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Effects of Inoculation with PGPR *Bacillus* and *Pisolithus tinctorius* on *Pinus pinea* L. Growth, Bacterial Rhizosphere Colonization, and Mycorrhizal Infection

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Abstract

The effect of co-inoculation with *Pisolithus tinctorius* and a PGPR belonging to the genus *Bacillus* (*Bacillus licheniformis* CECT 5106 and *Bacillus pumilus* CECT 5105) in enhancing growth of *Pinus pinea* plants and the changes that occurred in rhizosphere microbial communities and the degree of mycorrhization were evaluated. Both bacterial strains of *Bacillus* promote the growth of *Pinus pinea* seedlings, but this biological effect does not imply a synergic effect with mycorrhizal infection. However, the positive response to mycorrhiza in a longer-term experiment it could be expected. The introduction of both inocula causes an alteration in the microbial rhizosphere composition, despite the low levels of inocula that were found at the end of the assay.

Introduction

A wide variety of bacteria lives in proximity to roots and mycorrhizae, but understanding of the interactions between these groups is scant [29]. Furthermore, the knowledge about effects of these organisms on plant development is especially interesting if we consider co-inoculations of mycorrhizal fungi and PGPR (plant growth promoting rhizobacteria, [31]) as Chanway [9] described in a meticulous review of the current position.

For several decades PGPRs have been introduced into soil to improve plant growth [5, 8, 31, 34, 43]. There is an increasing interest in the application of beneficial bacteria, since PGPRs stimulate plant growth through several mechanisms [30], i.e., enhancing N_2 fixation [16]; exerting a biological control of soil-borne pathogens [6, 7] and frost injury [39]; or by means of improving plant growth, mainly through the production of plant hormones [25, 26].

Bowen and Theodorou [3, 4] were the first authors to report stimulatory and inhibitory effects of bacteria during growth in laboratory conditions and on root infectivity of *Pinus radiata* D. Don by ectomycorrhizal fungi, which belonged to the genera *Rhizopogon, Suillus,* and *Cenoccocum.* Similar works were developed by Garbaye and Bowen [21], McAfee and Fortin [32], and Garbaye and Bowen [22]. Garbaye and Duponnois [24] also demonstrated that the stimulatory effect of some bacterial strains was not plant-specific,

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but a striking degree of bacteria–fungus specificity was detected. Indeed, a specific bacterial enhancement in the number of *Laccaria lacctata* mycorrhiza on Douglas fir was reported by Duponnois et al. [13] after a 2-year nursery study.

From an ecological perspective, there has been little research on the effects of inoculation on indigenous members of microbial communities other than pathogens. Nurmiaho-Lassila et al. [36] have developed some interesting ideas about where bacterial communities of mycorrhizosphere were studied, but there is actually very limited information about the effect of a joint bacteria–mycorrhizae inoculation on the structure of bacterial communities.

With regard to the assayed genera of PGPR on co-inoculation, there are numerous investigations where the bacterium used belongs to the genus *Bacillus* [10, 45, 46, 47]. Generally, in these works, bacteria are studied as MHB (mycorrhization helper bacteria), where those bacteria have been considered unable to stimulate plant growth directly, which means in the absence of an appropriate mycorrhizal fungus [12, 13]. Clearly, these microorganisms enhance seedling growth by increasing the number of mycorrhyzal root tips [23]. Fitter and Garbaye [15] suggested that many PGPRs may really be MHB because of the prominence of pseudomonads and bacilli in both groups. In addition, PGPR studies often exclude the evaluation of the number and types of mycorrhizae. However, Shishido et al. [47] observed no effect of inoculation with *Bacillus* PGPR on mycorrhizal root tip formation of *Pinus contorta* var *latifolia* and *Picea glauca x engelmannii*. Furthermore, the size of both conifer species in the seedling growth promotion was similar in mycorrhizal and non-mycorrhizal plants, indicating that *Bacillus* PGPR may promote plant growth through a mechanism unrelated to mycorrhizal fungi.

The aim of the present study is (i) to evaluate the effect of co-inoculation with *Pisolithus tinctorius* and a PGPR belonging to the genus *Bacillus* (*Bacillus licheniformis* CECT 5106 and *Bacillus pumilus* CECT 5105) in enhancing the growth of *Pinus pinea* plants, and (ii) to study the changes that occurred in rhizosphere microbial communities and the degree of mycorrhization of treated plants in order to evaluate the interaction between both groups of microorganisms.

Materials and Methods

Inoculants

Two strains of *Bacillus* PGPRs (*Bacillus licheniformis* CECT 5106 and *Bacillus pumilus* CECT 5105) and mycorrhizal fungi *Pisolithus tinctorius* were used throughout this study.

The *Bacillus* strains were isolated, identified [40], and characterized as PGPR through production of indole acetic acid–like compounds [26] and gibberellin compounds (GA_4, GA_1, GA_3 and GA_{20}, unpublished data). The PCR-RAPDs profiles obtained from pure cultures of the bacteria grown on a nutrient broth (28°C, 24 h) are shown in Fig. 1. The method used to obtain these profiles is described below (see DNA isolation and PCR amplification).

The *Pisolithus tinctorius* (pers.) Coker and Couch [Syn = P. arhizus (Scop.:Pers) Rauschert] inocula used was MycorPlant. This is a commercial inoculum whose composition per 100 g is 10^8 fungi spores, 45 g of acrilamide, 10 g of silica sand, and 45 g of humic acids (leonardite humates).

Seed and Plant Substrates

Pinus pinea seeds were obtained from the Centro Nacional de Mejora Forestal "El Serranillo" managed by Dirección General para la Conservación de la Naturaleza, Spain. Seed lots originated from sites located close to the rivers Tietar and Alberche. Until use, seeds were stratified at 4°C in wet sand for 30 days and surface sterilized by floating them in 2.5% NaOCl for 5 min before sowing.

A 1:1 (w:w) peat:sand mixture was used as the plant substrate during the experiment. Peat composition was 90% black peat, 8% plant compost, clays, and sand, pH 6.0, 200 mg N L^{-1}, 200 mg P_2O_5 L^{-1}, and 150 K_2O mg L^{-1}. Before use, the substrate was autoclaved three times at 120°C for 20 min each.

Plant Growth and Inoculation Conditions

Seeds were sowed in plastic trays with autoclaved vermiculite and watered with sterile tap water. The environmental conditions during germination were 12 h light at 22°C. One week after germination, seedlings were transferred to 250 mL plastic pots (forest containers, Full-Pot, Mollerusa, Spain) with a 2 mm diameter mesh at the base. Plants were grown in a greenhouse (day 18°C, 16 h, and

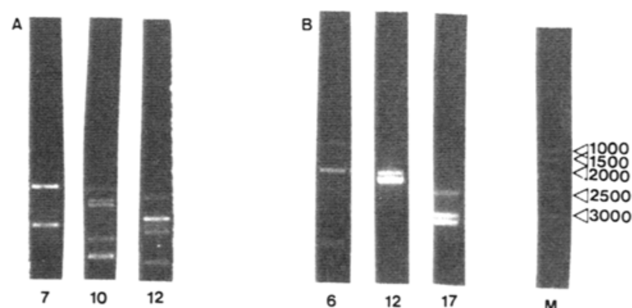

Fig. 1. Banding pattern by RAPDs-PCR of assayed bacteria. (A) *Bacillus licheniformis;* (B) *Bacillus pumilus;* M, synthetic marker (bands correspond to 1,000, 1,500, 2,000, 2,500, and 3,000 base pairs). Numbers correspond to primers of Kit B (random primers, Operon Technologies Inc., CA). Three primers were used for identification of bacteria in each sampling time: 7, 10, and 12 for *Bacillus licheniformis* and 6, 12, and 17 for *Bacillus pumilus.*

night 15°C, 8 h) for 3 weeks until inoculation. Throughout the experiment, plants were mantained under the same environmental conditions and watered twice a week with tap water.

The following treatments were used: *Pisolithus tinctorius* (Pt), *Pisolithus tinctorius* and *Bacillus licheniformis* (Bl + Pt), *Pisolithus tinctorius* and *Bacillus pumilus* (Bp + Pt), and the uninoculated control (C). Inoculation was carried out when the plants were 3 weeks old. Bacteria were stored on 0.2% TSA at 4°C. Twenty-four hours before inoculation, bacteria were transferred to a liquid medium (Nutrient Broth, Pronadisa, Spain). The culture was centrifuged and washed with sterile Nutrient Broth, and pellets were resuspended in sterile NaCl 0.9% solution to obtain 10^2 CFU g^{-1} soil. For treatments with *Pisolithus tinctorius,* a commercial powder noculum (1.92 g) was suspended in bacterial suspensions (100 mL) prepared in the same way as previously described (PGPR-fungi co-inoculations), or in sterile 0.9% NaCl (fungal inoculation) to obtain a concentration of 6×10^5 spores g^{-1} soil. The bacterial, fungi, or bacterial–fungi suspensions, in a final volume of 10 mL per plant, were spread homogeneously on the soil surface. Control plants were watered with sterile 0.9% NaCl solution.

Plant Harvest and Analysis

Seedlings were destructively harvested at 30 (sampling time 1, S1), 90 (sampling time 2, S2), and 150 (sampling time 3, S3) days after inoculation. Six plants were used for each treatment. Three out of the six plants were gently pressed in filter paper for the subsequent biometrical study (see below). The root systems of the remaining three plants were separated from their shoots and frozen at −70°C prior to ergosterol and chitin analyses (see below). One g of rhizosphere soil of the previouly referred to plants was obtained by softly washing the roots in 1 mL of sterile distilled water; 0.1 mL of this suspension was reserved for plate counting (colony-forming units, CFUs) and the other 0.9 mL used for the phospholipid fatty acids (PLFA) analysis (see below).

i. Effects on Plants. Biometrical data of plants were determined at each sampling time. The parametres studied were aerial surface (AS), root system surface (RS), aerial length (AL), and root system length (RL). Biometric analysis was carried out using an image analyzer Delta-T System with DIAS software. After plants had been heated at 55°C for several days, the dry weight (DW) was also measured. Three replicates were used for each measurement.

ii. Chitin and Ergosterol Analysis. Chitin was measured according to the method described by Ekblad et al. [14], with modifications. Roots (1 g) were crushed in liquid N_2 to obtain a fine powder. The extract was resuspended in 3.0 mL of methanol and centrifuged at 4,500 rpm for 20 min at 4°C. This procedure was repeated twice. Supernantants were assigned to analyze the ergosterol content, whereas the pellets were subjected to chitin analysis. Each washed and freeze-dried pellet was treated with 0.2 N NaOH to remove proteins and amino acids that could interfere with glucosamine determination. An acid hydrolisis (6 N HCl, v/v) was performed at 80°C for 6 h in order to release glucosamine residues, and followed by neutralization with 3 M sodium acetate. Glucosamine residues were evaluated colorimetrically at 653 nm.

Ergosterol was measured according to Salmanovicz and Nylund [42] and Nylund and Wallander [37], with modifications. Free ergosterol and that bound forming sterol esters, contained in the supernatant, were measured together. The sample processing, in outline, consisted of an evaporation of the methanolic fraction, saponification (KOH 4% in ethanol, 80°C for 30 min) and a final partition with cyclohexane (4 mL). The alkaline ethanolysis was stopped with 2 mL of the mixture Na_2HPO_4 and KH_2PO_4 (0.1 g ml^{-1}). The organic phase was evaporated under a stream of N_2 and the final dried residue was stored at −20°C and dissolved in 200 µL of methanol. Ergosterol was separated by HPLC with a C18 reversed phase column (150 × 4.5 mm, 5 µm i.d.) and detected with a UV detector at 282 nm. The mobile phase was 100% methanol (HPLC grade) with a gradient flow rate that began with 1.5 mL min^{-1} for 3 min, to be decreased to 1.0 mL min^{-1} for 5 min. The chromatographic run was ended after 12 min.

iii. Plate Counts. CFUs were determined at all sampling times. CFUs were determined on soil agar plates [40] after an appropriate dilution in sterile distilled water and were counted after a 48-h incubation at 28°C. Three plates per dilution were prepared, and those dilutions with 20–100 colonies per plate were used.

iv. DNA Isolation and PCR Amplification. In order to assess bacterial root colonization, 10 colonies from the plates used for CFU counts were randomly recovered and subjected to a RAPD-PCR analysis. For this purpose, plates with 30–40 colonies were used at each sampling time and treatment. Purity of isolates was tested by a Gram stain. Thus, DNA was isolated from bacteria grown in a nutritive culture broth by shaking (350 rpm) for 24 h at 28°C. Bacteria were separated by centrifugation and lysed following the method of Noller and Hartsell [35].

Amplification reactions were carried out with three different primers (random 10-mers, Kit B, Operon Technologies, Alameda, CA), 7, 10, and 12 for *Bacillus licheniformis* and 6, 12, and 17 for *Bacillus pumilus,* and were performed in a Perkin-Elmer Cetus DNA Thermal Cycler. Amplification products were analyzed by electrophoresis in 1% agarose gels, visualized by ethidium bromide staining, and compared with the PCR-RAPDs profiles obtained from pure cultures of PGPR bacteria (see Fig. 1). Program ADA v 1.0 (Análisis de datos Avanzados, TDI S.A., Madrid, Spain) was used to compare the patterns of isolates whith those of the inoculated strains.

v. Phospholipid Fatty Acid Analysis. For each sampling time and treatment, 1 g of rhizosphere soil was obtained by gently washing the roots in 1 mL of distilled water, 0.1 mL of the suspension was used for the plate counting and 0.9 mL for the analysis of PLFAs. Plates with 30–40 colonies were used. Three mL of 0.15 M citrate buffer (pH 4.0) was added to the plate, the agar surface was gently scraped with a glass rod, and 1.5 mL of each bacterial suspension was recovered for the PLFAs assay.

Lipids were extracted by a procedure previously described by Frostegård et al. [20], which is based on the method of Bligh and Dyer [2]. The extracted lipids were fractionated on silicic acid

(100–200 mesh, Unisil) columns by eluting with chloroform, acetone and methanol. The polar lipids (containing phospholipids) were subjected to a mild alkaline methanolisis [11], which transformed the fatty acids of the phospholipids into free fatty acid methyl esters. These were analyzed using gas chromatography, according to the methods described by Frostegård et al. [20]. All solvents used were HRGC-grade.

Fatty acids were designated as the total number of carbon atoms: number of double bonds, followed by the position of the double bond from the methyl end (ω) of the molecule. *Cis* and *trans* configurations are indicated by *c* and *t*, respectively. The prefixes *a* and *i* indicate anteiso and iso branching, respectively; *br* indicates an unknown methyl branching position, *10Me* indicates a methyl group on the 10th carbon atom from the carboxyl end of the molecule, and *cy* refers to cyclopropane fatty acids.

Statistics

Two-way analyses of variance (ANOVA) followed by LSD tests ($p < 0.05$) were used to detect treatment effects on biometrical data and chitin–ergosterol content. In these parametres studied, the average of the three sampling times was used. The mol percent of PLFA values from rhizosphere and bacterial suspension recovered from plates were $\log_{10}$ transformed before being subjected to a principal component analysis (PCA) [27] to elucidate the major variation patterns. Two PCAs were made for PLFAs samples, one for total PLFAs and the other for culturable bacteria PLFAs. In each case the matrix subjected to multivariate analysis was 12×37 (4 treatments $\times$ 3 sampling times $\times$ 37 PLFAs analyzed). The multivariate calculations, ANOVA, and LSD were performed by using the computer program SYSTAT v. 5.05 for Windows.

Results

Biometrical results are shown in Figs. 2A to 2E. Treated plants showed higher values han control, with the exception of Pt treatment. Those plants treated only with the fungus behaved quite similarly for those values corresponding to aerial organs (Figs. 2A and 2C), or showed even lower values in relation to root system (Figs. 2B and 2D). Bp + Pt treatment significantly increased both aerial and root system parameters, not only compared with the control but also with other treatments. An analogous result was observed when evaluating the dry weight of inoculated plants in respect of non-inoculated plants (Fig. 2E). The treatment Bl + Pt yielded significant differences when the aerial parameters were evaluated.

Chitin and ergosterol results are shown in Figs. 3A and Fig. 3B. ANOVA carried out with the data showed a strong effect of the Pt inoculant. For all data shown in Figs. 3A and 3B, the amount of these metabolites was the result of subtracting the background noise from the blank, which consisted of bulk soil. Both chitin and ergosterol contents were significantly ($p < 0.05$) higher in Pt treatments. In contrast, the presence of bacteria in the inoculation medium reduced the accumulation of ergosterol (Fig. 3B).

Results obtained from plate counts are summarized in Table 1. In the case of inoculating plants with any of the assayed bacterial strains, the number of colony forming units (CFUs) seemed to decline twofold between S1 and S2 sampling times. After that time, a recovery of CFUs was observed. Both in plants treated with *Pisolithus* and in those left untreated, the number of colonies remained steady during the experiment. Obviously, bacterial population recovered in these two treatments contained neither *B. licheniformis* nor *B. pumilus,* as deduced from PCR analysis (Table 1). Bacterial root colonization is also shown in Table 1 as a percentage calculated by comparing PCR profiles of plate colonies with those observed in pure cultures of these PGPRs. Optimal colonization (around 6%) took place at the first sampling time, to be subsequently diminished.

Principal component analysis of all PLFAs partitioned from each rhizosphere and sampling time resulted in a separation along the first component that explained 60.68% of the variation up to the second component, which absorbed 30.76% of the variation (Fig. 4). Score plots of PCA showed a clear difference between S1 and S2–S3. S1 plots are located at the highest values of axis I and the lowest ones of the second principal component. In contrast, both S2 and S3 plots appeared to be gathered at the lowest values of first principal component and at the highest of the second axis. At the first sampling time, there was no dispersion between the control and treatments, whereas at S2 and S3, controls seemed to be quite different from the inoculated samples. To investigate those shifts associated to the PLFA pattern, values for the individual PLFAs were also plotted in Fig. 4. On the one hand, the fatty acids 18:2ω6, i17:0, and a17:0 became connected to those plots proceeding from S1, and on the other hand, the PLFAs br16:0, 17:1ω8, and 18:1ω9 were located near S2 and S3. Finally, cy17:0 seemed to be associated with controls from S2 and S3.

The study of PLFAs obtained from plates used previously for examining CFUs represents only a fraction of the whole soil community. In fact, these strains are not only the culturable fraction, but also the most metabolically active in the soil at the time of sampling. PCA of those PLFAs is shown in Fig. 5. This analysis reveals a separation of the different plots along the first two principal components, which explains 60.54% and 30.76% of the variation in the data. S1 plots are

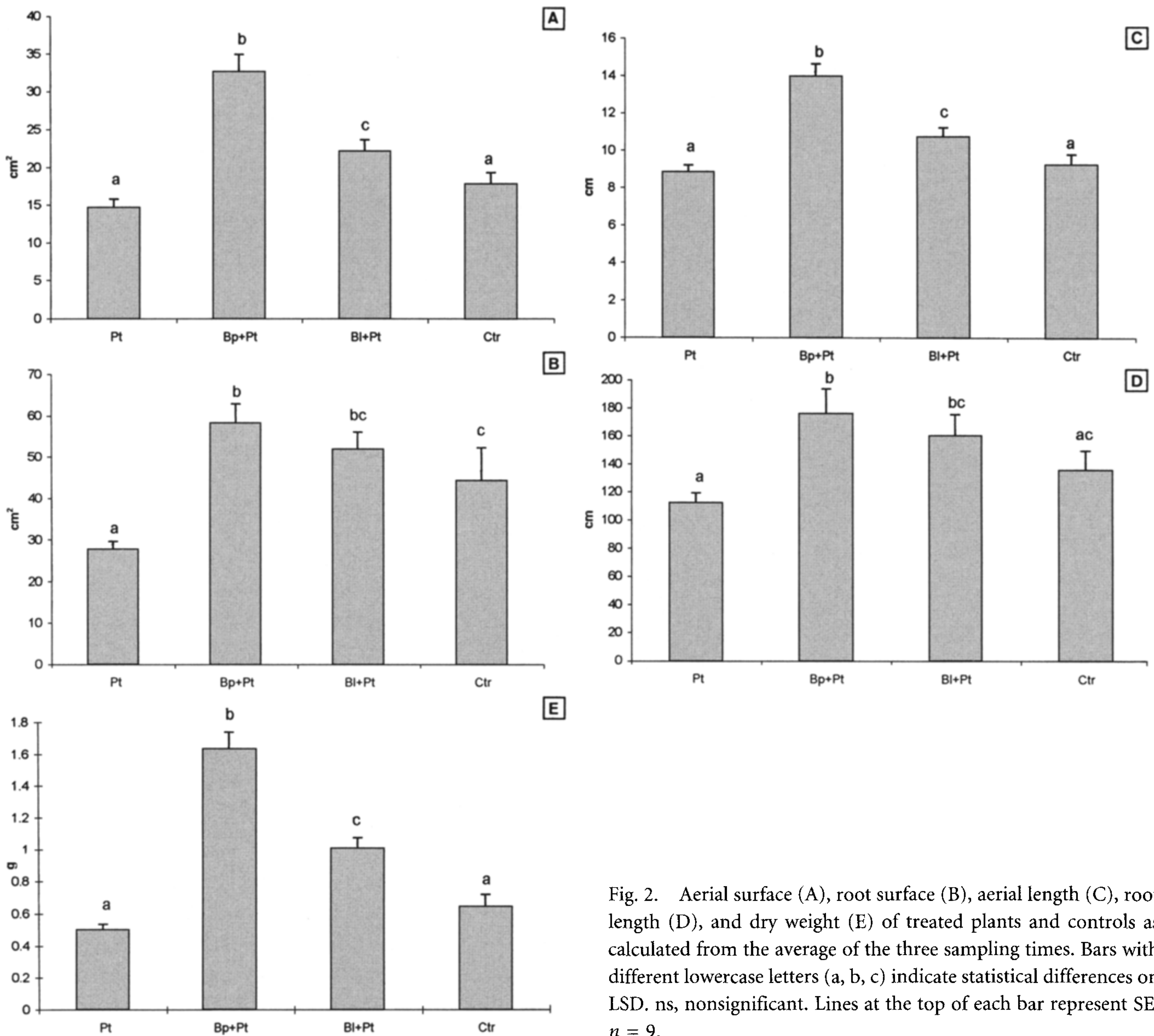

Fig. 2. Aerial surface (A), root surface (B), aerial length (C), root length (D), and dry weight (E) of treated plants and controls as calculated from the average of the three sampling times. Bars with different lowercase letters (a, b, c) indicate statistical differences on LSD. ns, nonsignificant. Lines at the top of each bar represent SE. $n = 9$.

situated at the highest values of the second component. In addition, the control of this sampling time deviates substantially from the plots corresponding to inoculated plants. Something similar was achieved for S2 scores, which are located close to the zero coordinate of the second principal component. There is another group of dispersal data, which corresponds to the plants sampled at S3. In a similar way as was represented for soil PLFAs, in this case (plate PLFAs), the most dispersed loadings for the individual fatty acids are graphed against each other in Fig. 5. The fatty acid i17:0 is close to the plots drawn for S1, whereas cy19:0 remains in the center of the graph, which approximates to those samples connected to S2 and S3. The PLFA cy17:0 and a17:0 are located at the lowest values of PC 1, where CS1, PtS3, and B1 + PtS3 are found.

Discussion

The maximal biological response is found in those plants treated with bacteria, especially when treated with Bp (Fig. 2). This results could be indicative of its potential role as a MHB [23]. Nevertheless, considering ergosterol and chitin results (Fig. 3), it is possible to rule out a synergic effect between bacteria and fungi since (i) an insufficient effect on biometry is found in plants treated only with Pt, and (ii) the

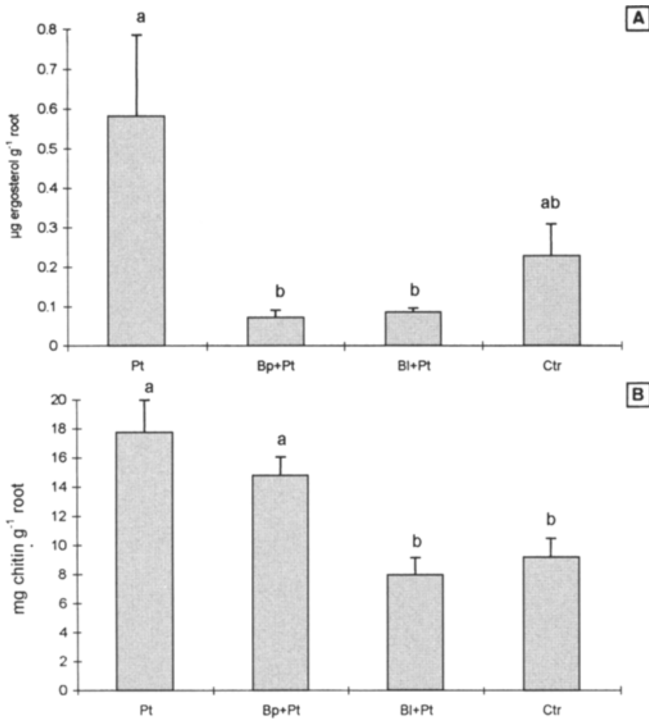

Fig. 3. Chitin (A) and ergosterol (13) analysis results for treated plants and controls as calculated from the average of the three sampling times. Bars with different lowercase letters (a, b, c) indicate statistical differences on LSD. ns, nonsignificant. Lines at the top of each bar represent SE. n = 9.

fungal inoculation with bacteria and Pt does not stimulate a significant accumulation of ergosterol (Fig. 3A). Ergosterol as a component of membranes, is considered to be a good measure of metabolically active fungal biomass [37]. The content of chitin, as a component of the cell wall, reflects all fungal biomass living or dead, and thus can be considered as

Table 1. Total bacterial counts (log CFUs) (A) and percentage of inoculated *Bacillus* detected by PCR-RAPDs (B) in the different treatments and sampling times (n = 3)

A	S1	S2	S3
C	7.8	7.8	7.9
Pt	7.4	7.3	7.5
Bp + Pt	9.2	6.6	7.6
B1 + Pt	9.3	6.7	7.6
B	**S1**	**S2**	**S3**
C	0	0	0
Pt	0	0	0
Bp + Pt	6.0	5.0	<5
B1 + Pt	6.6	6.4	<5

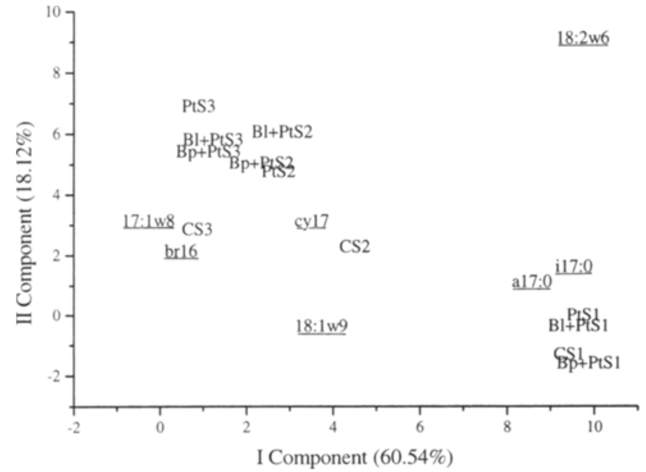

Fig. 4. PCA showing variation in scores of soil PLFAs and loading values for some individual PLFAs (underlined) of treated plants and controls at the various sampling times.

an indicator of the integration of the fungus over the life span of the root system.

These parameters have been identified in roots colonized by some ectomycorrhizal species in forest plants [14, 17]. Our results showed that bacterial inoculum activates plant growth, but the decrease in ergosterol and chitin concentrations reveals that somehow bacteria interfere with mycelium survival. The loss of fungal viability observed by inoculation with either of the two *Bacillus* strains suggests that mycorrhizal formation process and bacterial root colonization are coupled. In light of these results, it can be deduced that plant growth promotion is not due to mycorrhiza but is due to the

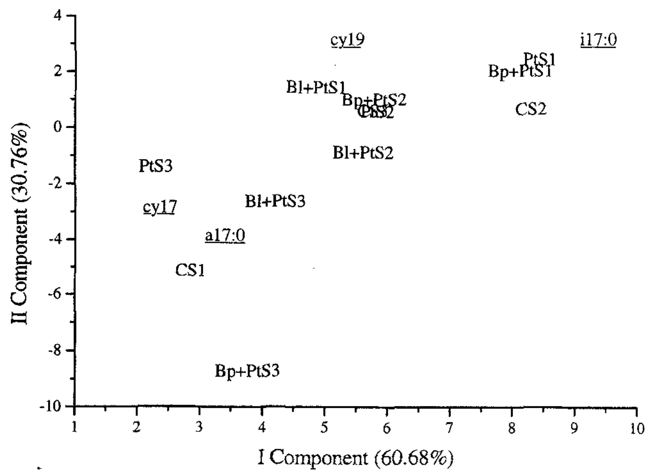

Fig. 5. PCA showing variation in scores of culturable bacteria PLFAs and loading values for some individual PLFAs (underlined) of treated plants and controls at the various sampling times.

two *Bacillus* strain assayed. Our results were consistent with other findings demonstrating that conifer seedling growth promotion by *Bacillus* PGPRs strains was not dependent on mycorrhizal fungi [46, 47]. Despite this fact, we cannot rule out a positive long-term effect on mycorrhizal infection due to the presence of the coinoculated bacteria. We hypothesize this because the treatment of plants produces an increase in root surface and, consequently, a major development of root hairs that may become ensheathed by mycorrhizal root tips (Marlekola, 1996, PhD Thesis; [41]). According to Frey-Klett et al. [18] it could be speculated that the concentration used for both of the two bacteria assayed in the inoculation (10^8 CFU g^{-1} soil) may not be optimal for mycorrhizal formation. Indeed, lower doses of some other PGPRs stimulate mycorrhization [12]. Previous reports show that the mechanism by which PGPRs stimulated pine seedling growth is through phytohormone production [9, 28].

Regarding the biometrical response, the increase detected in aerial length of treated plants cannot be attributed to IAA-like compounds by bacteria (Fig. 2A). Our earlier findings show that the two strains of *Bacillus* are able to synthesize these metabolites [26]. The increase of aerial length is achieved by a growth of internodal zones, but not by an increase in the number of nodes. In contrast, the biological effects could be related to the production of gibberellins by either *B. pumilus* or *B. licheniformis* (unpublished data). However, the previous effect does not correspond exactly to GA_3, because this gibberellin develops taller and more slender phenotypes with longer and narrower leaves and stems (Fig. 2C) (Vidal et al., 1999, unpublished). As opposed to the results obtained with the aerial parts, the effects observed on the root system could be explained in terms of the action of an IAA-like compound, since these hormones play an important role in plant physiology, i.e., increasing root surface [26, 44]. Biometrical analysis also reveals an increase in the dry weight of bacteria-treated plants. In addition, these plants showed a healthy aspect and none of them appeared with any pathological symptoms such as chlorosis or weakness of shoots.

The considered growth parameters in these plants inoculated with bacteria seem to be the resultant of a balanced development of photosynthetic apparatus, which is coupled with a vigorous root system able to supply the plant with all nutrients, as a result of a "sink effect" caused by production of plant growth regulators by bacteria.

Concerning bacterial colonization patterns, a decrease in the amounts of the inoculated bacteria was obteined during the experiment (Table 1). Frey-Klett et al. [19] found similar results in experiments carried out by co-inoculating mycorrhiza and bacteria. In any case, it can be concluded that colonization was successful, at least during the first two sampling times. In those times the percentage of the inoculated *Bacillus* strains were 5% for Bp (which represents 2.1×10^5 CFU g^{-1} rhizosphere) and 6% for Bl (which represents 3.4×10^5 CFU g^{-1} rhizosphere). Other reports show even lower amounts of bacteria (around 10^3) after 10 weeks of inoculation, which could possibly be due to drainage of bacterial inoculum from the beginning of the experiment [19]. A similar consideration could be used to explain the decrease observed in the bacterial population from S2 to S3. In the same manner, Wiehe and Höflich [48], in their studies of PGPR bacterial inoculation in cereals and leguminous plants, considered that values lower than 10^3 in CFUs are sufficient to estimate a successful colonization.

The presence of mycorrhiza had no marked effect on CFUs (Table 1A), although it tended to reduce the number of inoculated bacteria in the experiment (Table 1B). Most reports have indicated a depressive effect on bacterial numbers by mycorrhizal roots compared to non-mycorrhizal ones [1, 33].

The PLFA analysis allows us to make clear that the introduction of inoculum does not notably alter the rhizobacterial community structure (Fig. 4). The fatty acids i17:0 and a17:0 are commonly present on gram positive bacteria [38] and appear associated with S1 samples. Otherwise, S2 and S3 scores have no differences between them and are graphed sufficiently far from the controls. These results suggest that the rhizobacterial community of treated plants has followed a successional behavior different from that of untreated control plants, even when the number of inoculated bacteria are lower than 5% (Table 1B). Nevertheless, the study of PLFAs corresponding to the culturable bacterial fraction (Fig. 5) reveals a significant switch in the community from S1, because of the heterogeneity observed among PLFA composition on different treatments. A striking result is that at S2 (Fig. 5) the structure of bacteria from the rhizosphere seem to be homogeneous but different from the control. However, a major heterogeneity among treatments is detected at S3. Consequently, it could be thought that the depressive result in bacterial amounts up to S2 implies a simplification of the system, which envolves a greater homogeneity. From this homogeneous system, rhizobacterial communities trend to be diversified, and this change occurs in a different manner depending on each treatment.

We conclude that both bacterial strains of *Bacillus* promote the growth of *Pinus pinea* seedlings, but this biological

effect does not imply a synergic effect with mycorrhizal infection. However, this fact does not allow us to discard a positive response of mycorrhiza in a long-term experiment. The introduction of both inocula causes an alteration in the microbial rhizosphere composition, despite the low levels of inocula that were found at the end of the assay.

Acknowledgments

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