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Three native *Pseudomonas fluorescens* strains tested under growth chamber and field conditions as biocontrol agents against damping-off in alfalfa

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ABSTRACT

Alfalfa (*Medicago sativa*) is one of the most important crops used in Uruguay for livestock feeding. Seedling diseases, particularly damping-off, are a critical factor which limits its establishment. Three native *Pseudomonas fluorescens* strains, UP61.2, UP143.8 and UP148.2, previously isolated from *Lotus corniculatus*, were evaluated to determine their efficacy as biological control agents for alfalfa seedling diseases in the field. Their compatibility with the alfalfa-*Sinorhizobium meliloti* symbiosis was also assessed. In growth chamber conditions seed inoculation with *Pseudomonas* strains did not affect different parameters of alfalfa-rhizobium symbiosis as shown by nodulation rate and shoot dry weight of plants. The presence of the commercial inoculant strains of *S. meliloti* did not impair colonization by the *P. fluorescens* and vice versa. In field trials the dynamics of rhizobial rhizospheric populations were not affected by the presence of *P. fluorescens*. Each *P. fluorescens* strain successfully colonized alfalfa roots at adequate densities for biocontrol activity. Results showed that *P. fluorescens* strains provided a 10–13% increase in the number of established plants relative to the control, an intermediate result compared to the fungicide treatment (24%). The alfalfa above-ground biomass was increased by 13% and 15–18% in the presence of the fungicide and *P. fluorescens* strains, respectively. Therefore, results from this study demonstrated that the three *P. fluorescens* strains provided effective control against soil-borne pathogens and suggest a potential use in the development of a commercial inoculant to be applied for the control of legume seedling diseases.

1. Introduction

Forage legumes play an essential role in the productivity and sustainability of Uruguayan production systems. Their symbiotic association with rhizobia makes the atmospheric nitrogen available for themselves and other crops in the rotation. Alfalfa (*Medicago sativa*) is an important forage crop owing to its unique characteristics: high yield of excellent quality forage, hydric-stress tolerance and good persistence. However, rapid seedling emergence and adequate pasture establishment are crucial to maximize its potential. Seedling diseases caused by soil-borne pathogens, primarily *Pythium* spp. and other Oomycetes, are a critical factor which limits alfalfa establishment causing pre- and/or post-emergence seedling damping-off. The most favourable environmental conditions for disease development are low soil temperature and high soil moisture until 20–30 days after sowing (Martin and Loper, 1999; Pérez et al., 2000). After this period seedling is mature enough to have defenses against damping-off pathogens (Altier and Thies, 1995).

The indiscriminate use of chemical fungicides is not recommended for the management of alfalfa diseases because of their collateral adverse effects on the environment, along with negative effects on animal and human health. Moreover, their efficacy has been reduced by the appearance of microbial resistance (Cook and Zhang, 1985; Sanders, 1984) and their detrimental effect on the biological nitrogen fixation by rhizobia (Altier and Pastorini, 1988). A high-density sowing practice is usually the strategy followed by farmers to cope with alfalfa damping-off, but this approach significantly increases pasture establishment costs. Alternatively, biological control is a viable strategy for crop protection to be used in the framework of an integrated pest management program, by means of a rational use of natural resources, leading to a decline in synthetic pesticides usage and in sowing density.

Pseudomonas spp. are one of the most promising groups of rhizospheric inhabitants which are able to control pathogenic soil-borne microorganisms (O'Sullivan and O'Gara, 1992). They

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show antagonistic activity against diverse phytopathogens such as *Pythium* spp. (Loper, 1988), *Rhizoctonia* spp. (Howell and Stipanovic, 1979; Nagarajkumar et al., 2005), *Fusarium* spp. (Olivain et al., 2004) and *Gaeumannomyces* spp. (Thomashow and Weller, 1988; Sari et al., 2006). Little research has been carried out on the use of rhizospheric microorganisms to control legume diseases caused by soil-borne pathogens (Kraft and Papavizas, 1983; Marten et al., 1989; Parke et al., 1991; Pérez et al., 2000). However, the use of *Bacillus* spp. (Handelsman et al., 1990), *Streptomyces* spp. (Jones and Samac, 1996; Xiao et al., 2002) and *Pseudomonas* spp. (Orlandini and Signorini, 1993; Villacieros et al., 2003) has been previously described for alfalfa.

Our group have selected three native *Pseudomonas fluorescens* strains (UP61, UP143 and UP148), isolated from the rhizosphere of birdsfoot trefoil (*Lotus corniculatus*) plants, collected from different agro-ecological regions in Uruguay. These strains presented a wide range of *in vitro* antagonism against bacteria and phytopathogenic fungi and were able to protect birdsfoot trefoil from the infection caused by *Pythium ultimum* and *Rhizoctonia solani in vivo*, under controlled conditions (Bagnasco et al., 1998). They produce siderophores and hydrogen cyanide (HCN) and the production of other antimicrobial factors by two of them was showed by chromatography and spectrometry techniques. Strain UP148 produces a phenazine-derivative antifungal compound not previously described, which is involved in its biocontrol activity (Bajsa et al., 2005). *P. fluorescens* UP61 produces the antibiotics 2,4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin (De La Fuente et al., 2004). Under controlled conditions, the simultaneous inoculation of birdsfoot trefoil with *Mesorhizobium loti* and strains UP61, UP143 or UP148 showed no effect in shoot dry weight nor in the rate of nodulation by the diazotroph (Bagnasco et al., 1998; De la Fuente et al., 2002). No effect was detected in rhizobial colonization of birdsfoot trefoil or alfalfa rhizospheres when co-inoculated with UP61 (De La Fuente et al., 2002). Previous data from field trials demonstrated that inoculation of birdsfoot trefoil seeds with these *P. fluorescens* strains resulted in an increased dry matter yield compared to an un-inoculated control (Pérez et al., 2000).

This background suggests the potential of these *P. fluorescens* strains to be used as biological control agents against damping-off in forage legumes. The main objective of our research is to cope with seedling diseases of major forage legumes in our country, by means of an environmental-friendly innovative technology. Owing to the rhizobial inoculation practice widespread among Uruguayan farmers, this technology would be rapidly adopted.

The aim of this study was to validate in alfalfa, the efficacy of the three native *P. fluorescens* strains which showed biocontrol activity in the birdsfoot trefoil-*Pythium ultimum* pathosystem.

2. Materials and methods

2.1. Microorganisms and culture conditions

Pseudomonas fluorescens strains UP61.2, UP143.8 and UP148.2 are spontaneous rifampicin (rif) resistant mutants of the native strains UP61, UP143 and UP148, which were previously isolated from *L. corniculatus* (Bagnasco et al., 1998; De La Fuente et al., 2002). These mutants showed the same growth characteristics in minimal medium and antagonistic activity as their parental strains. *Sinorhizobium meliloti* MCH3 and LP21 are commercial inoculant strains for alfalfa (Laboratorio de Microbiología de Suelos y Control de Inoculantes, Uruguay) and *S. meliloti* strains M10 and L5 are double spontaneous rif and kanamycin (kam) resistant mutants of each commercial strain, respectively. The strains M10 and L5 showed the same growth characteristics in minimal medium, nodulation rate and nitrogen fixation efficiency as their parental strains. *P. fluorescens* and *S. meliloti* were cultured in King's B (KB; King et al., 1954) for 2 days and Tryptone Yeast Extract (TY; Beringer, 1974) for 3 days, respectively. Media were sterilized in autoclave for 20 min at 121 °C. When corresponded, rif, kam and/or benomyl (ben) were added after sterilization at concentrations of 50 μg/ml for rif or kam, and of 500 μg/ml for ben. The latter was selected by its antifungal action when analyzing natural soil samples. Bacterial strains were cultured in an orbital shaker (150 rpm) at 27 °C or incubated at 30 °C and culture stocks were stored in glycerol (25% v/v) at −20 and −80 °C.

2.2. Growth chamber assays: *P. fluorescens*–*S. meliloti* interactions

2.2.1. Nodulation rate and shoot dry weight in test tubes

Seeds were sterilized and aseptically sown in glass tubes (20 × 2.5 cm) containing Jensen medium (Vincent, 1970) as described in Bagnasco et al. (1998). After a week three treatments were applied: co-inoculation with a mixture of *S. meliloti* (strains MCH3 and LP21) and with each of the *P. fluorescens* studied (strains UP61.2, UP143.8 or UP148.2), previously grown for 72 and 36 h in liquid medium, respectively. Each experiment included three controls: (i) without bacterial inoculants; (ii) inoculated only with the mixture of *S. meliloti* and (iii) un-inoculated control added with 0.05% w/v KNO_3. The final bacterial concentration was 2.0×10^7 CFU of each species per tube. Ten tubes (each containing two seeds) were used per treatment and three independent experiments were performed. The number of nodulated plants and the number of nodules per plant were daily registered for ca. a month. A plant was considered nodulated when it presented at least one nodule. Forty-three days after sowing, plants were harvested, dried at 60 °C overnight and their shoot dry weight measured.

2.2.2. Seed bacterization

Alfalfa seeds used for assays performed in soil (nursery tray, pot and field trials) were bacterized using methylcellulose. *P. fluorescens* and *S. meliloti* strains were plated onto KB or TY media. After 2 and 3 days, respectively, bacterial lawns were suspended in phosphate buffer (0.1 M, pH 7.0). Cells were harvested by centrifugation, re-suspended in 1 ml 1.5% w/v methylcellulose and mixed with 3 g of alfalfa seeds. Inoculated seeds were dried under laminar flow for 12 h and stored at 4 °C. Before sowing, the bacterial inocula present per seed were estimated by plating on KB^{rif} or $TY^{rif,kam,ben}$ serial dilutions made from suspensions of 30 seeds in 10 ml 0.15 M NaCl.

2.2.3. Shoot dry weight in pots

Fifteen bacterized alfalfa seeds (see Section 2.2.2) were sown in 250-ml plastic pots containing a mixture of commercial soil and sand (3:1). Treatments were the same as those described for test tubes: seeds co-inoculated with a mixture of *S. meliloti* (MCH3 and LP21) and also with each of the three *P. fluorescens* strains studied. Each experiment included two controls: (i) without bacterial inoculants and with methylcellulose added and (ii) inoculated only with the mixture of *S. meliloti*. Each treatment consisted of six replicates and the experiment was repeated three times. At sowing, bacterial concentrations were ca. 10^5 CFU/seed for each bacterial species. After three months shoot dry weight was determined.

2.2.4. Rhizosphere colonization and persistence in nursery trays

Seeds were bacterized as described in Section 2.2.2. with each *P. fluorescens* strain singly or together with a mixture of *S. meliloti* M10 and L5. A treatment containing only the M10-L5 mix (M) was included. At sowing, the bacterial density ranked from 7.8×10^4 to 4.5×10^6 CFU of *S. meliloti* per seed and 5.1×10^5 to 7.7×10^7 CFU of *P. fluorescens* per seed.

Five treated seeds per well were sown in a 72-well plastic nursery tray containing soil and sand as previously described for pots.