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Method to evaluate disease supression against *Pythium debaryanum* and alfalfa growth promotion by fluorescent *Pseudomonas*

Método para evaluar protección contra *Pythium debaryanum* y promoción del crecimiento de alfalfa por *Pseudomonas* fluorescentes

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Abstract

Fluorescent *Pseudomonas* have been extensively reported as effective biocontrol agents and can be used to manage *Pythium* seedling diseases in alfalfa. An *in vivo* methodology was developed to evaluate fluorescent *Pseudomonas* isolates for their ability to suppress disease and promote plant growth in the *alfalfa-Pythium* pathosystem. The standard method consists of a bioassay performed in growth chamber under controlled conditions of temperature and photoperiod. Alfalfa seeds were sown in commercial substrate and subsequently treated as follows: no *Pseudomonas,* no *Pythium debaryanum* (germination control); inoculated only with *P. debaryanum* (disease control); each one of the *Pseudomonas* isolates co-inoculated with *P. debaryanum.* An augmented experiment design was obtained by selecting a random complete blocks design for four checks systematically evaluated on 12 bioassays, and enlarging the blocks to accommodate up to 16 treatments per assay. Disease control recorded an emergence of 33.2% on day 15 after sowing date, as compared with germination control (100%). The 101 fluorescent *Pseudomonas* isolates showed a wide response on disease suppression ability. Twelve percent of tested isolates reasonably protected alfalfa plants against P. *debaryanum,* recording an emergence of over 60%. A similar procedure, in the absence of the pathogen, was used to evaluate alfalfa growth-promoting ability of selected *Pseudomonas* isolates. Five isolates exhibited a significant biocontrol activity against P. *debaryanum* and four of them also recorded a significant plant growth-promoting effect. Both bioassays were appropriate to identify candidate *Pseudomonas* isolates to be further tested under field conditions.

Keywords: biocontrol, Medicago sativa L., PGPR, Pseudomonas, Pythium

Resumen

Fluorescent *Pseudomonas* have been extensively reported as effective biocontrol agents and can be used to manage *Pythium* seedling diseases in alfalfa. An *in vivo* methodology was developed to evaluate fluorescent *Pseudomonas* isolates for their ability to suppress disease and promote plant growth in the *alfalfa-Pythium* pathosystem. The standard method consists of a bioassay performed in growth chamber under controlled conditions of temperature and photoperiod. Alfalfa seeds were sown in commercial substrate and subsequently treated as follows: no *Pseudomonas,* no *Pythium debaryanum* (germination control); inoculated only with *P. debaryanum* (disease control); each one of

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Palabras clave: control biológico, Medicago sativa L., promoción del crecimiento vegetal, Pseudomonas, Pythium

INTRODUCTION

Forage legumes bring great benefits to agricultural production systems. Not only do they represent a high-quality feed source for livestock, but they also supply nitrogen to soils through symbiotic association with rhizobium, which can be harnessed by other components of mixed pastures or by rotating agricultural crops. Among forage legumes, alfalfa *(Medicago sativa* L.) shows outstanding qualities. It has a high nutritional content and maintains high-quality values throughout its cycle. It is a summer-growing crop, very persistent and tolerant to drought, which allows achieving excellent productive yields. For these reasons, alfalfa has been revalued by agricultural producers, especially dairy farmers and intensive greenhouse producers. (Rebuffo, 2000).

Alfalfa crop establishment requires a high investment due to the price of the seed, sowing densities and herbicides and phosphorus requirements. Consequently, the crop establishment stage is critical to amortize the cost of implantation and capitalize on the advantages offered by this legume (Formoso, 2000; Rebuffo, 2000). Emergence failures and seedling death are frequently observed, associated with infections caused by oomycetes of the genus *Pythium*, responsible for the 'damping-off' (Altier, 1996; Hancock, 1996, Stuteville & Erwin, 1990). In the pre-emergence stage, the infection causes the seed to rot, preventing seed germination. Post-emergence infection is characterized by necrosis of the radicle and hypocotyl and loss of turgidity, leading to the collapse of the plant (Hancock, 1996). These diseases occur when environmental conditions are unfavorable for the rapid emergence and establishment of plants, such as excessive rainfall, moisture and low soil temperatures (Altier, 2000).

Losses caused by alfalfa implantation diseases have accounted for recent research on possible management strategies (Altier & Thies, 1995; Jones & Samac, 1996; Larkin and others, 1995). Diseases can be controlled by direct seed treatment with agrochemicals such as metalaxyl (Apron Novartis, commercial formulation), which is a systemic action fungicide that has no adverse effects on rhizobium (Leath and others, 1996). However, isolates of *Pythium* resistant to this fungicide have been reported (Cook & Zhang, 1985; Sanders, 1984) so its effectiveness may be compromised.

Moreover, the harmful effects on the environment and animal health must be added to this.

The use of genetically resistant cultivars and biological control of pathogens are the possible alternatives for disease management in integrated and organic production systems or in those where traceability of the final product is required, and the exploitation of natural resources is rational and environmental conservation is taken into account. Obtaining germplasm with marked resistance to *Pythium* has not yet yielded promising results (Altier& Thies, 1995; Hancock, 1996). Biological control is therefore a viable alternative and less aggressive for the environment than the use of agrochemicals. A large number of microorganisms have been described as plant pathogen controllers, including those belonging to the genus *Pseudomonas* (Martin & Loper, 1999). Within this genus, some species of fluorescent *Pseudomonas* can be classified as PGPR *(Plant Growth Promoting Rhizobacteria*). *P. fluorescens, P. putida* and *P. aureofasciens* are frequently found in the rhizosphere and have a great capacity to colonize roots (Palleroni, 1984), which makes them good candidates for the production of inoculants.

These bacteria can control different genera of phytopathogenic fungi such as *Pythium* (Loper, 1988; Martin & Loper, 1999), *Rhizoctonia* (Bagnasco and others, 1998), *Gaeumannomyces* (Thomashow & Weller, 1988; Weller and others, 2002) and others, indirectly favoring plant growth. Rhizobacteria can promote plant growth through more direct

mechanisms such as the production of plant hormones and increasing availability of soil nutrients, among others (Gerhardson & Wright, 2002).

IIBCE Microbial Ecology Laboratory has a collection of native fluorescents *Pseudomonas* that were isolated from the rhizosphere of healthy alfalfa plants, collected from three different regions of Uruguay, within the framework of the IFS project No. C/2945-1 "Native fluorescent *Pseudomonas* as biocontrol agents of alfalfa seedling diseases". The 702 isolates in the collection were characterized regarding their *in vitro* antagonism capacity against *Pythium* spp. and the presence of genes for the biosynthesis of antibiotics with biocontrol activity.

In vitro characterization of microbial interactions does not necessarily reflect the natural processes that occur in the rhizosphere of plants, so it is essential to develop a standard methodology that allows the evaluation of disease protection capacity from bacterial isolates at a more complex level of the interaction plant-pathogen-biological control agent. This methodology should be efficient and provide reproducible and reliable results while maintaining similar conditions to the natural environment where such interactions take place. In this way, it is possible to broaden existent knowledge of native rhizobacteria - a largely unexplored natural richness of our soil (Fabiano and others, 1994). The approach of study systems involving the affected plant, pathogen and beneficial microorganism has been reported for alfalfa and soy, co-inoculated with *Phytophthora* spp. and *Streptomyces* spp. (Xiao and others, 2002). In this work, the tests were carried out in tubes with sterile vermiculite, under controlled conditions of temperature and photoperiod.

The objectives of this study were to:

(i) develop a method for evaluating the capacity of isolated disease protection of fluorescent Pseudomonas in the alfalfa-Pythium debaryanum system; (ii) develop a method for evaluating the plant-growth promotion capacity of fluorescent Pseudomonas isolates; (iii) characterizing the aforementioned collection of native fluorescent Pseudomonas using both methods

MATERIAL AND METHODS

Microorganisms and crop conditions

The 101 isolates used in this study were selected from a collection of 702 isolates of native fluorescent *Pseudomonas*, obtained from the rhizosphere of healthy alfalfa plants collected in influence areas of INIA La Estanzuela (National Institute of Agricultural Research, Colonia), EEMAC (Experimental Station "Dr. Mario A. Cassinoni", Paysandú) and UEG-INIA Tacuarembó (Glencoe Experimental Unit, Paysandú), within the framework of the IFS project No. C/2945-1 "Native fluorescent *Pseudomonas* as biocontrol agents of alfalfa seedling diseases". The selection was based on the ability to inhibit the growth of *Pythium debaryanum in vitro,* and the presence of genes for the biosynthesis of antibiotics that have been reported in *Pseudomonas fluorescens* as responsible for biocontrol activity. The strains of *P. fluorescens* UP61, UP143, and UP148, originally isolated from the rhizosphere of healthy plants of Lotus corniculatus L., were also included (Bagnasco and others, 1998; Pérez and others, 2001). Isolates and strains of P. fluorescens were kept in 25% glycerol at -20°C and grown in King B Medium (KB, King and others, 1954). Pythium debaryanum Pyl strain belongs to the INIA Las Brujas collection of pathogenic microorganisms. This isolate is kept in Corn Meal Agar discs (CMA, Difco®) in sterile water.

Preparation of fungal inoculum

The fungal inoculum (mycelium and sporangium) was obtained as follows: a 3mm diameter disc was extracted from *Pythium debaryanum* mycelium grown 2-3 days in CMA, and placed in the center of a Petri dish containing 1.5% Water Agar (WA). It was incubated at 22°C for 3 days and, once the surface was colonized, the agar was homogenized in a blender (8 plates/1000 ml of sterile water).

Preparation of bacterial inoculum

The bacterial inoculum was obtained by incubating the isolates of fluorescent Pseudomonas in tubes containing 10 ml of KB with stirring at 27°C overnight, up to a concentration of approximately 1.5 x 109 cells per ml of culture.

Finally, the 10 ml of culture were diluted in 140 ml of sterile water.

Disease protection capacity caused by P. *debaryanum*

Disease protection tests were performed in growth chamber at 12º C, with a photoperiod of 10 hrs. Plastic foam (BROMYROS) was used for seedlings, whose cells have an opening of 4 x 4 cm, a depth of 8 cm, and an approximate volume of 50 ml. The experimental unit consisted of 12 cells with a rectangular arrangement of 4 x 3 cells. Ten alfalfa seeds were seeded per cell, containing commercial plant substrate (Floragard, TKS 1 Instant, Germany). This substrate, 100% fine-structured white peat, has all the necessary nutrients and oligonutrients at low

concentrations [N (70-150 mg/L), P_2O_2 (80-180 mg/l) K_2O (140-220 mg/l), salt (0.5-1.1 g/l)], an optimal pH of 5.2-6.0 (CaCl2), and an *instant* system (patented) that allows immediate absorption and uniform distribution of water.

The treatments were as follows: without *P. debaryanum* and without *Pseudomonas* (germination control); with *P debaryanum* and without *Pseudomonas* (disease control); each of the 101 isolates of *Pseudomonas* and the strains of *P. fluorescens* UP61, UP143 and UP148 co-inoculated with *P. debaryanum.*

To inoculate with *P. debaryanum* , 5 ml of inoculum/cell was applied the day before sowing and 3 ml of inoculum/cell the day after sowing. For inoculation with *Pseudomonas* 3 ml of inoculum/cell (approximately 3 x 10^8 bacteria) was applied on the day of sowing. The irrigation was carried out daily with running water.

Twelve bioassays were performed, all of which were considered as an augmented experimental design (Federer, 1991; Federer & Raghavarao, 1975). This was obtained by selecting four systematically evaluated treatments among the 12 tests, in randomized complete blocks with 4 replications (corresponding to each shelf of the growth chamber). The block was expanded to locate the isolates of the collection until reaching a total of 16 treatments per bioassay. The systematically evaluated treatments were: germination control, disease control, T683 isolate with high disease protection capacity and T655 isolate with low protective capacity.

The number of healthy emerged plants was recorded at 7, 9, 11, 13, 15, and 22 days post-sowing. The recorded value of germination control was expressed as base 100, and the obtained emergence for each bacterial isolate was expressed as a percentage relative to base 100.

Plant growth promotion capacity

Alfalfa growth promotion tests were performed in a growth chamber at 12°C, in a photoperiod of 11 h. The same methodology as the one described for the disease protection trial was used, plastic foam seedling, 12-cell experimental unit (4 x 3 cell rectangular arrangement), experimental design of randomized complete blocks with 4 replications, and sowing of 10 alfalfa seeds per cell on commercial plant substrate.

In this case, the treatments were: germination control (without *Pseudomonas)*and 14 isolates of fluorescent *Pseudamonas* that showed the best behavior in terms of disease protection capacity. For inoculation with *Pseudomonas* 3 ml of inoculum/cell (approximately 3 x10^8 bacteria) were applied on the sowing day. Irrigation was carried out daily with running water. Two consecutive tests with the same isolates were performed, therefore, it was not considered an augmented experimental design. Alfalfa plants were kept for 35 days under the described conditions, then they were harvested and carefully separated from the substrate. The number of emerged plants (NP) and biomass per experimental unit (fresh weight, FW). The material was oven dried at 60°C for 48 hours to get the dry weight (DW) per experimental unit.

Statistical analysis

The analyzed variables for the disease protection trial were emergence at day 15 (D5) and emergence at day 22 (D22). Number of plants at day 35 (NP), fresh weight per experimental unit (FW) and dry weight per experimental unit (DW) were the variables for the growth promotion trial.

For both trials, the mean was estimated and adjusted according to a mixed model that considers the treatment effect as fixed, and the trial effect, the nested block effect in trial and the interaction treatment x trial effect, as random:

$$y_{ijk} = \mu + \tau_i + \alpha_j + \beta_k(\alpha_j) + (\tau\alpha)_{ij} + \varepsilon_{ijk}$$

The model was analyzed using the SAS MIXED procedure, which uses REML *(Restricted Maximum Likelihood)* to estimate variance components of random effects. "PROC MIXED for *Recovering Interblocking Information"* (Wolfinger and others, 1997) was used for the drafting of the augmented design program. Treatment means were separated using Fisher's protected LSD (P<0.05).

RESULTS

Disease protection capacity caused by P. *debaryanum*

The four replicated treatments in each of the 12 experiments differed significantly from each other, recording a wide range of emergence values (Table 1). No significant interaction between treatment and experiment was recorded, indicating a repeatable pattern of behavior in terms of accuracy and precision of the evaluated variables. The correlation between the observed records on day 15 and day 22 was very high (R^2 = 0.914); this result indicates that the number of plants on day 15 can be used as a discriminating variable, significantly reducing the length of the experiment.

Disease control (inoculated with *P. debaryanum)*recorded an emergence of 33.2%, compared to the uninoculated control (base 100).

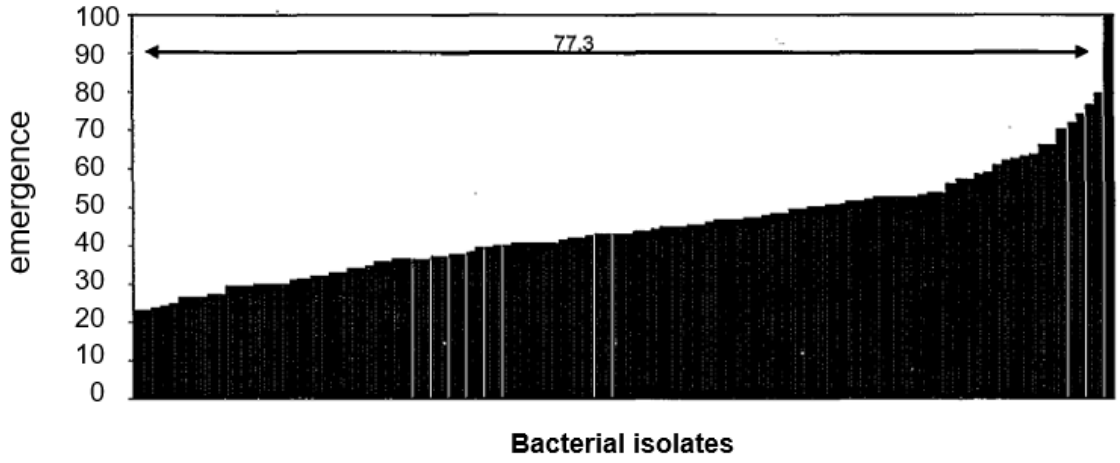
The 101 isolates of native fluorescents *Pseudomonas* showed great diversity in terms of protective capacity, expressed as a percentage of emergence at day 15 (Figure 1). Approximately 12% of the isolates showed a high protective capacity that allowed records of alfalfa emergence over 60% (Figure 2). The five treatments with the highest percentage of emergence recorded at day 15, P388, T633, P271, CI 19, and T688, were significantly superior to the disease control, inoculated only with *P. debaryanum.* The strains of *Pseudomonas fluorescens* UP61, UP143, and UP148, whose protective capacity was reported for implantation diseases of *Lotus corniculatus* (Bagnasco and others, 1998; Pérez and others, 2001), did not exhibit protective characteristics for alfalfa against the attack of *P. debaryanum* (Fig. 2). On the other hand, a significant percentage of isolates did not differ significantly from disease control, despite showing biocontrol characteristics in previous *in vitro* tests.

Table 1. Emergence (%) of alfalfa plants at day 15 and 22 post-sowing, for the four systematically evaluated treatments in 12 bioassays with an augmented experimental design; average values of the joint analysis. Germination control: *without Pseudomonas, without Pythium debaryanum* (base 100=98.2 plants/experimental unit); disease control: inoculated only with *P. debaryanum;* isolates T683 and T655 of fluorescent *Pseudomonas* co-inoculated with *P. debaryanum,* with high and low protective capacity of the 'damping-off', respectively.

Treatment	% emergence day 15	% emergence day 22
Germination Control	100.0 a	100.0 a
Disease Control	33.2 d	26.4 d
T683	65.9b	56.3b
T655	43.0 e	36.1 e
LSD (5%)	9.1	9.0

The treatments followed by the same letter do not differ significantly.

Figure 1. Variability of fluorescent *Pseudomonas* isolates in terms of protective capacity of the damping-off caused by *P. debaryanum* in alfalfa. Number of alfalfa plants emerged at 15 days post-sowing, expressed as a percentage. Average values of 12 bioassays joint analysis (augmented experimental design). Base 100 corresponds to the alfalfa plants emergence from germination control without inoculation (98.2 plants per exp. unit)

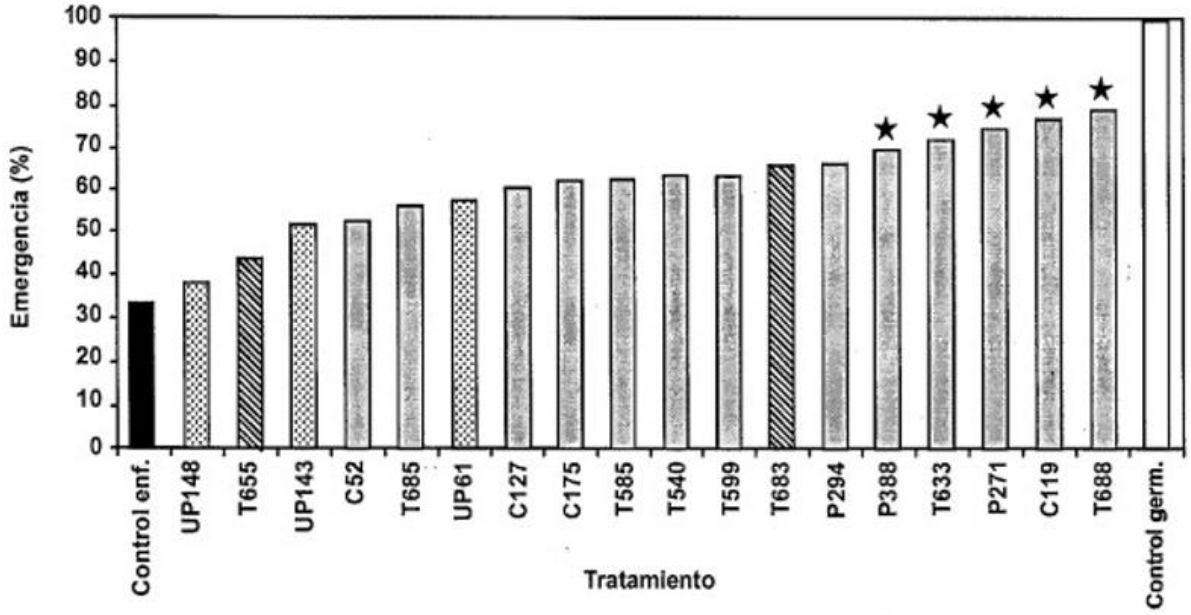


Plant growth promotion capacity

The interaction treatment by experiment was not significant for the three variables evaluated (NP, FW and DW), therefore, the trial replications added robustness to the analysis.

None of the fluorescent Pseudomonas treatments had negative effects on alfalfa implantation, since no significant differences in the control were observed regarding the number of plants emerged after 35 days (Fig. 3A). The biomass expressed as fresh weight or dry weight allowed the characterization of Pseudomonas isolates in terms of their growth-promotion capacity. Although little diversity was observed between the evaluated isolates, three isolates (T633, P271, P388) showed a significantly higher promotion capacity (biomass expressed as fresh weight), when compared to the control without bacterization. When the biomass expressed as dry weight was analyzed, isolation P388 did not differ significantly from the control, and isolation T688 did (Fig. 3B and C).

Figure 2. Effect of fluorescent *Pseudomonas* isolates on alfalfa emergence in the presence of *P. debaryanum.* Number of alfalfa plants emerged on day 15 post-sowing, expressed as a percentage. White bar: germination control, without inoculation (Base 100=98.2 plants/ exp. unit); gray bars: isolates of native fluorescent *Pseudomonas* with better behavior; dotted bars: strains of *Pseudomonas fluorescens* isolated from *Lotus corniculatus* plants; striped bars: checks of high (T683) and low (T655) protective capacity; black bar: disease control, inoculated only with *P. debaryanum.*The isolates that significantly increased the number of emerged plants compared to disease control are indicated by an asterisk (LSD, P≤0.05).



DISCUSSION

The applied methodology was adequate to characterize fluorescent *Pseudomonas* isolates, in terms of their protective capacity against disease and their ability to promote plant growth. It is resource-efficient as it allows working with a high number of isolates in a short period, and generates repeatable and reliable results. The statistical analysis of the evaluated variables confirms that these were discriminating variables and that the augmented experimental design proposed by Wolfinger and others (1997) is an appropriate tool if it is based on the selection of control treatments of known behavior.

Slow plant growth is imposed through bioassays on seedlings under controlled temperature (12° C) and humidity conditions, which favors the action of the pathogen, reproducing disease occurrence in the field (Altier, 2000). In these conditions, it is possible to select isolates that have a disease-protective effect. Among 101 evaluated isolates, T633, P271, P388, T688 and C119 were rated as promising for evaluation in greenhouse trials, using different soil types and in subsequent field trials. The first four are not only able to protect alfalfa plants from "damping-off", caused by *P. debaryanum,* but also promote their growth.

The methodology also allowed a significant proportion of the evaluated isolates to be discarded, which had originally been selected for having bio-controlling characteristics *in vitro* trials. These results are consistent with those reported by other authors, in that they indicate that experimentation *in vitro* is deficient as the only method to identify antagonists (Xiao and others, 2002).

Currently, there is little knowledge about microbial processes that occur at the rhizosphere level, where the microbial population has a higher density compared to the rest of the soil (Fabiano and others, 1994). The reported methodology offers another tool to interpret the interactions that take place therein, which can bring important benefits to the agricultural production system. It can also be applied to other plant-rhizobacteria-pathogen systems, modifying the conditions for each case, to evaluate the beneficial or harmful interactions that may occur between the participating organisms.

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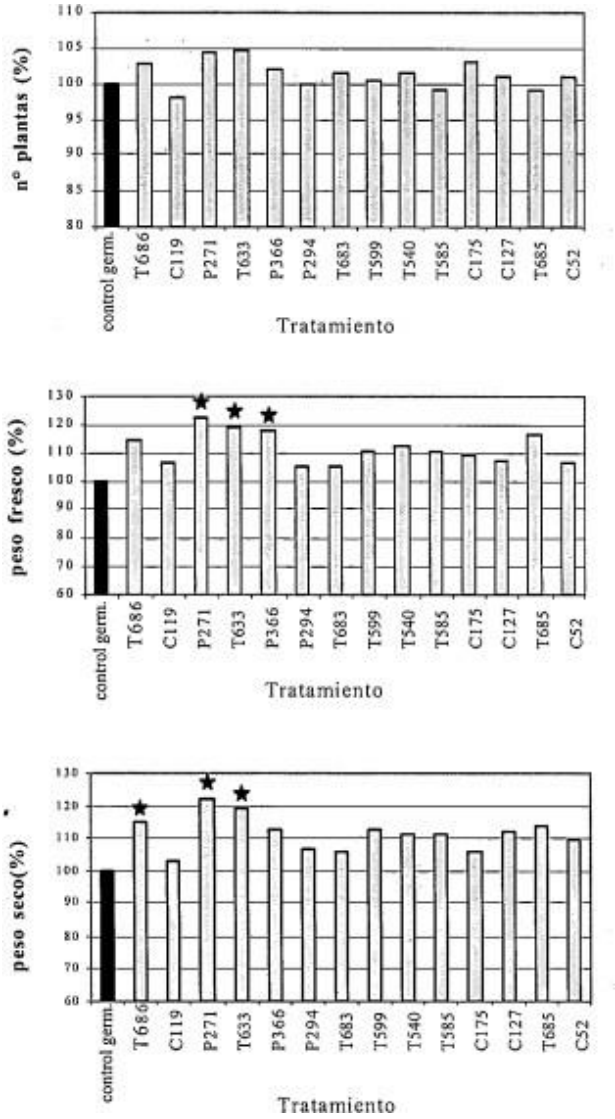
Figure 3. Effect of fluorescent *Pseudomonas* isolates on the emergence and growth of alfalfa plants, in the absence of the pathogen. A: Number of alfalfa plants emerged at day 15 post-sowing, expressed as a percentage. In black, germination control is indicated, without bacterization (Base 100=93.6 plants/exp. unit). B: Biomass expressed as fresh weight of alfalfa plants 35 days post-sowing. In black, germination control is indicated (base 100=4.10g/exp. unit). Isolates that produced a significant increase in the fresh weight of alfalfa are indicated with an asterisk (LSD, P≤0.05). rc=C:rc=Biomass expressed as fresh weight of alfalfa plants 35 days post-sowing. In black, germination control is indicated, without bacterization (Base 100=0.34g/exp. unit). The isolates that significantly increased the dry weight of alfalfa are indicated by an asterisk (LSD, P≤0.05).



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