undoubtly due to interactions between the plant roots, the mycosymbiont and the pathogen.

Discussion

The approach developed in this study was successfully used to characterize the receptiveness of two forest soils. The high percentage *L. bicolor* mycorrhizal association on seedlings grown on Pine and Douglas autoclaved soils indicates that both soils are very receptive to the introduced fungus, from a physical and chemical point of view, in spite of the differences between the soils, especially P content.

Control nonmycorrhizal seedlings transplanted into Pine soil developed more native mycorrhizas after 2

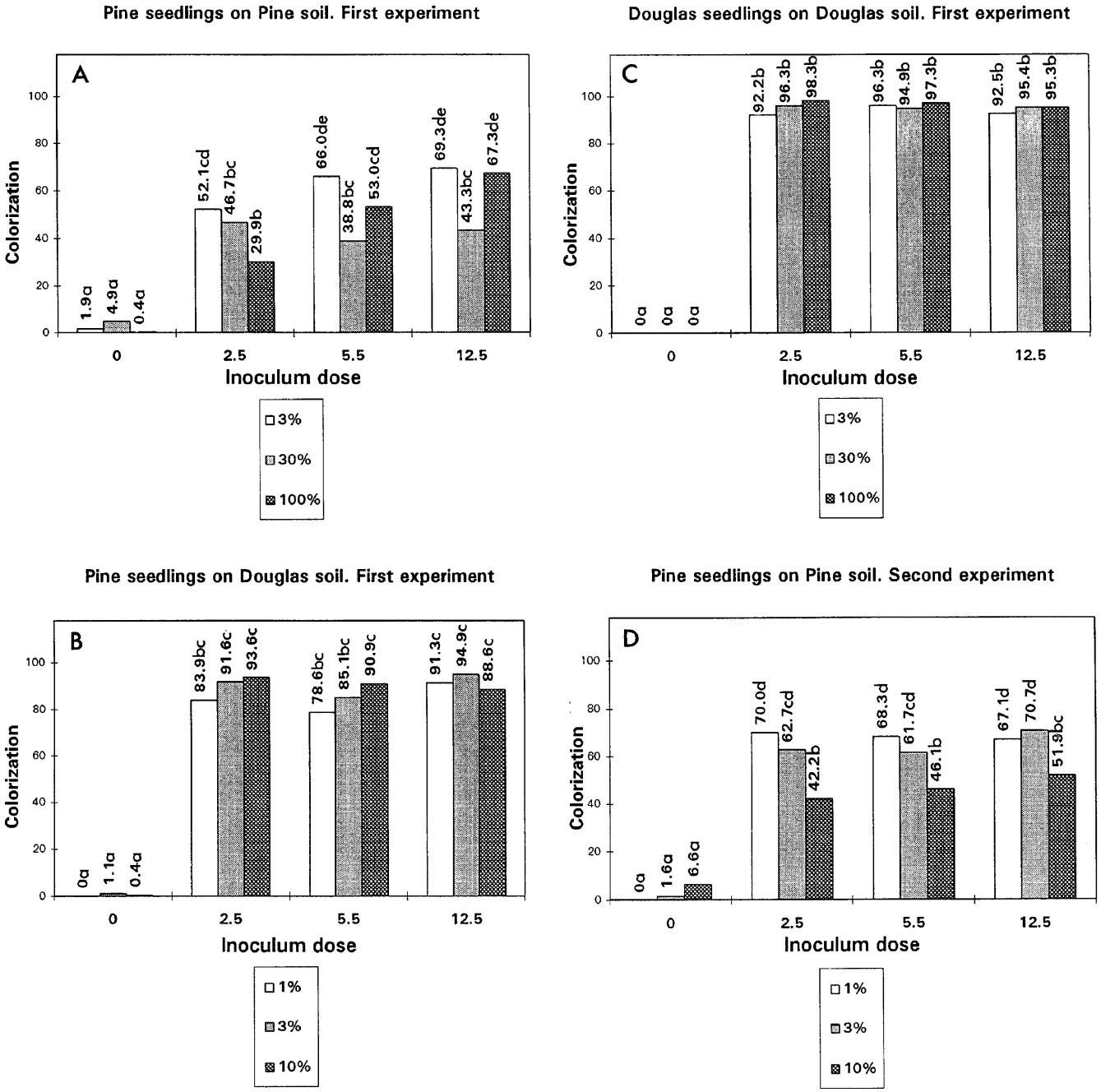

Fig. 1A–D Relationship between the final *Laccaria bicolor* mycorrhizal colonization, inoculum dose and nonautoclaved soil concentration. **A, C** First experiment with 3, 30 and 100% nonautoclaved soil; **B** first experiment with 1, 3 and 10% nonautoclaved soil; **D** second experiment with 1, 3 and 10% nonautoclaved soil. Within individual experiments, means of values followed by the same letter do not differ significantly ($P<0.05$)

months than in Douglas soil. These findings are consistent with the concept of soil infectivity of Perrin et al. (1988), sometimes named inoculum potential (Hornby 1990), in that Pine soil showed high infectivity by indigenous mycorrhizal fungi, whereas the infectivity of Douglas soil was very low. The decrease in *L. bicolor* mycorrhizal infection with increase in the proportion of nonautoclaved soil, which occurred in the Pine soil, is no doubt related to competition from the native ectomycorrhizal population which was strong in Pine soil and low in Douglas soil. Indeed, it has previously been reported that native mycobionts colonize short roots in the region of new root growth, whereas inoculated symbionts tend to extend directly from mycorrhizas developed prior to transplanting (McAfee and Fortin 1986). *L. bicolor* mycorrhizal formation on seedlings grown in Douglas soil tended not to vary, either with soil dilution or inoculum level. This probably results from physical and chemical properties of the soil favourable to *L. bicolor* and the low competition by native mycosym-

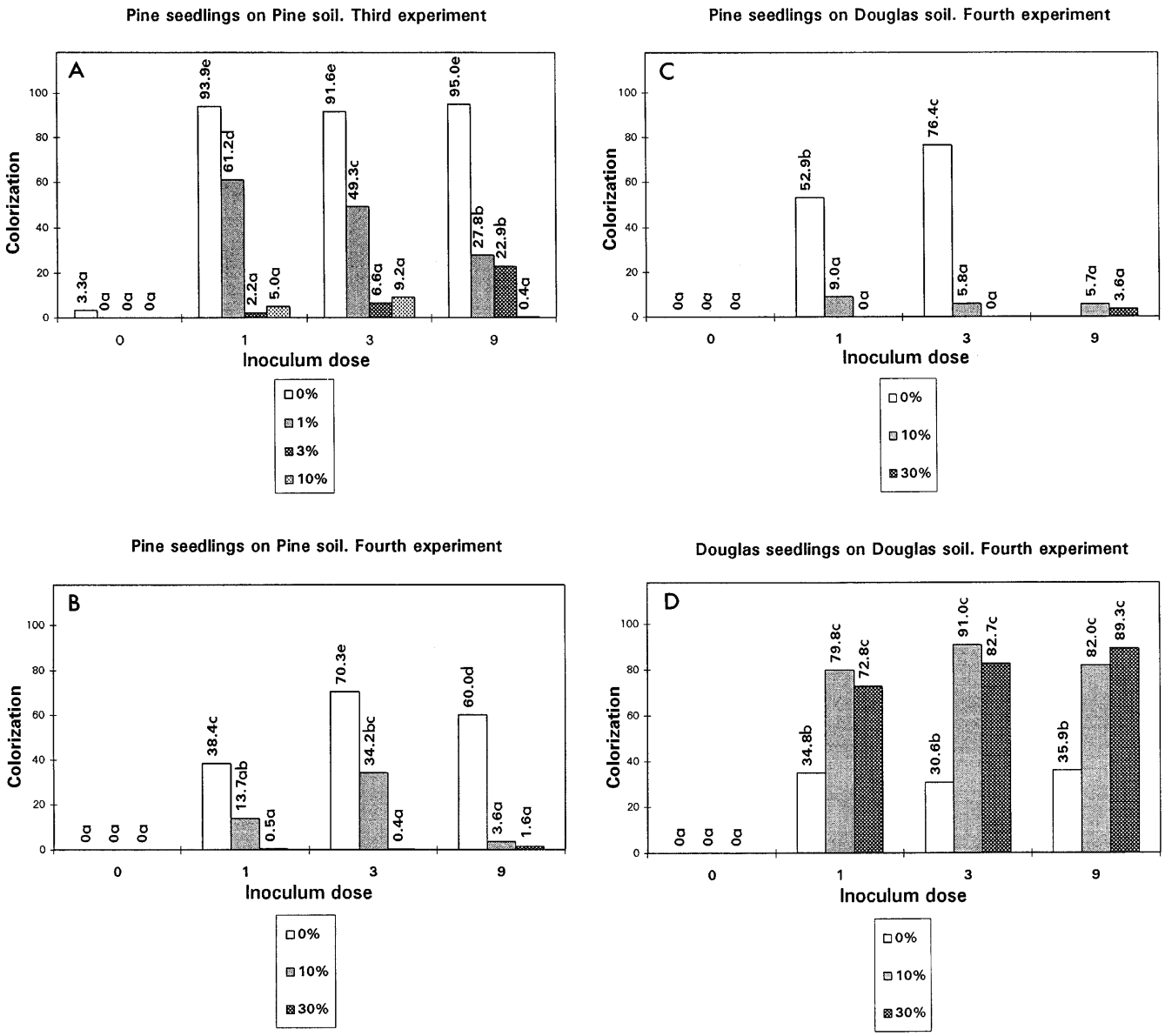

Fig. 2A–D Relationship between the final *L. bicolor* mycorrhizal colonization, inoculum doses and nonautoclaved soil concentration. **A** Third experiment with 0, 1, 3 and 10% nonautoclaved soil; **B, C, D** fourth experiment with 0, 10 and 30% non autoclaved soil. Within individual experiments, means of values followed by the same letter do not differ significantly ($P<0.05$)

bionts. In contrast, a low percentage of Pine soil (<10%) appeared to be sufficient to provide high competition pressure by native mycorrhizas, which prevented the formation of *L. bicolor* mycorrhizas. Thus, in the outplanting of seedlings the behaviour of inoculated mycorrhizas formed in the nursery depends mainly on their ability to compete with native mycorrhizal fungi. Furthermore, *L. bicolor* is little affected by high levels of fertilizer (Molina and Chamard 1983) and this confer the fungus with an ecological advantage over native ectomycorrhizal fungi in the high P soils. Our results substantiate the great importance of the biological component, especially native mycorrhizal fungus populations, in the receptiveness of forest soils to ectomycorrhiza fungal inoculation.

The ability of ectomycorrhizal fungi to survive after clearcutting varies with geographical location and the history and preparation of sites. Most authors have reported decline or loss of the native ectomycorrhizal fungi in soils after clearcutting (Perry et al. 1987). Soil infectivity decreases rapidly with time and the proportion of ectomycorrhizal fungal types shifts after disturbances. We observed this in the Douglas soil, which was cleared of ectomycorrhizal plants several years before planting Douglas fir. In contrast, researchers from northern Europe reported the survival of indigenous mycorrhizal fungi for 1–5 years after clearcutting of trees (Dahlberg and Stenstrom 1991).

It is very likely that ectomycorrhizal populations will be low or nonexistent in many sites to be planted, especially in fields set-aside from agriculture and devoid of an indigenous ectomycorrhizal inoculum. The receptiveness of the soil will then depend more on its physical and chemical properties than on the microbial component, and inoculation with a selected mycosymbiont could be advantageous. The success of controlled inoculation of ectomycorrhizal species will, therefore, depend on disturbances in mycorrhizal soil infectivity in the absence of host plants and on the ecological competence of the species or strains selected for inoculation at the nursery level.

Few studies have examined the behaviour of introduced mycobionts after outplanting, or the receptiveness of forest soils to mycorrhizal fungi. Stenstrom et al. (1990) reported that different inoculated fungi remained on seedling roots for 1.5 years after outplanting but were later slowly replaced by native mycorrhizal species. Dahlberg and Stenstrom (1991) also observed 2 years after outplanting that Scots pine seedlings hosted an average of 1.8 indigenous mycorrhizal types and 0.95 nursery types, comprising 35 and 65% of the mycorrhizal roots, respectively.

Only two studies have dealt with time-saving methods for testing ectomycorrhizal isolates. Garbaye (1983) developed an experimental model for quantifying the competitivity of ectomycorrhizal fungi, but the approach was designed for the simulation of inoculation at the nursery stage rather than for outplanting situations. McAffee and Fortin (1986) presented a rapid field method to evaluate the competitive performance of selected ectomycorrhizal isolates. Their method proved to be very efficient both for evaluation of soil infectivity and the study of competitive interactions of introduced mycobionts under field conditions. However, because large environmental variation exists in the field, this method does not permit analysis of the factors involved in the competition between selected strains and indigenous fungi, which itself determines the postplanting performance of the introduced mycobiont. The bioassay presented in the present study, carried out under controlled conditions, was designed to study the receptiveness of forest soils to mycorrhizal associations. It could still be improved by producing test plants with a wider range of initial mycorrhizal colonization, and by using an inoculum formula such as small amounts of vegetative mycelia entrapped in a calcium alginate gel (Le Tacon et al. 1983); this would allow accurate dosing of the quantity of mycelium to be introduced into a substrate. Furthermore, for very aggressive strains, such as *Laccaria* species, the use of less-receptive substrates should permit the better control of mycorrhiza development levels (Perrin unpublished data).

The method used in the present study, associated with previously developed methods to measure soil infectivity or soil receptiveness to inoculation (Perrin et al. 1988), provides a useful basis for an inoculation strategy aimed at selecting fungal species or strains for the capacity to enhance the adaptation of inoculated seedlings to destined planting sites.

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