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SCREENING OF FLUORESCENT RHIZOBACTERIA FOR THE BIOCONTROL OF SOILBORNE PLANT PATHOGENIC FUNGI[1]

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ABSTRACT - The biocontrol of soilborne plant pathogens represents a promising approach from the environmental and practical points of view. Fluorescent pseudomonad rhizobacteria are well known by their antagonistic capacity towards several plant pathogens due to a diversity of antimicrobial metabolites they produce. This study was conceived to select and characterize rhizobacteria having antagonistic potential towards the pathogenic fungi *Rhizoctonia solani* and *Sclerotium rolfsii*. A total of 94 bacterial strains isolated from the rhizospheres of four vegetable species under organic cultivation were evaluated. Twenty-two strains which predominate in lettuce and rudbeckia rhizospheres showed identical biochemical profiles to *Pseudomonas fluorescens,* while in kale and parsley rhizospheres identical profiles to *Pseudomonas putida* (subgroups A and B) strains prevailed. Two types of antagonism were verified *in vitro* and defined as competition and inhibition of mycelial growth. Sixty percent of the evaluated strains showed antagonistic potential and, among those, 24 strains expressed antagonism to both target fungi, with *P. fluorescens* being the most representative bacterial species. This work clearly identified a number of strains with potential for use as plant growth-promoting and biocontrol of the two soilborne fungal pathogens in vegetable crops production systems.

Keywords: *In vitro* antagonism. *Rhizoctonia solani. Sclerotium rolfsii*. Plant growth promoting bacteria.

SELEÇÃO DE RIZOBACTÉRIAS FLUORESCENTES PARA O BIOCONTROLE DE FITOPATÓGENOS HABITANTES DO SOLO

RESUMO - A utilização de agentes para o biocontrole de fitopatógenos habitantes do solo representa uma alternativa promissora tanto do ponto de vista ambiental quanto prático. Rizobactérias do grupo das *Pseudomonas* spp. fluorescentes são reconhecidas por sua capacidade antagônica a diversos fitopatógenos, graças à diversidade de metabólitos com ação antimicrobiana que produzem. Este estudo teve por objetivo selecionar e caracterizar rizobactérias com potencial antagônico aos fungos *Rhizoctonia solani* e *Sclerotium rolfsii*. Foi avaliado um total de 94 estirpes bacterianas isoladas da rizosfera de quatro espécies de hortaliças cultivadas em sistema orgânico. Vinte e dois isolados, que apresentaram perfil bioquímico idêntico ao de *Pseudomonas fluorescens*, foram mais abundantes nas rizosferas de alface e rúcula, em comparação a couve e salsa, em cujas rizosferas prevaleceram estirpes com perfil idêntico ao de *Pseudomonas putida*, subgrupos A e B. Duas formas de antagonismo foram verificadas e definidas como competição e inibição do crescimento micelial. Sessenta por cento das estirpes avaliadas apresentaram potencial antagônico e, dentre essas, 24 estirpes expressaram antagonismo para ambos os fungos-alvo, sendo que a principal espécie antagonista foi *P. fluorescens*. Os resultados abrem possibilidades da utilização de estirpes selecionadas para uso como agentes de biocontrole dos fitopatógenos mencionados em sistemas de produção de olerícolas.

Palavras-chave: Antagonismo *in vitro*. *Rhizoctonia solani. Sclerotium rolfsii.* Bactérias promotoras do crescimento de plantas.

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INTRODUCTION

Agriculture still relies upon the usage of chemicals to control plant diseases and Brazil is the one of the largest pesticide users in the world. Although farmers know the risks to their health, they are exposed to several harmful substances during pesticide handling (PASIANI et al., 2012). The steady price elevation of pesticides along with the concern of a sustainable environment has lead to approaches aiming at the reduction or even the replacement of chemical control by biological control of plant pathogens (COMPANT et al., 2005).

Studies involving isolation and selection of microorganisms with biocontrol potential are relevant especially concerning soilborne fungi, due to a difficulty in pathogenic microbial populations reduction with the use of chemical substances, because of some pathogens to obtain resistance to commonly used pesticides (HAAS; DÉFAGO, 2005; LUGTENBERG; KAMILOVA, 2009). As an alternative, the use of fluorescent pseudomonads rhizobacteria may be effective to prevent deleterious effects of plant pathogens through the production of antimicrobial compounds and siderophores (CHOUDHARY et al., 2009; RAAIJMAKERS et al., 2009) or by induction of systemic resistance against pathogens in several plant species (BENEDUZI et al., 2012). Besides that, fluorescent pseudomonads show high rhizosphere competence, produce indole acetic acid and solubilize insoluble phosphate (LUGTENBERG ; KAMILOVA, 2009).

A strategy for selection of efficient biocontrol agents is their isolation from agro- ecological production units, which are enhanced in microbial diversity and soil suppressivity conditions (ALTIERI, 1999). The commonly abundant presence of benefic microorganisms in organic farming systems depicts the opportunity of bacterial strains selection for utilization against plant pathogens. Furthermore, antagonistic bacterial strains, in combination with organic fertilizer, could effectively suppress soil diseases that the organic fertilizers provided the antagonistic bacteria with carbon and other nutrients (BORRERO et al., 2006; DING et al., 2013). Thus, it is extremely useful for future development of field biological control agents, to apply adequate methods to evaluate *in vitro* the bacterial response (BERG et al., 2005).

The present paper aimed at the characterization and selection of rhizobacteria isolated from vegetable crops cultivated under organic production system for acting against plant pathogenic soilborne fungi, *Rhizoctonia solani* and *Sclerotium rolfsii*. Therefore, the strategy was to isolate bacteria from the rhizosphere of the target plant species kale, lettuce, parsley and rudbeckia grown under organic management, as a starting point in the selection of beneficial bacteria.

MATERIAL AND METHODS

Rhizobacteria were isolated from kale (*Brassica oleacea* var. *acephala L.*), lettuce (*Lactuca sativa L.*), parsley (*Petroselinum sativum L.*) and rudbeckia (*Eruca sativa* Miller) cultivated on an experimental area (SIPA - Integrated Agroecological Production System) located in Seropédica, lat 22º 45´S, long 43º 41´N in Rio de Janeiro State, Brazil. This area represents a collaborative research effort between Embrapa Agrobiologia, Universidade Federal Rural do Rio de Janeiro and Pesagro-Rio (NEVES et al., 2005). Three plants of each vegetable species were harvested at the last third part of the vegetative cycle and the roots shaken to remove the loosely attached soil. The adhering soil of the three plants was pooled and considered as the rhizosphere soil. Rhizosphere soil (1 g) from each vegetable species was mixed with 9 ml of distilled water and serial dilutions were prepared. One hundred microliters of each dilution were spread on plates containing King's B agar (KING et al., 1954) amended with the antibiotics as described by Pinton et al. (2010). Fluorescent colonies presenting growth characteristics of pseudomonad were isolated and stored in glycerol at -80 ºC for further analysis.

Strains were biochemically characterized using the commercial Kit API system 20NE (BioMérieux Laboratory, France). This kit enables non enteric gram negative bacteria identification through enzymatic tests and use of different carbon sources. The identification of bacterial species is done by comparing the numeric profiles obtained to the ones from "Profile Analytic Index" database.

Data referring to each strain of rhizobacteria selected with the aid of API 20NE kit were submitted to similarity calculations based on the SM coefficient ("Simple Matching"). Later on, the data from SM were used for construction of a dendrogram by the "Unweighted Pair Group Method with Arithmetic Mean". Similarity matrix and dendrogram were both obtained from the program NTSYSpc 2.10t version (Applied Biostatistics Inc.).

In vitro antagonism tests were carried out with the pathogenic fungi *Rhizoctonia solani* Kühn and *Sclerotium rolfsii* Sacc. *Rhizoctonia solani* was isolated from kale plant hypocotyls with damping off symptoms and identified through hypha morphology and sclerotia formation typical of the species. *S. rolfsii* was isolated on BDA medium from sclerotia collected from green pepper (*Capsicum annum* L.) with sclerotium wilt disease (AGRIOS, 2005).

R. solani and *S. rolfsii* were grown in King's B medium during 4 days at 29 $^{\circ}$C. Then, a mycelium disc of 0.5 in diameter was cut and transferred to another Petri dish containing the same medium. This fragment was placed in the center of the plate whereas rhizobacterial cells were spread next to the border according to modified method proposed by Amorim and Melo (2002). The plates were then incubated in a B.O.D. regulated at 29 °C and the evaluations carried out in different periods of time: after 4, 6, and 9 days of incubation. Each treatment had triplicates, including the control represented by plates containing only the plant pathogenic fungi. The antagonistic test was repeated ever since results for the triplicates did not fit in the same type as described below. Three types were considered to evaluate the interaction between fungus and bacterium: absence of antagonism, when mycelium growth surpassed the boundaries of bacterial growth; competition, when mycelium growth did not surpass the boundaries of bacterial growth; and inhibition, when macroscopic reduction of fungal colony development occurred with consequent formation of the classic inhibition zone.

Inhibition or competition during the three periods of evaluation (4, 6, and 9 days of incubation) were considered to place the rhizobacteria in different classes. Three following levels of antagonism also were considered: absent (-), weak (+), and strong (++). The weak level corresponded to temporary competition or temporary inhibition, i.e., disappearing right after the first evaluation proceeded. The strong level corresponded to inhibition lasting until the second or third time period of evaluation.

RESULTS AND DISCUSSION

The aim of this study was to isolate and characterize fluorescent pseudomonads able to acting as biocontrol agents against plant pathogenic soilborne fungi, *Rhizoctonia solani* and *Sclerotium rolfsii*, that cause several losses in vegetable crop yield.

A total of 94 fluorescent bacterial strains from lettuce, kale, rudbeckia, and parsley rhizospheres were characterized by API 20NE System. From this total, 25% of strains showed an identical biochemical profile to *P. fluorescens* and the rest of then to *P. putida*. The origin and distribution of these strains in relation to rhizospheres of the four plant species studied are shown in Table 1.

Table 1. Number of strains characterized as *Pseudomonas fluorescens* and *Pseudomonas putida* obtained from rhizospheres of four vegetable crops grown under organic cultivation.

		P. fluorescens		*P. putida*	
Species	Number of isolates (n)	(n)	(%)	(n)	(%)
lettuce	8	4	50.0	4	50.0
kale	39	5	12.8	34	87.2
rudbeckia	25	9	36.0	16	64.0
parsley	22	4	18.2	18	81.8
Total	94	22	23.4	72	76.6

The research has shown that *P. putida* strains characterized by API profiles prevailed among the strains regardless of the plant species, particularly, kale and parsley rhizospheres showed more than 80% of this bacterial species. On the other hand, for lettuce and rudbeckia rhizospheres, 50 and 36% of the strains were identified as *P. fluorescens*. Such observations could possibly mean discrimination of these two bacterium groups relating to rhizospheres of vegetable crops, due to distinct composition of root exudates or specific molecular signs. In that sense, organic exudates have been appointed as main components of descriptive models concerning microbial rhizosphere dynamics (STRIGUL; KRAVECHENKO, 2006). Several authors have found *P. fluorescens* or *P. putida* populations in association with different plants, stages of plant cycles, and microhabitats. Latour et al. (1996) studied the phenotypic and genotypic diversity of fluorescent pseudomonads populations from two soil types, cultivated and not cultivated with tomato, concluded that the plant itself had great selective influence upon these bacterial communities. Berg et al. (2005) working with isolates from potato determined that each microhabitat was colonized by a specific bacterial community, with pseudomonads species predominant in rhizosphere while gram-positive bacteria prevailed in the phyllosphere.

A successful biological control by using antagonistic microorganisms will need adequate understanding of biotic and non-biotic factors involved in the complexity of plant diseases suppression. It also requires the knowledge of bacterial community dynamics and composition in association with plants, as well as the mechanisms which take part in microhabitats colonization (BERG; SMALLA, 2009).

Figure 1 illustrates the dendrogram showing the 94 strains characterized. Two main groups were identified: group I gathered identical profile to *P. fluorescens* strains revealing 70% of similarity, while group II had predominance of identical biochemical profile to *P. putida* strains.

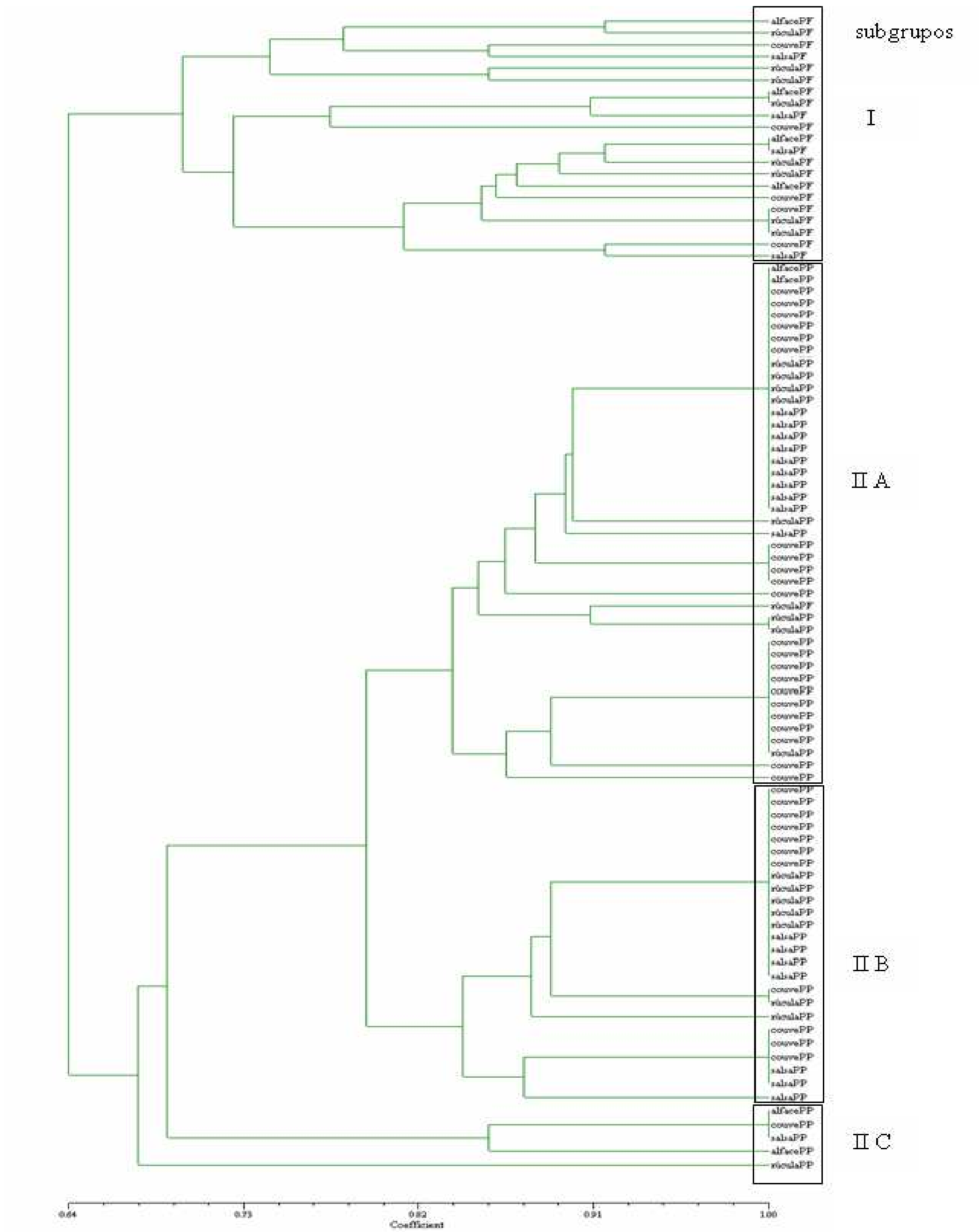

Figure 1. Grouping of fluorescent bacteria isolated from the rhizosphere of organically grown vegetables crops, based upon biochemical characteristics (PF: *Pseudomonas fluorescens*; PP: *Pseudomonas putida*).

The main biochemical characteristics for separation of these two species were utilization of mannitol and gelatinase activity, both positive for *P. fluorescens* and negative for *P. putida*, which also shows negative reaction to gelatin hydrolysis, normally used to differentiate them from *P. fluorescens*, *Pseudomonas clororaphis*, *Pseudomonas aureophaciens* and *Pseudomonas aeruginosa* that belonged to the same homology group (PALLERONI, 1984). Group II could be divided into major subgroups A and B, both constituted by *P. putida* strains but with intra-similarities of 83.5 and 84.4%, respectively. The main difference between these subgroups relies on the capacity of arabinose utilization, exclusively by subgroup A. A third subgroup, IIC, composed of five strains closer to *P. putida* differed from A and B by the capacity of arabinose utilization and phenyl acetate assimilation, respectively. These data are in accordance with results of ZAGO et al. (2011) that showed a wide variation the use of carbon compounds and enzymatic activity by fluorescent pseudomonads which might be an important feature to select strains of this bacterial group as integrants of inoculants to use in the agriculture.

Two types of interaction have been already characterized and defined as competition and inhibition. Figures 2A and 2B exemplify the competitive type meanwhile Figures 2C and 2D illustrate inhibition, when there was macroscopic reduction of mycelial growth of *S. rolfsii* and *R. solani*, respectively.

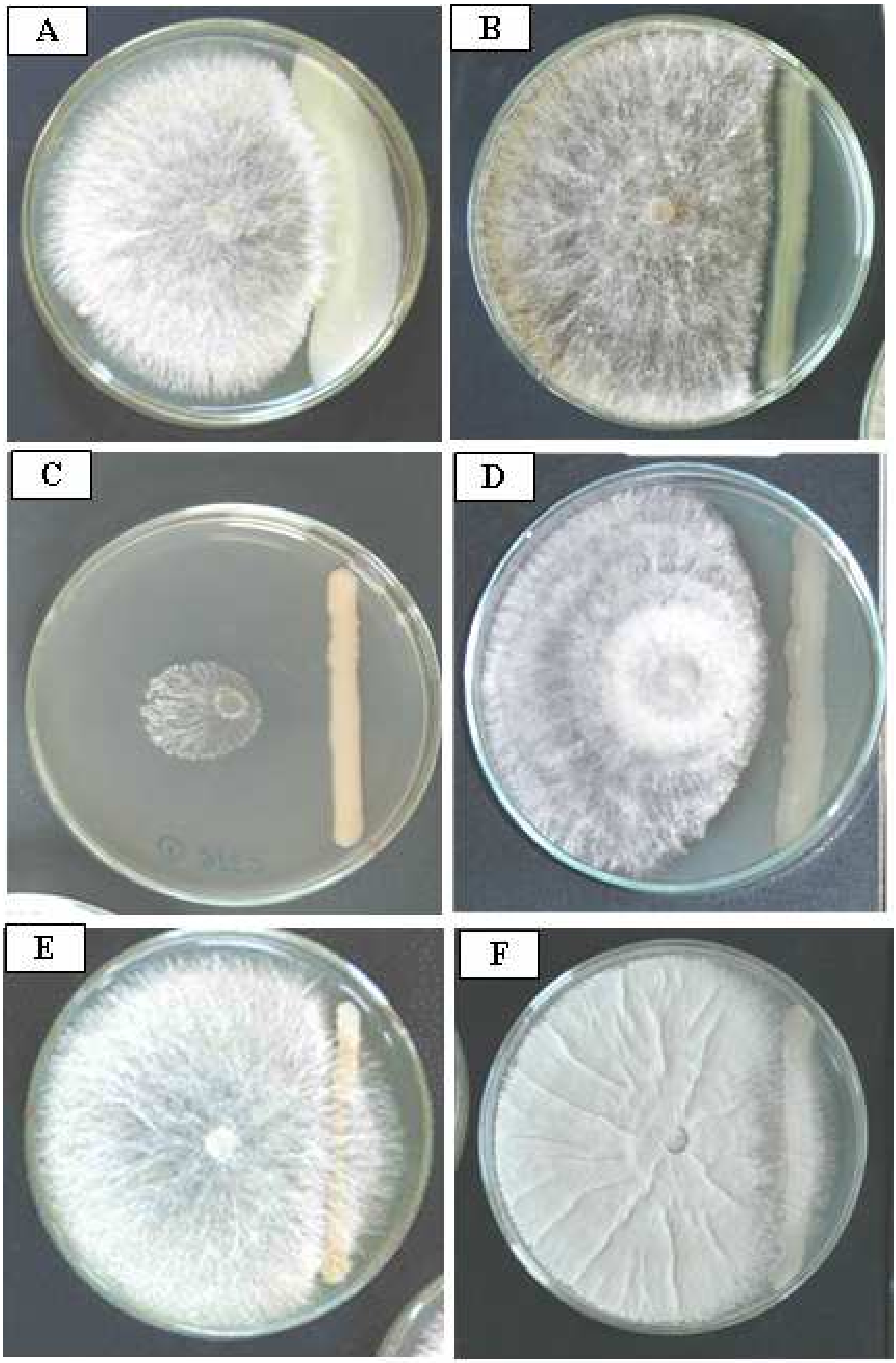

Figure 2. Types of antagonism of fluorescent pseudomonads rhizobacteria towards plant pathogenic fungi: (A) competition with *Sclerotium rolfsii*; (B) competition with *Rhizoctonia solani*; (C) growth inhibition of *S. rolfsii*; (D) growth inhibition of *R. solani*; (E) absence of antagonism to *S. rolfsii*; and (F) absence of antagonism to *R. solani*.

From the strains tested, 57 of them (60%) were capable of some type of antagonism against at least one fungal species. This universe was represented by 53% of strains that showed identical profile to *P. putida* and by 87% of strains characterized as *P. fluorescens.* Nineteen strains (all *P. putida*) presented antagonism solely for *R. solani*; fourteen of then (10 *P. putida* and 4 *P. fluorescens*) presented antagonism towards *S. rolfsii* only, and twenty-four (9 *P. putida* and 15 *P. fluorescens*) were antagonistic to both plant pathogens. Only 14% of *P. fluorescens* strains did not show antagonism to any of the target fungi. As for subgroups A and B of *P. putida*, absence of antagonism was observed in 40% and 56% of respective strains (Figure 3A). Strains from subgroup A showed a higher proportion of antagonism by competition (53%) comparatively to subgroup B (28%). Figure 3B shows the proportion of antagonistic response, both type and period of evaluation, by the pseudomonad strains considering the two fungi. Regarding *S. rolfsii* the inhibition was more common while, for *R. solani*, competition prevailed. Just two strains were capable to inhibit *R. solani* growth. Furthermore, the antagonistic response observed for each fungus tends to be maintained during the entire period of evaluation.

Fluorescent pseudomonads produce several substances with antimicrobial effect, worth highlighting six classes of antibiotics, diffusible or volatile, and a range of siderophores and lytic enzymes. Possibly, these results are due to qualitative and / or quantitative differences in the production of these metabolites that may: (i) provide greater specificity of action resulting in increased efficiency or (ii) be produced in greater amounts in response to the presence of target-specific fungus. It should also be considered the presence of carbon sources, amino acids, iron and phosphorus in the culture medium which exerts selective influence on the spectrum of antimicrobial substances produced by these bacteria (BERG et al., 2009; COMPANT et al., 2005; HAAS; DEFAGO, 2005; RAAIJMAKERS et al., 2009).

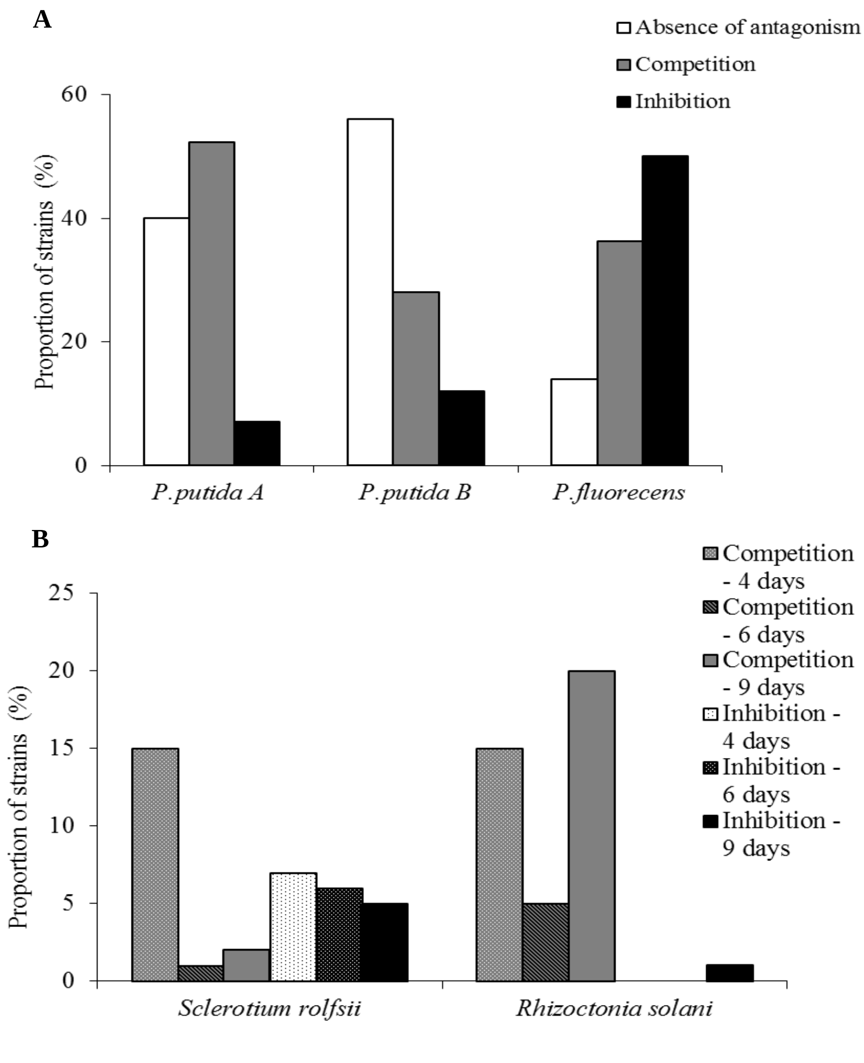

Figure 3. *In vitro* antagonism towards *Sclerotium rolfsii* and *Rhizoctonia solani* fungi by fluorescent pseudomonads rhizobacteria: (A) proportions of characterized *P. putida* strains (subgroups A and B), and of *P. fluorescens* strains based on the type of interaction (competition and inhibition); (B) percentage of antagonistic rhizobacteria to *S. rolfsii* and *R. solani* fungi, according to type of interaction (competition or inhibition) and time of evaluation (4, 6, and 9 days of paired cultures incubation).

The organic farming management at SIPA undergo ecological principles since 1993. The biota preservation in soil-plant systems has been a major priority through the use of conservative agricultural practices such as green manuring, no-tillage cultivation and organic matter amendments (NEVES et al., 2005). The high percentage of antagonism among the rhizobacteria strains now evaluated could be the reflex of microbial diversity, leading to natural soilborne plant pathogens suppression, what has been clearly observed along years in this area.

Seven classes (Table 2) were established according to the three types of antagonistic activity: absent (-), weak (+), and strong (++). Thirty-seven strains (class I) representing 38% of the rhizobacteria collection did not demonstrate any type of antagonism to the named fungi. Classes II, III, and IV comprized 33 strains that were antagonistic to only one of these fungi, indicating specificity of interaction. Classes V, VI, and VIII clustered 24 rhizobacteria strains with antagonistic activity towards both studied fungi.

Table 2. Classes of fluorescent *Pseudomonads*, isolated from the rhizospheres of vegetables, according to the level and type of *in vitro* antagonism to *Rhizoctonia solani* and *Sclerotium rolfsii* fungi. Symbols: (-) absence of antagonism; (+) weak antagonism (competition or inhibition disappearing four days after paired culture incubation); (++) strong antagonism (mycelial growth inhibition remaining for nine days of incubation).

	Rhizoctonia solani		*Sclerotium rolfsii*		
Class	*in vitro* antagonism				Number of strains
I	-	-	-	-	37
II	-	+	-	-	19
III	-	-	-	+	12
IV	-	-	++	-	2
V	-	+	-	+	15
VI	-	+	++	-	8
VII	++	-	++	-	1
				total	94

The group of strains which displayed strong antagonism deserves special attention concerning biotechnological potential. This group includes 10 strains (6 *P. fluorescens*; 3 *P. putida* subgroup A; and 1 *P. putida* subgroup B) having strong antagonism against *S. rolfsii*, and one strain (*P. fluorescens)* which demonstrated similar strong antagonism to either *S. rolfsii* or *R. solani.*

Regarding biocontrol mediated by microorganisms, three types of interaction can occur: competition, antagonism and parasitism (RAAIJMAKERS et al., 2009). The frequency of antagonistic strains among fluorescent pseudomonads is variable and possibly related to complex factors such as intraspecific diversity and pathological system at each location. Berg et al. (2002) tested the antagonistic ability of bacteria isolated from different potato microhabitats towards *Verticillum dahliae* Kleb. and *R. solani* and concluded that *P. putida* represented the majority of species among 40 strains. Berg et al. (2005) evaluated 2,648 bacterial strains and only 14% of them showed antagonism to one or both above mentioned fungi. Suppressive soils are considered natural sources of microorganisms capable of controling pathogenic populations. Therefore, Adesina et al. (2007) isolated bacteria having antagonistic potential against *R. solani* and *Fusarium oxysporum* from soils with and without suppressiveness records; the results showed a greater proportion of antagonists related to suppressive soils to the plant pathogens mentioned.

Despite the recognized variability, ubiquity of *P. fluorescens* and *P. putida* pointed out to a high potential for plant pathogenic fungi biocontrol. With respect to *R. solani* and *S. rolfsii*, different levels and types of antagonism indicated specific mechanisms that could be present in a given bacterial strain. Nevertheless, that hypothesis must be confirmed in order to achieve viability in terms of selection of efficient biocontrol agents.

CONCLUSIONS

Around 60% of fluorescent pseudomonads strains isolated from the rhizosphere of organically grown vegetable crops show antagonistic potential towards *R. solani* or *S. rolfsii* and 28 of these strains demonstrate antagonistic action upon both soilborne plant pathogens;

Strains showing similar biochemical profiles to *Pseudomonas fluorescens* respond for a greater proportion of antagonism concerning those fungi in comparison to *P. putida.*

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