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Colonisation of *Pinus halepensis* roots by *Pseudomonas fluorescens* and interaction with the ectomycorrhizal fungus *Suillus granulatus*

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Abstract

Colonisation of *Pinus halepensis* roots by GFP-tagged *Pseudomonas fluorescens* Aur6 was monitored by epifluorescence microscopy and dilution plating. Aur6-GFP was able to colonise and proliferate on *P. halepensis* roots. Co-inoculation with the ectomycorrhizal fungus *Suillus granulatus* did not affect the bacterial colonisation pattern whereas it had an effect on bacterial density. Bacterial counts increased during the first 20 days of seedling growth, irrespective of seedlings being mycorrhizal or not. After 40 days, bacterial density significantly decreased and bacteria concentrated on the upper two-thirds of the pine root. The presence of *S. granulatus* significantly stimulated survival of bacteria in the root elongation zone where fungal colonisation was higher. The number of mycorrhizas formed by *S. granulatus* was not affected by co-inoculation with Aur6-GFP. Neither Aur6-GFP nor *S. granulatus* stimulated *P. halepensis* development when inoculated alone, but a synergistic effect was observed on seedling growth when bacteria and fungus were co-inoculated.

Keywords: *Pseudomonas*; Root colonisation; Ectomycorrhizae; *Suillus granulatus*; *Pinus halepensis*

1. Introduction

The rhizosphere is a dynamic soil environment formed by living plant roots and their associated microflora. Root exudates (sugars, amino acids and organic acids) are the driving force for nutrition and growth of bacterial and fungal communities [1]. Special attention has been given in rhizosphere research to bacteria and fungi showing positive effects on plant growth and health, with potential application in soil amelioration programmes [2–4]. Among free-living bacteria, two groups can be distinguished: (a) the plant growth-promoting rhizobacteria (PGPR) that can influence plant growth directly or indirectly by releasing mineral nutrients and phytohormones [2] and (b) the mycorrhizal helper bacteria (MHB) that indirectly affect plant development by stimulating hyphal growth and improving root colonisation by mycorrhizal fungi [5]. Ectomycorrhizae are symbiotic associations between fungi and roots of many forest trees. Benefits of ectomycorrhizae to trees include protection against pathogens, improved mineral and water uptake and enhanced tolerance to stresses [6]. Plant growth promoting rhizobacteria, including *Pseudomonas fluorescens*, have been applied as biological control agents against soil-borne

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diseases in agricultural crops [7], and undoubtedly have great potential in the production of forest trees [2].

A better understanding of the microbial colonisation processes in the rhizosphere is necessary to ensure optimal efficacy of plant production or bio-control applications of micro-organisms. Bacterial colonisation of plant rhizosphere is a complex process dependent on many different biotic and abiotic factors [8]. Motility and the ability to grow on root exudates or to synthesize molecules that promote attachment to the root are relevant characteristics for the establishment of effective and enduring root colonisation by bacteria [9,10]. Attachment kinetics of bacteria in the colonisation process is important, since fast colonizing strains will leave fewer attachment sites available for competitors [9]. Most colonisation studies have been carried out on plant–bacteria combinations related to agriculturally interesting plant species [11]. Studies on forest trees, by contrast, are scarce [2]. The composition and the colonisation ability of the rhizospheric bacterial community can be highly influenced by ectomycorrhizal fungi [12,13]. A large proportion of the carbon derived from photosynthesis in plants is transported to the external mycorrhizal mycelium, which can promote bacterial growth in the soil and ensure the maintenance of introduced bacteria [14,15]. Inoculation of mycorrhizal helper bacteria such as *P. fluorescens* strain BBc6 have been reported to improve mycorrhization and growth of Douglas fir in nursery [16].

The use of auto-fluorescent proteins in bacterial transformation and subsequent monitoring by epifluorescence (EFM) and confocal laser scanning microscopy (CLSM) are valuable tools for studying bacterial colonisation patterns and interaction with other micro-organisms in the rhizosphere [17,18].

Understanding the interactions of beneficial PGPR bacteria and mycorrhizal fungi will contribute to the development of more efficient methods for the production of mixed inocula and their application for plant growth promotion or soil amelioration purposes [19,20].

The aim of this work was to describe the colonisation pattern of *Pinus halepensis* roots by GFP-tagged *P. fluorescens* strain Aur6 and to determine whether co-inoculation with an ectomycorrhizal fungus could modify the bacterial colonisation behaviour. The effects of bacterial and fungal inoculation on early seedling growth were assessed independently and in co-culture.

2. Material and methods

2.1. Pseudomonas fluorescens *strain, transformation and inoculum preparation*

Strain Aur6 was firstly isolated from the rhizosphere of *Lupinus hispanicus* [21], and was identified as *P. fluorescens* by FAMEs (Microbial ID, Inc. Newark). Aur6 was transformed by electroporation (2.5 kV, 25 μF, 200 Ω, pulse duration 4.5 ms) with plasmid pHC60 that promotes constitutive expression of the green fluorescent protein GFP-S65T [22], and confers resistance to tetracycline. Transformed bacteria were cultured in Luria–Bertani (LB) plates supplemented with tetracycline (10 $\mu g\,ml^{-1}$) (LB-Tet). To obtain the bacterial inoculum, a single transformed *P. fluorescens* Aur6-GFP colony was transferred to 3 ml of liquid LB-Tet medium and incubated at 28 °C and 200 rpm overnight. This pre-inoculum was diluted with fresh liquid LB-Tet medium (1:20) and incubated at 28 °C and 200 rpm. Bacteria were collected by centrifugation (9820*g*, 10 min) and washed twice with sterile water to eliminate antibiotic excess. Bacteria were suspended in PBS buffer without antibiotic to achieve a final inoculum concentration of 10^8 cfu ml^{-1}.

Plasmid stability was tested in vitro by sub-culturing three times (20 bacteria generations) a chosen transformant colony in LB without antibiotic [23]. Plasmid stability in the rhizosphere of *P. halepensis* was also tested. Seedlings were inoculated with 5 ml of bacterial inoculum (10^8 cfu ml^{-1}) and roots were harvested 15 and 40 days after seedling inoculation. Bacterial suspensions from roots were obtained as described below (see Section 2.6) and plated in selective and non-selective LB.

2.2. Suillus granulatus *strain and inoculum preparation*

Suillus granulatus (L:Fr) O. Kuntze strain ccma-1 was isolated from sporocarps collected in a *P. halepensis* forest in Rivas-Vaciamadrid (Madrid, Spain). Pure cultures were grown in MMN medium [24], at 25 °C for one month. To obtain fungal inoculum, plugs of actively growing mycelium collected from the edge of the colonies were placed into liquid MMN medium (containing 5 $g\,l^{-1}$ glucose) and incubated at 25 °C for three weeks, with weekly manual shaking.

2.3. *Effect of* P. fluorescens *on fungal growth*

The effect of Aur6-GFP on *S. granulatus* growth was assayed in a factorial experiment using different glucose concentrations. Fungal plugs (one per Petri dish) were grown on a cellophane sheet, in 90 mm Petri dishes in MMN medium containing 0.1, 1 or 10 $g\,l^{-1}$ glucose. Once the fungus had started to grow, 10 μl of Aur6-GFP inoculum (10^8 cfu ml^{-1}) were added on each of the four radial axes of the plate, 2 cm from the fungal plug. Controls were performed in the same way by adding 10 μl of PBS. Fungal radial growth (the mean of the four radii values for each colony), and fresh weight were recorded after two weeks.

2.4. Short-term attachment assay

To check the influence of mycorrhization on the attachment ability of bacteria to pine roots, a short-term experiment was carried out as previously described [25]. Mycorrhizal seedlings were obtained using an in vitro system in Petri dishes [26]. *P. halepensis* seedlings were inoculated by placing four plugs of *S. granulatus* directly on the tap-root. The main root of the seedling was colonised by the fungus within two weeks, showing a well-developed mantle and Hartig net. Seedlings were grown in a climate chamber with a 15 h photoperiod of 250 μmol photon $m^{-2} s^{-1}$ and day/night temperature of 25/20 °C. After three weeks, similarly sized control non-mycorrhizal and mycorrhizal roots (selected when the main root was about 30% covered with fungal mantle) were submerged in Aur6-GFP inoculum (10^8 cfu ml^{-1}) and incubated at room temperature for up to four hours. Four roots were removed per treatment at different times (0, 15, 30, 60, 120 and 240 min), individually washed in 50 ml PBS five times by gentle shaking and ground in 1 ml PBS using pestle and mortar. In order to detect any contamination, controls were performed with roots that were not submerged in bacterial inoculum. Samples were serially diluted in PBS, and 100 μl aliquots were plated on LB-Tet medium. Plates were incubated for two days at 28 °C in the dark prior to colony counting.

2.5. Plant growth conditions and inoculation procedures

Pinus halepensis Mill. seeds were surface sterilized in 33% (v/v) H_2O_2 for 35 min and rinsed several times with sterile distilled water. Seeds were placed on 15% agar, stratified at 4 °C for 10 days and germinated at 15 °C in the dark. Axenic and pre-germinated *P. halepensis* seedlings (1–2 cm root) were transferred to 100 ml tubes filled with peat: vermiculite (1:10, v:v) and 25 ml of liquid MMN medium (containing 2.5 g l^{-1} glucose). Half of the seedlings were inoculated with *S. granulatus* inoculum. After ten days, when the fungus had colonised most of the substrate, half seedlings inoculated and non-inoculated with *S. granulatus* were supplied with 5 ml of Aur6-GFP inoculum (10^8 cfu ml^{-1}). Control seedlings were performed by adding 5 ml PBS per tube. Four seedling treatments with 25 replicates were established: (a) non-inoculated, (b) inoculated with *S. granulatus*, (c) inoculated with Aur6-GFP and (d) co-inoculated with *S. granulatus* plus Aur6-GFP. Tubes were wrapped in the bottom half with aluminium foil to protect roots and fungus from direct light and seedlings were incubated in a climate chamber with a 15 h photoperiod of 250 μmol photon $m^{-2} s^{-1}$ and day/night temperature of 25/20 °C.

2.6. Bacterial population dynamics on pine rhizosphere

Dynamics of bacterial colonisation were assessed by sampling five seedlings per treatment on day 1, 8, 20, 40 and 60 after inoculation. Roots were separated from shoots and cleaned free of substrate. Mycorrhizal percentages (mycorrhizal short roots/total number of short roots) were axenically assessed under the stereomicroscope. All seedlings were measured for tap-root length, number of lateral roots, epicoltyl length and number of needles. Seedling shoots were oven dried (60 °C, 48 h) to obtain shoot dry weights. The root of each seedling was divided in three parts: (a) shoot-root junction, (b) elongation and (c) apex, and a 1-cm root segment was sampled from each part, under axenic conditions. In parallel, 5 g of root-surrounding substrate were sampled per tube. Root segments and substrate samples were individually placed in tubes with glass beads filled with 2 or 25 ml PBS and vigorously shaken for two minutes. The resulting suspensions were serially diluted in PBS and 100 μl aliquots were plated in LB-Tet. Bacterial growth was recorded after two days at 28 °C in the dark. Dilutions yielding 30–100 colonies per plate were used for cfu determination. The persistence and distribution of bacteria on the root surface was monitored on the different root parts by epifluorescence microscopy (EFM) under blue light using a Zeiss Axiophot fluorescence photomicroscope, with a filter set consisting of a 450–490 nm band-pass excitation filter and a barrier filter with 590-long pass cut-off. Root samples were imbibed in PBS solution, covered with a glass slide and slightly pressed before observation under the microscope.

2.7. Statistical analysis

In all experiments, data were analysed by one-way ANOVA and differences among treatments were separated by DMS test ($P \leqslant 0.05$). Data of bacterial counts and percentages of mycorrhizas were log and arc-sin transformed prior to ANOVA.

3. Results and discussion

3.1. Transformation of P. fluorescens Aur6

Pseudomonas fluorescens Aur6 was readily transformed by electroporation with plasmid pHC60. Transformation did not affect bacterial growth rate compared with the wild strain (data not shown). The plasmid was highly stable in the rhizosphere of *P. halepensis*. Fifteen and 40 days after seedling inoculation, more than 94% of the bacteria recovered were carrying the plasmid, as evidenced by plating in selective medium. The plasmid pHC60 contains a 0.8-kb fragment of the stabilization region from the broad-host-range plasmid RK2, which

permits its maintenance in bacterial cells in the rhizosphere with no antibiotic selection [27]. Aur6 cells harbouring plasmid pHC60 strongly expressed GFP-S65T, thus allowing visualization of the bacteria on the root surface by epifluorescence microscopy.

3.2. Effect of P. fluorescens Aur6-GFP and glucose availability on fungal growth

The radial growth of *S. granulatus* was significantly increased as glucose concentration diminished (Fig. 1(a)). Fungal fresh weight was significantly reduced at the lowest glucose concentration (Fig. 1(b)). *P. fluorescens* did not affect fungal growth when glucose availability was high to moderate (Fig. 1(a) and (b)). However, at low glucose concentration the presence of bacteria significantly reduced fungal biomass (Fig. 1(b)). The relationship between the fungus and bacteria seemed to evolve from neutral to competitive depending on glucose availability. As suggested by different authors [5,28], the main mechanism involved in the Mycorrhizal Helper Bacteria (MHB) effect concerns the bacterial influence on the fungal growth. Our results indicated that Aur6 did not act as a Mycorrhizal Helper Bacteria (MHB) since it did not stimulated *S. granulatus* growth.

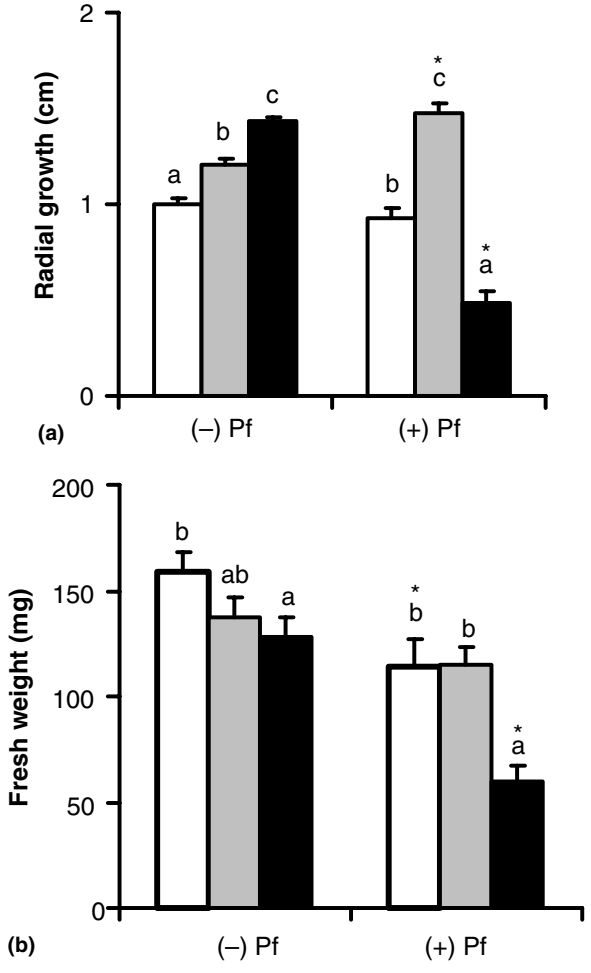

Fig. 1. Effect of *P. fluorescens* Aur6-GFP on radial growth (a) and fresh weight (b) of *S. granulatus* colonies, at different glucose concentrations (□ 10 g l^{-1}, ▩ 1 g l^{-1} and ■ 0.1 g l^{-1}), after 15 days. Different letters separately in treatments without (–) Pf and with (+) Pf bacteria denote significant differences among glucose concentrations. Asterisks denote significant differences between Pf treatments at identical glucose concentration, by DMS test ($P \leqslant 0.05$).

3.3. Short-term assay of bacteria attachment to pine roots

Pseudomonas fluorescens Aur6-GFP attached in significantly higher numbers to *P. halepensis* roots when they were mycorrhizal with *S. granulatus* (Fig. 2). In non-mycorrhizal seedlings, the number of root-attached bacteria remained constant at the different times, while in mycorrhizal roots it quickly decreased during the first 60 min to remain constant thereafter. Bacteria can immediately adhere in a non-specific way to solid surfaces, including inert ones, as a mechanism to avoid dispersion by soil watering [3]. Stable polysaccharidic and proteic links are then formed among bacteria, hyphae and root surfaces [29,30]. Our results indicated that hyphae within the mantle of *S. granulatus* are adequate for mechanical retention of bacteria. The intricate net of hyphae forming the mantle has been also demonstrated to improve not only bacterial attachment but also biofilm formation [20,29,31]. It is important to note that the percentage of root surface colonised by the fungus can highly influence the quantity of attached bacteria and data could vary with this parameter. In bacterial counts, we did not consider the fungal extramatrical mycelium, which usually provides an extensive habitat for bacteria proliferation [15].

3.4. Colonisation and localisation of P. fluorescens Aur6-GFP on the rhizosphere of P. halepensis

Microscopic observations of *P. fluorescens* Aur6-GFP colonisation of pine roots showed a similar distri-

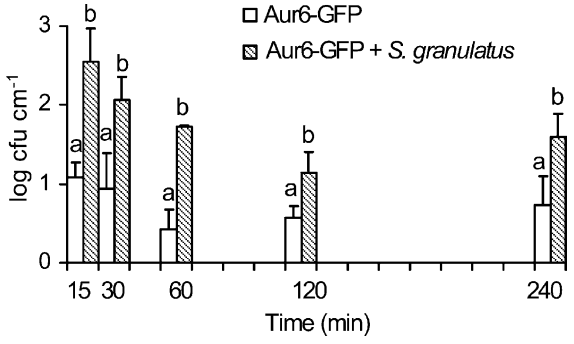

Fig. 2. Attachment of *P. fluorescens* Aur6-GFP to non mycorrhizal and mycorrhizal *P. halepensis* roots with the fungus *S. granulatus*. At each point in time, different letters denote significant differences between treatments, according to DMS test ($P \leqslant 0.05$).

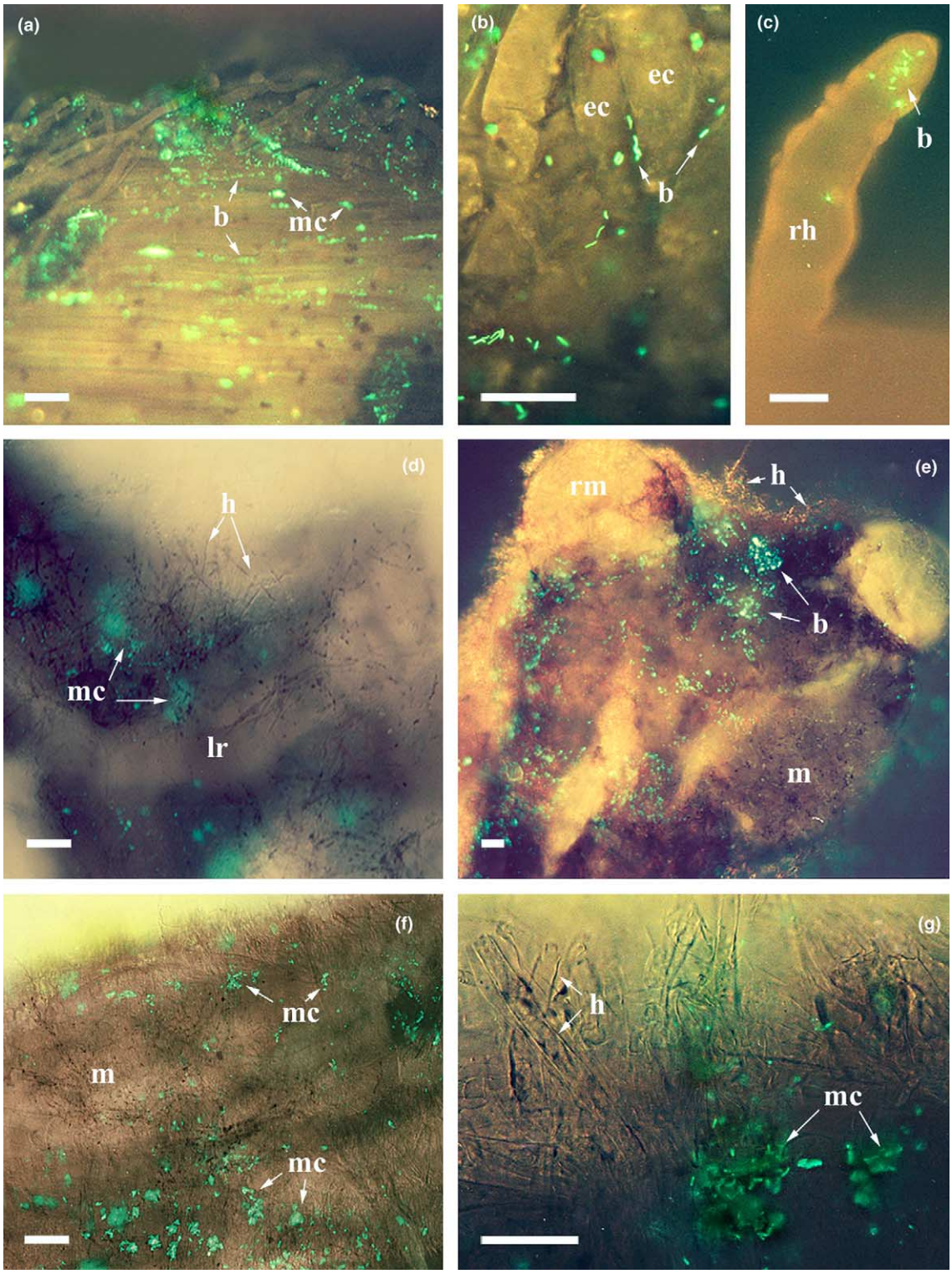

Fig. 3. Colonisation of *P. halepensis* roots by *P. fluorescens* Aur6-GFP in absence (a–c) or presence (d–g) of *S. granulatus*, monitored by epifluorescence microscopy one month after bacterial inoculation. (a) Bacteria located in rows, forming microcolonies on a non-mycorrhizal, lignified root; (b) bacteria between epidermal root cells; (c) bacteria on the apex of a root hair; (d) bacteria within the fungal mantle at the base of a lateral root; (e) bacteria forming microcolonies on a mycorrhizal, dichotomous lateral root; (f) and (g) detail of bacteria forming microcolonies within the mantle of a mycorrhizal root. b = bacteria, mc = microcolony, ec = epidermal root cells, rh = root hair, h = hyphae, rm = root meristem, lr = lateral root, m = mantle. Bar (a,d,e,f) = 50 μm. Bar (b,c,g) = 20 μm.

bution of bacteria on the three parts of the roots (shoot-root junction, elongation zone and apex) during the first 20 days of the experiment. After 40 days, most bacteria were located in the upper two-thirds of *P. halepensis* roots and were scarcer in the apex. The spatial colonisation pattern of *P. fluorescens* Aur6-GFP was not modified when roots were mycorrhizal with *S. granulatus*, indicating the compatibility between both microorganisms. Fungal-bacteria compatibility has been pointed out as an important aspect in the colonisation of plant mycorrhizosphere by bacteria [14–32]. In both mycorrhizal and non-mycorrhizal roots, bacteria were mainly located on long lignified roots (Fig. 3(a), (b) and (d)) and at the base of emerging lateral roots (Fig. 3(d) and (e)). When bacteria localised on long lignified roots in the absence of fungus, they formed rows and microcolonies between epidermal cells (Fig. 3(a) and (b)). In non-mycorrhizal roots, bacteria were occasionally observed on root hairs (Fig. 3(c)). In mycorrhizal roots, bacteria were located on and within the fungal mantle, usually forming microcolonies (Fig. 3(d)–(g)). Similar bacterial colonisation patterns have been described for other pine species using different microscopic techniques [14–35]. The presence of microcolonies was indicative of active bacterial growth on pine rhizosphere and mycorrhizosphere [12–36]. Intracellular localisation of bacteria described for other bacteria–conifer combinations [37,38] was not observed in our examinations.

Quantitative measurements of the bacterial population during the first 20 days of the experiment showed persistence of *P. fluorescens* Aur6-GFP on pine roots at densities close to 10^6 cfu cm^{-1}, irrespective of whether or not the root was mycorrhizal with *S. granulatus* (Fig. 4(a)–(c)). After 40 days, total bacterial population significantly decreased in both non-mycorrhizal and mycorrhizal roots. Root lignification and limited growth in the confined environment of the test tubes (due to limited space, depletion of nutrients and accumulation of excretion products) are possible causes for bacterial population diminution and redistribution on roots between days 20 and 40. Variations in bacterial densities along the root and over time have been related to patterns of root exudates composition and concentration [11] and reduction of *P. fluorescens* density in the rhizosphere has been often reported [19–36]. Interestingly, mycorrhizal roots showed higher bacteria densities than non-mycorrhizal ones (Fig. 5(a)–(c)), indicating that the fungal mantle provides an additional support and niche for bacteria. Similarly to what was observed with the roots, bacterial density in the substrate significantly decreased by two orders after 40 days in both mycorrhizal and non-mycorrhizal treatments (Fig. 4(d)). After 60 days, significantly higher bacterial counts were recovered from substrate of mycorrhizal roots compared to

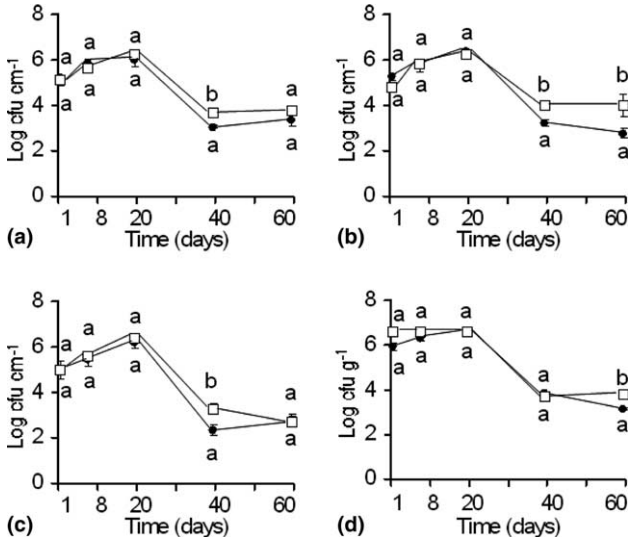

Fig. 4. Colonisation dynamics of *P. fluorescens* Aur6-GFP of non-mycorrhizal (●) and mycorrhizal (□) *P. halepensis* rhizosphere. (a) Shoot-root junction, (b) root elongation zone, (c) apical root zone and (d) rhizospheric soil. Different letters denote significant differences between simultaneous treatments, by DMS test ($P \leqslant 0.05$).

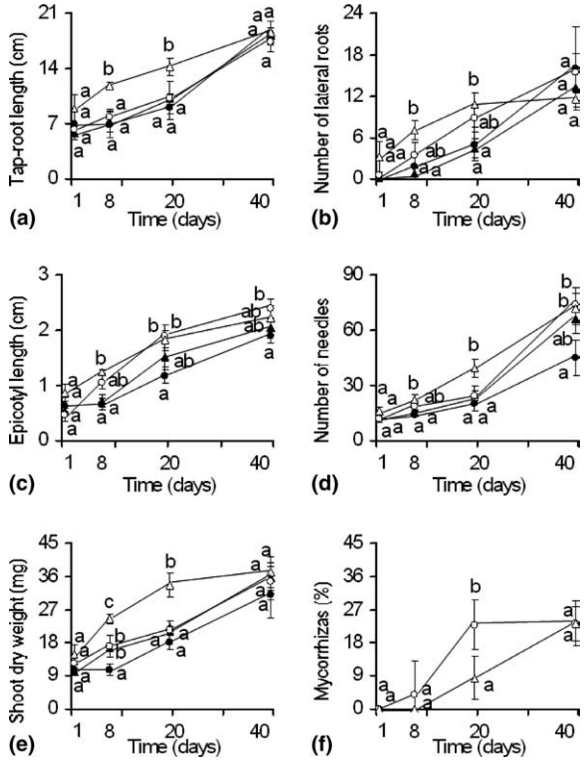

Fig. 5. Effect of inoculation and co-inoculation with *P. fluorescens* Aur6-GFP and *S. granulatus* on *P. halepensis* growth. (●) Non-inoculated control; (○) mycorrhizal with *S. granulatus*; (▲) inoculated with *P. fluorescens* Aur6-GFP; (△) co-inoculated with Aur6-GFP and *S. granulatus*. (a) Tap-root length, (b) number of lateral roots, (c) epicotyl length, (d) number of needles, (e) shoot dry weight and (f) percentage of mycorrhizas (mycorrhizal short roots/total short roots). In each time, different letters denote significant differences between mycorrhizal and non-mycorrhizal treatments, by DMS test ($P \leqslant 0.05$).

that of non-mycorrhizal ones (Fig. 4(d)). Root exudates can be quantitatively and qualitatively modified in the mycorrhizosphere [16–39], thus allowing bacteria to maintain a more active metabolism and survive longer on roots. Bacteria can also use fungus-derived energy sources exuded by the ectomycorrhizal fungus, and even use the senescing mycelium as nutrient source [15,40,41].

Microscopy observations and bacterial colonization dynamics indicated that *P. fluorescens* Aur6 was able to colonize the *P. halepensis* roots and to grow actively in the pine rhizosphere. The colonisation efficiency of this fluorescent pseudomonad strain has been previously described for several plant species such as lupin [21], pepper [42], pine and oak [43], demonstrating its non-specific character.

3.5. Effects of Aur6-GFP and S. granulatus on pine growth

At day 20, seedling tap-root length, the number of lateral roots, the number of needles, and the shoot dry weight were not affected by inoculation with Aur6-GFP or *S. granulatus* separately, whereas these parameters were significantly increased when both bacteria and fungus were co-inoculated (Fig. 5(a), (b), (d) and (e)). The epicotyl length was significantly stimulated by the fungus alone or when seedlings were co-inoculated with Aur6-GFP (Fig. 5(c)). After 40 days, the tap-root length, the number of lateral roots and the shoot dry weight were equalled in all treatments (Fig. 5(a), (b) and (e)).

Pseudomonas fluorescens Aur6 has been reported to promote pine growth under different culture conditions [43]. The strain Aur6 has been showed to produce auxin and siderophores able to mobilise iron from chelating substances [43]. In our experiments, neither *S. granulatus* nor Aur6-GFP promoted pine seedling growth when inoculated alone. The establishment of a large bacterial population may not be essential for bacterial promotion of plant growth, and other biotic and abiotic factors may influence this ability [44]. In our experimental conditions, the restricted extension of root and mycelium within the test tube and variations in the nutrient composition of the medium could have limited fungal and bacterial plant growth promotion abilities as well as the growth of the microorganisms directly. Interestingly, when co-inoculated, fungus and bacteria displayed a synergistic effect on plant growth during the first 20 days. Interaction between both microorganisms probably amplified plant growth promotion traits such as auxin production. Barazani and Friedman [45] suggested that auxin concentration can determine the extent of inhibition and promotion of plant growth by PGPR bacterial strains. We can hypothesize that fungus and bacteria together produced auxin at adequate concentration to stimulate plants growth. Synergistic effects of mycorrhizal fungus-pseudomonad associations have been described in numerous agronomical interesting plant species [4,19,36], and for some tree species [14,15,46]. When *P. halepensis* seedlings grew older (40 days), the synergistic effect of co-inoculation was not maintained. These results suggest that the initial non-competitive interaction between bacteria and fungus could have turned with time into a competitive one under conditions of nutrient depletion and root lignification. The competition for nutrients and root exudates not only would have led to a significant diminution of bacterial density, but it could also minimise the microbial effects on plant growth [19]. Root lignification could also limit the quantity and quality of exudates affecting bacterial numbers and probably fungal growth.

The number of ectomycorrhizae formed by *S. granulatus* on *P. halepensis* short roots after 20 days was significantly diminished when co-inoculated with Aur6-GFP. However, there were no significant differences between treatments at the end of the experiment (Fig. 5(f)). These results together with those obtained with both microorganisms growing in Petri dish, indicated that Aur6-GFP did not act as a mycorrhizal helper bacterium (MHB), opposed to what has been reported for other *P. fluorescens* strains [5,28,36].

Results from in vitro experiments cannot be extrapolated to non-sterile conditions where competition with other microorganisms can alter colonisation patterns and interactions. Further research under nursery and field conditions in the presence of indigenous microflora will be conducted. A better understanding of the complex biotic interactions in the rhizosphere is essential for efficient exploitation of microorganisms as biofertilizers, biocontrol agents and in soil amelioration.

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