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Characterization of rhizobacteria from Citrus rhizosphere to control *Phytophthora nicotinae* var.*parasitica* and *P.citrophthora*

Caractérisation des rhizobactéries de la rhizosphère de Citrus utilisables pour contrôler *Phytophthora nicotinae* var.*parasitica* and *P.citrophthora*

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Introduction

The root rot of citrus (*Citrus* sp) caused by *Phytophthora nicotianae* var. *parasitica* (Dastur) Waterhouse and *P.citrophthora* (Sm. & Sm.)Leon. is a disease that it has already been verified in all the areas producing of citrus of the country, causing significant losses in nurseries notedly in the São Paulo state.

According to Feichtenberger (1989,1990), Harris *et al*.(1994), the index of greenhouse contamination is high and when the roots are attacked by the fungus, it will can induce a drastic reduction of the total root area,total destruction and carrying out to seedlings death.

To reduce the contamination by pathogen fungi the nurseries are submitted to a fumigation process using the methyl bromide, however this product has been uneconomical because it can induce the arising of resistant pathogens (Harris, 1994). The search of viable alternative methods with less risks of failures and environmental contamination it has been needed.

Microorganisms from rhizosphere have received considerable attention as potential protecting agents against soilborne pathogens (Melo, 1991; Robbs, 1991). Antagonistic microorganisms has been evalueted as to control root rot caused by *Phytophthora* sp. in eucalyptus (Malajczuk *et al.*, 1977a), soybean (Sneth *et al.*, 1977) and citrus (May, 1994) as well as citrus growth-promoting in Australia (Wilkinson *et al*., 1989).

Since the evidence for the involvement of bacteria-mediated induced resistance against some fungi (Kloepper et al, 1993), the isolation of rhizobacteria from citrus rhizosphere

and the treatment with selected antagonistic bacteria will can induce plants to defense themselves against pathogen attack and at the same time the capacity and the potential of these microorganisms to colonize the radicular system it will can act as a barrier to entrance of the pathogens.
The objective of this study was isolation, characterization and identification of rhizobacteria to control of soil pathogen and capable to growth-promoting citrus seedling.

Material and Methods

Soil samples

Soil samples were collected in São Paulo state from different localities: citrus (São Pasquale/Sorocaba); bean (Macaúbas/Guaíra; cucumber (Campinas). The collected soils samples were stored at 4^{o}C until the use.

Isolation of antagonistic rhizobacteria

1,0 gram of the soil were mixed in 9,0 ml of saline solution (0,85% NaCl)). The soil suspension was shaken in order to remove the bacterial cells . Ten-fold serial dilution
(10^{-1} to10^{-9}) of the suspension was prepared asseptically for inoculation. The isolation was carried out in two different media. 0,1ml samples were spread onto Nutrient agar plates (Difco) and King's medium B (King et al. ,1954) and incubated at 28^{o}C for 48 h. A total of 136 colonies were isolated from the different rhizospheres and rhizoplanes.

Antagonism *in vitro* and *in vivo* of isolated rhizobacteria against *Phytophthora* sp.

The isolated colonies were inoculated on Nutrient agar plates and incubated at 28^{o}C during 5 days. In the middle of well grown developed bacterial cultures, spore suspension of *Phytophthora* sp. was sprayed. Then the plates were again incubated for more 7 days at 28^{o}C.

Test *in vitro*

Six selected colonies in the preliminary test were inoculated individually against *Phytophthora parasitica* (P9*) and Phytophthora citrophthora* (P38, P41, P45).
In an extremily of PDA (potato dextrose agar) plate, a agar disk of 5 mm with well growth *Phytophthora* sp. were put from 1,5 cm of the border and in the other extremity a selected bacterial colony was inoculated. The plates were put at 28^{o}C for 7 days, photoperiod 12/12h. After 7 days of the incubation period the decrease of the mycelial growth was measured.

Test *in vivo*

The experiment was carried out at random in factorial arrangement: 4 controls (without bacteria), 28 treatments formed by 7 strains isolated and 4 *Phytophthora sp.* combinations, total 96 treatments with 3 repetitions. The original data of number of dead plants were transformed in x+1, where x= % of dead plants. For effect of statistical analysis used the F test and the averages were compared through Tukey test at the probability 1% level, in accordng to Pimentel Gomes (1985).
A mixture of clay/sand/humus (2:1:1) was sterlized and left by three days before the use. The bacterial strains were selected from the results of the antagonism test in vitro.

Each isolate strain was cultivated on Nutrient agar plate at 28°C, 24 h. Bacterial cells were suspended in saline solution (0,85% NaCl) at 10^5 ufc/ml then citrus seedlings roots were immerged by 30 minutes.

The pots (1L) with a sterile mixture of clay/sand/humus were inoculated with a *Phytophthora* sp. spores suspension (50 ml spores/pot). The spores were obtained from the *Phytophthora* sp. cultivated in V8-$CaCO_3$ agar plates at 28°C in the dark for 10 days followed by the homogenization in 200ml of distilled water.

Characterization and identification of isolated rhizobacteria with potential control of *Phytophthora* sp.

To obtain data on the description and identification of the isolated strains, cultures were grown on Nutrient Agar medium. Gram-staining and motility by the hanging drop technique were examined by following method described by Case and Johnson, 1984. Cell shapes were observed in Gram-stained smear under a light microscope. Cytochrome-oxidase production was tested using paper oxidase test (Difco). Catalase activity was determined by the production of bubles after adding one drop of 3% hydrogen peroxide. For the oxidative-fermentative test (OF test), a test tube containing 8ml of Hugh -Leifson's glucose medium (Cowan and Steel's, 1995) was inoculated with the fresh culture by stabbing.

ID EB-20 and NF-18 (Nissui SA, Japan) test procedure

Bacteria were removed from a 24 hour culture on the Nutrient agar plate. The cells were suspended in 5 ml of 0,85% sterile NaCl solution to an opacity of the MacFarland 0,5 standard. The cell suspensions were distributed into the wells containing the test media. Cultures of the trays and observations were performed by following the directions of the system. Identification was conducted by consulting the Nissui profile index and when results could not be consulted with the index, traditional biochemical tests were done.

Preparation of bacterial DNA

Bacteria cultures were grown on the Nutrient agar plates for 48 hours at 15°C. The cells were carefully suspended with saline-EDTA and centrifuged at 10,000rpm for 20min at 4°C. The cells were suspended with saline- EDTA for lysing. Methods for extraction are based upon Marmur's procedure (Marmur, 1962).

Determination of guanine plus cytosine (G+C) content

The DNA dissolved in 0,1x SSC (saline-citrate) was heated at 100°C for 10 min and cooled rapidly in an ice bath. 10 ul of a nuclease P1 solution was added to 10 ul of the denaturated DNA solution. Then 10 ul of the mixture was applied to HPLC after one hour of incubation at 50°C. Analysis Standard was applied as the standard quantification. HPLC was performed by using a model LC-6A apparatus, chromatopac C-R4A (Shimadzu Corp.), under as follows conditions: column, Cosmosil 5C18 4.6x150mm; eluent: 20mM $NH_4H_2PO_4$-CH_3CN (20:1, v/v); flow rate, 1ml/min; detection at UV 270nm.

Results and Discussion

Selection of rhizobacteria and antagonism test *in vitro* and *in vivo*

From a total of 136 isolates from different soils, 33 isolates were from citrus and cucumber rhizosphere that showed antagonism to *Phytophthora* sp. through the inhibition zone formation. From this experiment were selected 7 strains: C1-1B, CIS/NA, RC2, RA2 and C2- 8C from citrus rhizosphere and OG and Santa Barbara from beans rhizosphere (Table1).

Table 1. Effect of selected rhizobacteria strains in the control *in vitro* of *Phytophthora* sp.

Strains/Treatment	mycelium growth (cm)	Inhibition %
Control*	7,50 a**	00,00
Sta Barbara[#]	4,85b	35,33
C28C[#]	4,58b	38,93
RA2[#]	4,55b	39,33
C1-1B[#]	3,82c	49,06
Cis/Na[#]	3,65c	51,70
OG[#]	3,63c	51,60
RC2[#]	3,55c	52,66
DMS= 1%	0,30	
CV=5,87		

* *Phytophthora* strains (P41, P45, P9 and P38).
** Averages of three repetitions. Averages followed with the same letter don't differ to each other for the Tukey test. [#] Selected isolated strains.

Table 2. Control of *Phytophthora* sp. by selected rhizobacteria in citrus seedling

	antagonism/pathogen (death%)				
Strains/ *Treatment*	P9	P45	P41	P38	DMS (5%)
Control*	77,7a* A	77,7a A**	88,8a A	77,7a A	3,93
Sta Barbara[#]	11,1bc A	22,2c A	22,2c A	22,2b A	3,93
C28C[#]	11,1b A	11,1c A	22,2c A	22,2b A	3,93
RA2[#]	44,3a A	44,4a A	55,5b A	22,2bc A	3,93
C1-1B[#]	00,0bc A	00,0b A	11,1bc A	0,00c A	3,93
Cis/Na[#]	00,0bc A	00,0b A	11,1b A	00,0c A	3,93
OG[#]	00,0c A	00,0c A	00,0c A	00,0c A	3,93
RC2[#]	22,2bc B	33,3a A	44,4b AB	22,2b A	3,93
DMS(5%) = 2,26					

* Averages followed with the same lower letter, in the vertical line, don't differ to each other for the Tukey test.** Averages followed wirh the same capital letter, in the horizontal line don't doffer to each other for the Tukey test.P9= *P.parasitica*; P45, P41, P38= *P.citrophthora*

Table 2 presents the results obtained in the control of *Phytophthora* sp. with selected rhizobacteria in citrus seedling growth. The OG strain provided the smallest citrus death percentage, indicating an efficient control of rot root disease although it doesn't present significant difference among C1-1B and CIS/NA isolates and it are similar to the ones isolates C2-8C and Santa Barbara. All these isolates significativelly inhibited the mycelium growth of *Phytophthora* sp.
It was also observed that the introduced strains OG and Santa Barbara that were selected as potential biocontrol agents of a different phytopathogens presented the same or higher antagonistic activity than C28C and RA2 isolates. These results indicate that

rhizobacteria from different cultures can be used for different plant-host system. The same results were observed by Malajczuk et al., 1977b with *P. cinnamomi.*

From the table 2 we can observe that some of those rhizobacteria isolates from citrus rhizosphere differ in ability to colonize the root system of plants. In agreement with Brown, 1974 some characteristics as high microbial population in the soil, growth capacity, resitance to antibiotics and competitive ability with the indigenous microorganisms can be the key for the success by an isolate microbial to establish in the rhizosphere. Jacobsen, (1997) verified that the antagonistic substances, fast growth, capacity to use carbon sources and the extracellular enzyme production can have been providing a base for the fast colonization in maize root system by *Burkholderia cepacia* (formely *Pseudomonas cepacia).*

Some works support the great potential of *Bacillus* sp. in the control of soil pathogens. Melo et al. (1995a) observed that strain OG isolated from the bean rhizoplane in Guaira/SP was a potent antagonistic strain against some pathogens of bean rot root *P.solani* f.*phaseoli* Kendrick and Snyder, *Sclerotium rolfsii* (Melo et al., 1995b ; Melo & Valarini, 1995 and Valarini *et al*., 1995).

Characterization and grouping of strains

The seven selected isolates were characterized.and identified. Some of the characteristics of the strains are listed in table 3. Cells of the strains were mostly rod-shaped and differed in size.

Table 3. Characterization tests of selected strains.

Test\ Isolates	C28C	RC2	RA2	CIS/NA	C1-1B	SB	OG
Gram	-	+	-	-	-	-	+
OF-test -	F	I	O	I	O	I	I
Motility	-	-	+	-	+	-	+
Oxidase	-	+	+	+	+	+	+
Catalase	-	+	-	+	-	-	+
Fluorescence pigment	-	-	-	-	+	-	-
Growth at 4^{o}C	-	-	+	-	-	+	-
NO_3 toNO_2	+	+	+	-	+	+	+
VP test	-	-	-	-	-	-	-
Aesculin hydrolysis	-	+	-	-	-	-	+
ONPG	+	+	-	+	-	-	+
Gelatin production	-	-	+	-	-	-	-
Carbohydrate acid from:							
arabinose	+	+	-	-	-	-	+
galactose	+	-	+	-	+	+	-
glicose	+	-	-	+	+	-	-
lactose	-	-	-	-	-	-	-
xylose	-	-	+	+	+	+	-
%G+C			60,6	37,8	61,4		

F=fermentative;O=oxidative; I=Inert

The seven selected strains which had been classified into fermentative, oxidative and inert group were further identified using the ID test-EB-20 and NF-18 (Nissui SA, Japan) identification strips. The results were confirmed by DNA base composition of the isolates. It is well known that in soil there are various kinds of bacteria which are functionally and taxonomically diverse. This may sometimes account for the low applicability of ID system, which has been mainly used for medical or food isolates that are not diverse compared to those from soil. So it is very important to do additional tests to confirm the identification. Therefore the knowledge of bacteriological characteristics of the soil isolates, as well as a modification in usage of the system, we can use the ID
According to Bergey's Manual of Determinative Bacteriology (Krieg et al., 1984 and 1994) and Cowan and Steel's, 1995 the strains were identified as:
C1-1B- *Pseudomonas putida biovar A,*Santa Barbara- *Pseudomonas putida biovar B,* CIS/NA- *Flavobacterium* sp., C28C- *Escherichia coli,* RA2- *Pseudomonas fluorescens,* RC2-*Bacillus subtilis* and OG- *Bacillus subtilis.*
Recent progress in the purification and identification of bacterial mainly pseudomonad metabolites has to led the consideration that in several cases, disease supression relied production of antibiotic and associated with competition for nutrients and iron in the rhizosphere. In this context, siderophores produced by root-colonizing fluorescent pseudomonads have been the focus of great interest, mainly because they could allow a more mobilization of iron than fungal siderophores (Piga *et al*., 1997).
Beneficial or plant-promoting rhizobacteria have been isolated and demonstrated to protect the roots of certain root crop plants (Burr and Caesar, 1984).
Santa Barbara (*Pseudomonas putida*) and OG(*Bacillus subtilis*) isolates have been used as growth-promoting in cucumber and tomato seedlings (Mantonavelo & Melo, 1991, Melo 1991, Melo *et al.*, 1995a). According to Thomashow and Weller, 1990, especially strains of *Bacillus* sp. and fluorescence *Pseudomonas* sp. have been effective in reducing diseases in field trials.
A better understanding of root colonization and disease supression mechanisms is needed before suitable strains can be directly selected.

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