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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.