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Bacterial selection for biological control of plant disease: criterion determination and validation



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ABSTRACT

This study aimed to evaluate the biocontrol potential of bacteria isolated from different plant species and soils. The production of compounds related to phytopathogen biocontrol and/or promotion of plant growth in bacterial isolates was evaluated by measuring the production of antimicrobial compounds (ammonia and antibiosis) and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and phosphate solubilization. Of the 1219 bacterial isolates, 92% produced one or more of the eight compounds evaluated, but only 1% of the isolates produced all the compounds. Proteolytic activity was most frequently observed among the bacterial isolates. Among the compounds which often determine the success of biocontrol, 43% produced compounds which inhibit mycelial growth of *Monilinia fructicola*, but only 11% hydrolyzed chitin. Bacteria from different plant species (rhizosphere or phylloplane) exhibited differences in the ability to produce the compounds evaluated. Most bacterial isolates with biocontrol potential were isolated from rhizospheric soil. The most efficient bacteria (producing at least five compounds related to phytopathogen biocontrol and/or plant growth), 86 in total, were evaluated for their biocontrol potential by observing their ability to kill juvenile *Mesocriconema xenoplax*. Thus, we clearly observed that bacteria that produced more compounds related to phytopathogen biocontrol and/or plant growth had a higher efficacy for nematode biocontrol, which validated the selection strategy used.

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Introduction

Concerns regarding food safety and the environment have led to reduced use of agrochemicals and the development of sustainable agriculture. In this context, the focus of biological control studies reflects the desire of several sectors to develop

sustainable methods for plant disease control.¹ However, efficient antagonists must be obtained for biological control to become a reality.

Soil microorganisms coexist in association with plant roots and interfere with plant performance and microbial community structure. Bacteria are estimated to occupy between 7%

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and 15% of the total root surface area.² Of these, some bacteria positively affect plants and have been designated as plant growth-promoting rhizobacteria (PGPR).³

Rhizobacteria can indirectly or directly promote positive effects on plants. Indirectly, they suppress pathogens mediated by competition and the production of antimicrobial compounds and lytic enzymes. Directly, they solubilize minerals and cause a wide range of changes in the rhizosphere, which promotes higher efficiency in the absorption of water and macro- and micronutrients by plants and changes in phytohormone concentrations, nitrogen fixation, and siderophore production.^{4–6}

In addition to the rhizosphere, the phylloplane is a source of antagonists; however, bacteria from this area are still underused as biological control agents, especially compared to rhizobacteria.⁷ Kishore et al.,⁸ found that both rhizoplane and phylloplane bacteria promote peanut seedling growth. In addition, bacteria that colonize the shoots have a better chance of surviving and multiplying in a nutrition-rich environment such as the soil, whereas in the phylloplane, they are exposed to high temperatures, moisture content fluctuations, and limited nutrient availability.

In vivo biocontrol agent selection is not a simple task due to the diversity of agents and interactions with the host plant, and therefore, efficient search methods are required. Thus, it is necessary to develop efficient selection strategies to reduce costs and increase the possibility of selecting organisms that can be produced in a large scale at low cost and that maintain their viability and efficiency for long periods. In 1997, Schisler and Slininger⁹ divided the selection process into three categories: (i) choosing the appropriate pathosystem, (ii) choosing the adequate method, and (iii) characterizing the isolates and evaluating efficiency. In recent years, this concept has evolved, and other groups of researchers have proposed initial selection criteria based on evaluations in the absence of the host.^{10,11}

In this sense, in vitro tests are appropriate during the initial selection steps due to the large number of microorganisms that can be evaluated and, especially, their low cost. Thus, due to the need for alternative management strategies for difficult-to-control pathogens, and considering that the initial steps for biocontrol agent selection should be performed in the absence of the host, the present study aimed to (i) characterize bacteria to determine their in vitro potential for the production of compounds related to phytopathogen biocontrol and/or promotion of plant growth (CRBPGs); (ii) select bacterial isolates with the highest number of CRBPGs; and (iii) validate the selection process by studying the effect of the bacteria selected on the ringed peach nematode.

Material and methods

Origin of the bacteria

A total of 1219 bacteria that belonged to the collection of the Plant Bacteriology Laboratory (Laboratório de Bacteriologia Vegetal – LBV) at the Universidade Federal de Pelotas, Brazil, were used. The bacteria were obtained from different niches (phylloplane, rhizosphere, and soil) and grouped according to

their isolation source: fig tree (*Ficus carica* L. – 55), Gramineae (72), Leguminosae (151), Liliaceae (219), peach tree (*Prunus persica* L. – 297), *Tagetes* sp. (51), non-rhizospheric soil (309), and others (65 – tomato plant (*Solanum lycopersicum* L.), Brassicaceae, and culture medium contaminants).

Evaluation of CRBPG production

The abilities to produce antimicrobial compounds (antibiosis and ammonia production) and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and to solubilize phosphates were evaluated. Bacteria that previously exhibited or did not exhibit the ability to produce each compound studied were used as positive and negative controls, respectively.

The ability of bacteria to produce antibiotic compounds against *Monilinia fructicola* (Winter) Honey, the causal agent of peach brown rot was evaluated. Four bacteria arranged equidistant at the edges were streaked on each Petri dish containing 523 medium¹² and in the center, a mycelial disk containing the fungus previously grown in a potato dextrose agar (PDA). After seven days at $22 \pm 2^\circ\text{C}$, scores were assigned according to the inhibition zone of the fungal growth as follows: 0 – no inhibition; 1 – ≤ 10 mm; 2 – ≥ 11 and ≤ 20 mm; and 3 – ≥ 21 mm).

Ammonia production was observed by the presence of a yellow-orange precipitate after five days of incubation at 28°C .¹³

Starch hydrolysis was evaluated according to the method of Schaad (1988). After four days of incubation at 28°C , the addition of Lugol's Iodine allowed the visualization of clear zones around the bacterial colony, indicative of starch hydrolysis, and the bacteria were classified using the scale described for antibiotic compounds.

Lipid hydrolysis was evaluated in medium containing 1% Tween 80, according to the method used by Fahy and Persley,¹⁴ after four and seven days of incubation at 28°C and verified by the presence of a milky white precipitate surrounding the colonies.

Two substrates were used to evaluate the ability of the bacteria to hydrolyze proteins, Litmus® milk (Difco) and 5% gelatin medium, as described by Schaad.¹⁵ After four and ten days of incubation at 28°C , the medium changed from milky to translucent (Litmus) or became liquefied after being refrigerated at 4°C for 1 h (gelatin). Chitin hydrolysis was evaluated using 0.5% chitin medium as the sole carbon source¹⁶ and calcium phosphate solubilization was evaluated in NBRIP culture medium,¹⁷ both activities were observed at seven, 14, and 21 days of incubation at 28°C to verify the degradation zone of each compound.

The hydrolysis of chitin, lipid and proteins (Litmus® milk and gelatin) was determined in a semiquantitative way, using different incubation times for evaluating. The intensity of production was low for positive reaction after 10 (lipids and proteins) or 21 days (chitin) of incubation; middle if the positive reaction happened after 14 days of incubation (chitin); and high when the positive reaction occurred after four (lipids and proteins) or seven days of incubation (chitin).

Table 1 – Percentage of bacteria producing compounds related to biocontrol and/or promoting growth according to number of produced compounds and total isolated by grouping as the origin of the bacterial isolates (*Ficus carica*, Gramineae, Leguminosae, Liliaceae, *Prunus persica*, soil, *Tagetes* and others – total = 1219 isolates).

Source groups/(no. isolates)	Number of the compounds produced									
	0	1	2	3	4	5	6	7	8	
<i>Ficus carica</i>	10.9*	18.2	23.6	14.5	9.1	10.9	9.1	3.6	0.0	
Gramineae	8.3	11.1	16.7	15.3	11.1	6.9	20.8	8.3	1.4	
Leguminosae	7.9	13.9	17.9	7.9	12.6	15.9	17.9	6.0	0.0	
Liliaceae	2.3	6.4	8.2	16.9	13.7	19.2	17.8	15.5	0.0	
<i>Prunus persica</i>	10.8	16.8	18.2	20.5	12.5	12.1	6.1	2.7	0.3	
Soil	11.0	12.0	11.0	17.5	12.9	14.6	13.9	5.5	1.6	
<i>Tagetes</i>	0.0	2.0	23.5	21.6	19.6	15.7	13.7	3.9	0.0	
Others**	6.2	10.8	18.5	20.0	12.3	10.8	13.8	7.7	0.0	

* Percentage values for 1219 bacterial isolates tested.

** Others = *Solanum lycopersicum*, Brassicaceae, and culture medium contaminants.

Validation of biocontrol bacterial selection – evaluation of nematicide activity

At this step, the bacteria that produced at least five of the eight CRBPGs analyzed were used, as well as DFs1985 (which did not produce any of the compounds evaluated) and DFs2180 (which produced one compound), for contrast effects. The ringed nematode (*Mesocriconema xenoplax* (Raski) Loof and de Grisse) species associated with Peach Tree Short Life disease was chosen as the target species to evaluate the efficiency of bacterial biocontrol.

Mobile forms (juvenile and adult) of the nematode (25 per plot) were treated with a bacterial suspension ($A_{540} = 0.5$) or with saline solution (control) at a 1:1 ratio (v/v). The microtiter plates containing the treatments were incubated at 25 °C for 24 h and arranged in a completely randomized design (86 bacterial treatments in four replicates). The number of dead nematodes was evaluated by adding 10 μ L of 1 N NaOH, according to Chen and Dickson.¹⁸ The values were $\sin \arcsin \sqrt{x/100}$ transformed, subjected to analysis of variance, and grouped by the method of Scott and Knott.¹⁹

Bacterial identification

The bacteria with best control were identified by their 16S rRNA sequences. Total DNA was extracted with Wizard genomic DNA purification Kit (Promega Corporation, USA). Partial amplicon of 16S rRNA of samples were produced by PCR with primers 27F and 1492R.²⁰ The amplicons were cleansed with Wizard SV Gel and PCR Clean-up kit (Promega) and sent for sequencing by Ludwig Sequenciamento.

Results

Of the 1219 bacterial isolates studied, 92% were able to produce one or more of the eight compounds evaluated. However, only 1% of the isolates produced all the compounds. The ability of the bacteria to produce compounds was called 'production of compounds related to biocontrol and/or promoting growth (CRBPG)', and most isolates (17%) produced three CRBPGs (Table 1).

Upon analysis of the source groups and the number of compounds produced (Table 1), three groups exhibited bacterial isolates that produced all the compounds evaluated (soils – 1.6%, grasses – 1.4%, and peach tree – 0.3%). Additionally, a large number of isolates from Liliaceae (15.5%) were able to produce at least seven CRBPGs. The isolates from *Tagetes* comprised the only group in which 100% of the isolates exhibited production of at least one of the compounds. In contrast, the group of isolates from soils exhibited the highest percentage of isolates, without producing any of the compounds, followed by isolates from the fig tree and peach tree.

In general, the groups exhibited different percentage distributions among the numbers of compounds produced. The highest percentage for bacteria isolated from fig tree, leguminous plants and *Tagetes* was observed for two compounds and for the bacteria isolated from peach tree, soil and others, took place in three compounds. The isolates obtained from the soil had a more homogeneous distribution, ranging from 11% to 17.5% for zero to six compounds produced, in contrast to *Tagetes*, which concentrated the distribution between two and six compounds produced (85.9%).

When observing each of the evaluated compounds (Fig. 1A), it is noteworthy that proteolytic activity was most frequently observed for both substrates, and chitin hydrolysis was least frequently observed. In the other tests, the quantity of isolates that produced compounds varied between 36% (amylolytic) and 50% (lipolytic). However, when comparing the eight groups originating from the bacterial isolates (Fig. 1B), it is possible to distinguish which isolates have higher production potential for each of the compounds evaluated. The *Tagetes* group exhibited the highest percentage of phosphate solubilizers, protease-producers in gelatin, ammonia-producers, and amylase-producers. The bacterial isolates from Liliaceae were notable as protease-producers and for their antibiosis activity against *M. fructicola*. The bacterial isolates from grasses proved to be the best lipase-producers, and those from legumes were the best chitinase-producers.

The semiquantitative component of the evaluations, through the assignment of grades or evaluations during distinct incubation periods (Fig. 2), allowed us to summarize the results of compound production intensity into negative, low (type I zone or slowest isolates), medium (type II zone or isolates with positive reaction after intermediate incubation

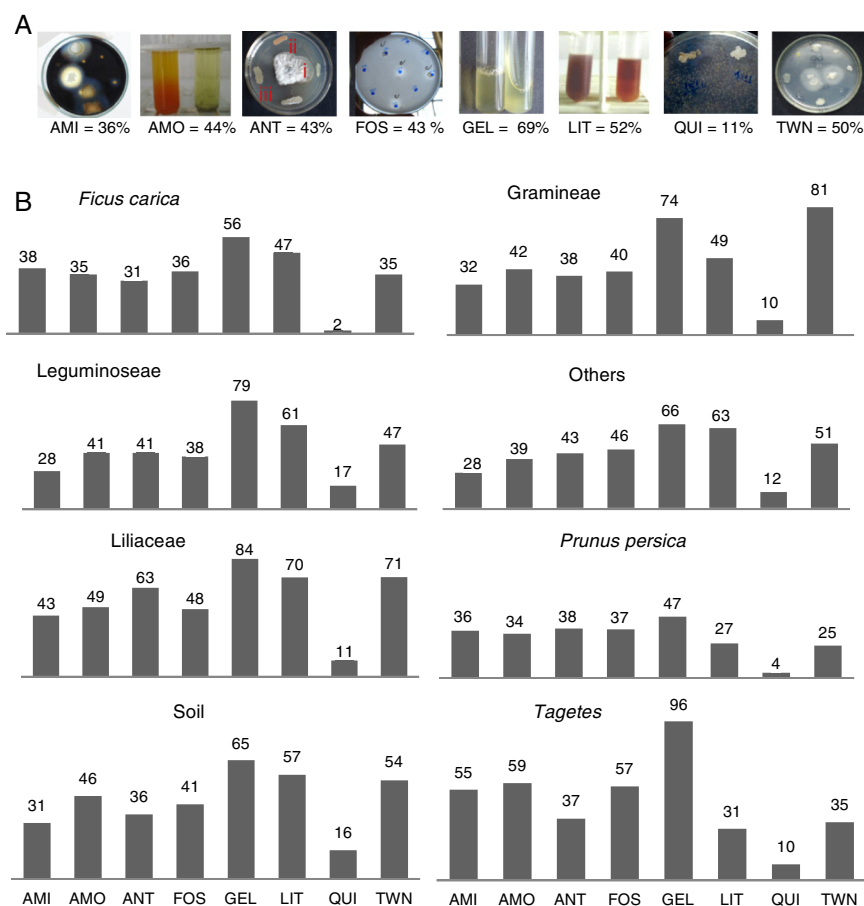


Fig. 1 – Percent bacteria producing compounds related to biocontrol and/or promoting growth depending on the total isolates number. (A) AMI, amylase; AMO, ammonia; ANT, antibiotics (halos type I – ≤ 10 mm; II – ≤ 20 mm; and III – ≥ 21 mm); FOS, phosphate solubilization; GEL, proteases medium gelatin; LIT, proteinase medium Litmus®; CHI, chitinase; TWN, lipases medium Tween 80. (B) Groups formed as the isolation origin (*Ficus carica*, Gramineae, Leguminosae, others, Liliaceae, *Prunus persica*, soil, *Tagetes*).

time), and high (type III zone or fast isolates) categories. Thus, protease-producing isolates (both substrates) predominated upon the negative ones. Among the positive results, the high intensity was more frequent to isolates that hydrolyzed lipids, gelatin and milk or solubilized phosphate (Fig. 2).

In contrast, considering only the isolates that produced each compound and their isolation niches (Fig. 3), rhizospheric isolates predominated for all the compounds (ranging from 53 to 38%), except for chitinase (Fig. 3i) and lipase production (Fig. 3ii), where the three niches exhibited practically the same percentage.

Thus, upon determination of the isolates ability to produce CRBPGs, it was possible to select 86 bacteria that produced from five to eight compounds evaluated (three bacteria that produced eight, 32 that produced seven, 23 that produced six, and 28 that produced five compounds).

Evaluation of ringed nematode mortality upon exposure to a bacterial suspension for 24 h allowed us to group the isolates into 10 groups with different efficiency levels (a–j, Table 2). Eight of these groups positively differed from the control, i.e., comprised bacteria that caused mortality in juvenile *M. xenoplax* (groups a–h); one was equal to the control (group i); and

another reduced their mortality (group j). Of these, 89.4% of bacteria were effective, and most bacteria (42.5%) resulted in at least 96.7% mortality (group a).

In contrast, when considering the bacteria grouped by number of CRBPGs produced, we clearly observed that bacteria that produced more CRBPGs had a higher nematocidal percentage (Table 2). Thus, all isolates evaluated that produced eight CRBPGs resulted in 100% nematode mortality; producers of seven CRBPGs had 43.7% of their isolates in the superior group; producers of six CRBPGs had 34.8% of their isolates in group a; and among those that produced five CRBPGs, only 21.4% exhibited high efficiency.

The partial 16S rRNA gene sequencing (Table 3) resulted 85–100% identity and 76% isolates identified belonging to the genus *Bacillus*.

Discussion

In general, all the plant species and soils used to isolate bacteria were host candidates for pathogen biocontrol. Each source group exhibited different percentages for each number

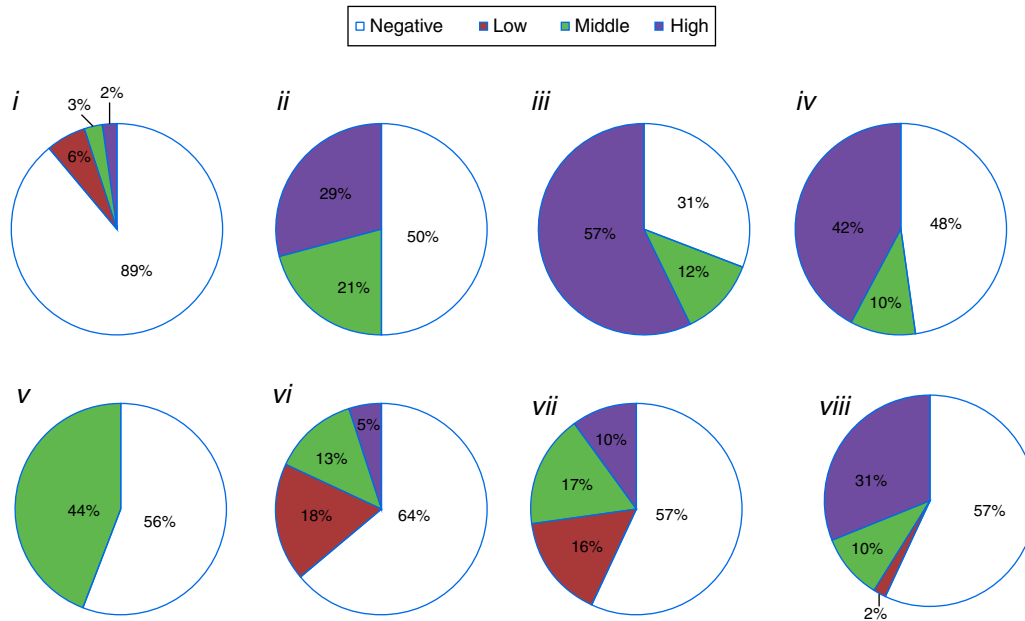


Fig. 2 – Percentage of isolates (total = 1219) grouped as reaction intensity: (i) chitinolytic; (ii) lipolytic; (iii) proteolytic on gelatin medium; (iv) proteolytic Litmus® milk medium; (v) ammonia-production; (vi) amylase-production; (vii) antibiotic against *Monilinia fructicola*; (viii) phosphate-solubilization.

of CRBPGs produced. The isolates that originated from grasses, legumes, and Liliaceae exhibited higher percentages for production of six or seven CRBPGs, although the two first groups comprised approximately 20% of the isolates that did not produce any or one of these compounds.

However, the group of isolates obtained from *Tagetes* is noteworthy because different species from these plants are considered antagonists to species of *Pratylenchus* and

Meloidogyne.²¹ There are no reports of antagonism of *Tagetes* bacterial isolates against species of *Mesocriconeuma*. Additionally, studies in the 1990s showed that using isolates from roots of antagonistic plants increased the chances of finding good candidates for biocontrol agents by up to six times in in vivo tests.^{22,23} Thus, the high percentages of CRBPG producers (98% between 2 and 7 CRBPGs) isolated from the *Tagetes* rhizosphere observed in the present study reflect this premise.

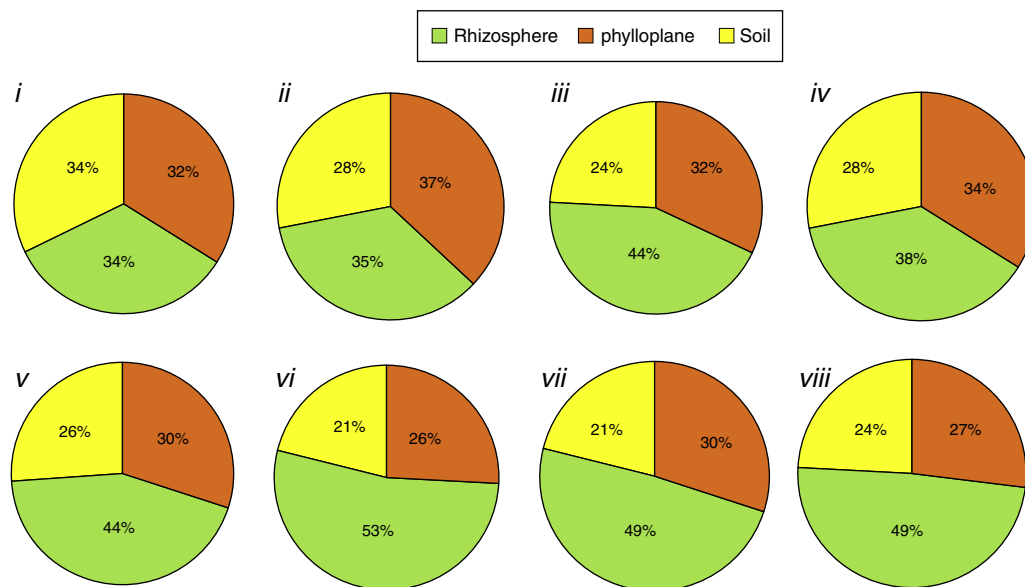


Fig. 3 – Percentage of isolates with positive reaction grouped as isolation niche (phylloplane, rhizosphere and soil): (i) individually chitinolytic (135); (ii) lipolytic (604); (iii) proteolytic on gelatin medium (839); (iv) proteolytic medium Litmus® milk (630); (v) ammonia-producers (531); (vi) amylolytic (443); (vii) antibiotics producing (529); (viii) phosphate solubilizers (530). Values between brackets represent the number of isolates with positive results.

Table 2 – Mortality percent of *Mesocriconema xenoplax* due the action of bacteria producing at least five compounds related to biocontrol and/or promotion growth (CRBPGs) after 24 h incubation at 25 °C.

Bacterial treatment	Mortality (%)	Bacterial treatment	Mortality (%)	Bacterial treatment	Mortality (%)
<i>Producers eight compounds CRBPGs</i>					
DFs1391	100.00a*	DFs1486	100.00a	DFs19866	100.0a
<i>Producers seven compounds CRBPGs</i>					
DFs0066	100.00a	DFs2247	100.00a	DFs0081	55.67f
DFs0390	100.00a	DFs2251	100.00a	DFs1021	49.08g
DFs0695	100.00a	DFs0439	97.95a	DFs0582	46.87g
DFs0886	100.00a	DFs1887	93.15b	DFs1985	31.56h
DFs1256	100.00a	DFs1039	86.87b	DFs1421	29.63h
DFs1258	100.00a	DFs0419	80.64c	DFs0706	22.57i
DFs1341	100.00a	DFs0023	78.33c	DFs2137	19.25i
DFs1947	100.00a	DFs1219	72.15d	DFs1967	17.77i
DFs2049	100.00a	DFs0756	69.56e	DFs1320	12.65j
DFs2108	100.00a	DFs0377	59.51f	DFs1344	9.38j
DFs2124	100.00a	DFs2121	58.73f		
<i>Producers six compounds CRBPGs</i>					
DFs1983	100.00a	DFs2191	90.23b	DFs2172	50.00g
DFs2007	100.00a	DFs2122	85.42b	DFs1934	28.37h
DFs2126	100.00a	DFs2175	82.89c	DFs1923	22.55i
DFs2156	100.00a	DFs0032	81.68c	DFs2162	22.33i
DFs2227	100.00a	DFs1691	71.94d	DFs2048	16.11i
DFs2229	100.00a	DFs2265	67.09e	DFs2062	13.29j
DFs2236	100.00a	DFs2052	65.06e	DFs0422	7.09j
DFs2110	96.74a	DFs2246	62.75e		
<i>Producers five compounds CRBPGs</i>					
DFs2008	100.00a	DFs2148	82.55c	DFs2155	54.17f
DFs2099	100.00a	DFs2113	81.03c	DFs1955	45.71g
DFs2109	100.00a	DFs0418	79.02c	DFs1970	40.91g
DFs2133	100.00a	DFs2168	77.11d	DFs1932	37.29h
DFs2147	100.00a	DFs1650	76.06d	DFs2193	37.08h
DFs1928	98.04a	DFs2060	74.54d	DFs1935	19.46i
DFs1899	91.92b	DFs1909	69.88e	DFs1950	13.92i
DFs2250	90.77b	DFs1902	69.61e	DFs2051	9.17j
DFs2136	83.94c	DFs2027	68.60e	DFs1985**	31.56h
DFs1958	83.90c	DFs2089	64.12e	DFs2180**	6.01j
CV (%)	10.02			Control 16.08i	

* Original values were converted to $\arcsin \sqrt{x/100}$ whose original means followed by the same letter in the column do not differ by Scott–Knott test at 5% probability.

** Isolated used as contrast: DFs1985, not produces evaluated compounds and DFs2180, producing one compound.

In contrast, approximately 90% of the isolates obtained from peach trees produced some CRBPGs. Although these isolates are not among those with highest CRBPG production, they should receive special attention because peach trees are affected by the ringed nematode, and biocontrol agents from the hosts are desirable because they have been adapted to survive in this habitat. This aspect was successfully exploited in the control of the ringed nematode.^{24–26} For most of the source groups, the isolates from the rhizosphere exhibited higher production of the evaluated compounds, as well as higher intensities of CRBPG production. These results reflect the richness of the rhizospheric environment, which is responsible for the occurrence and preference of several organisms, including those that exhibit antagonistic effects against several pathogens, such as nematodes.²⁷

It has been previously established that enzymes, especially lytic enzymes, are synthesized by microorganisms and also act as an antagonistic mechanism, which, in many situations, may partially explain the biological control of plant disease.²⁸

The isolates studied here exhibited a higher frequency for protein and lipid hydrolysis, which is an important result, as these compounds are present in the membranes surrounding the eggs and in the cuticle of mobile forms of different nematode species.²⁶ Thus, these lytic activities have been associated with the biological control of nematodes.²⁹

Chitinases are also important in controlling phytopathogens, as chitin is the main constituent of nematode eggs^{26,30} and the fungal wall.³¹ Thus, chitinolytic bacteria are potential biocontrol agents for both pathogen groups.^{32–34} However, just over 10% of the isolates studied produced chitinases, which indicates that this ability is more common in actinomycetes and less common in bacteria and fungi present in the soil.³⁵

Several researchers consider antimicrobial compound production one of the most important antagonist mechanisms. Antibiotics and toxic compounds produced by the bacteria can act on both the hatching and development of nematodes.^{26,36} The isolates studied here (43%) inhibited mycelial growth of

Table 3 – Bacterial identification by homology of partial sequence of 16S rRNA using 27F and 1492R primers.

Bacterial Treatment	Description	Identity	Accession
DFs066	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> strain KISR-1 16S ribosomal RNA gene, PS ^a	98%	KM068047.1
DFs390	<i>Bacillus thuringiensis</i> strain BT-EM14 16S ribosomal RNA gene, PS	99%	KT588443.1
DFs439	<i>Bacillus subtilis</i> strain CI1 16S ribosomal RNA gene, PS	99%	KU557409.1
DFs695	<i>Bacillus</i> sp. AR493 partial 16S rRNA gene, strain AR493	100%	LN829576.1
DFs886	<i>Bacillus thuringiensis</i> Bt407 16S ribosomal RNA, CS ^b	98%	NR_102506.1
DFs1256	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> strain DSM 10 16S ribosomal RNA, PS	98%	NR_027552.1
DFs1258	<i>Bacillus subtilis</i> strain 1-3 16S ribosomal RNA gene, PS	97%	HQ693082.1
DFs1341	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168 strain 168 16S ribosomal RNA, CS	99%	NR_102783.1
DFs1928	<i>Paenobacillus polymyxa</i> gene 16 S RNA, PS	99%	AB689755.1
DFs1947	<i>Bacillus cereus</i> strain CF-S30 16S ribosomal RNA gene, PS	97%	KJ781400.1
DFs1983	<i>Bacillus thuringiensis</i> Bt407 16S ribosomal RNA, CS	99%	NR_102506.1
DFs2008	<i>Pseudomonas poae</i> RE*1-1-14 strain RE*1-1-14 16S ribosomal RNA, CS	97%	NR_102514
DFs2049	<i>Pseudochrobactrum saccharolyticum</i> strain ALK626 16S ribosomal RNA gene, PS	96%	KC456591.1
DFs2099	<i>Bacillus cereus</i> strain YLB-P5 16S ribosomal RNA gene, PS	99%	KF376341.1
DFs2108	<i>Pseudomonas</i> sp. VS-2 16S ribosomal RNA gene, PS	98%	JF699699.1
DFs2109	<i>Pseudomonas fluorescens</i> strain M5 16S ribosomal RNA gene, PS	99%	KT767962.1
DFs2110	<i>Bacillus cereus</i> strain NCIM 2106 16S ribosomal RNA gene, PS	100%	KU218541.1
DFs2124	<i>Bacillus cereus</i> ATCC 14579 strain ATCC 14579 16S ribosomal RNA, CS	99%	NR_074540.1
DFs2126	<i>Bacillus</i> sp.	85%	
DFs2133	<i>Bacillus</i> sp. SNG-3 16S ribosomal RNA gene, PS	99%	KP992135.1
DFs2147	<i>Pseudomonas putida</i> strain BB3 16S ribosomal RNA gene, PS	98%	KP314235.1
DFs2229	<i>Bacillus cereus</i> ATCC 14579 strain ATCC 14579 16S ribosomal RNA, CS	100%	NR_074540.1
DFs2236	<i>Bacillus cereus</i> strain FT9, CG ^c	97%	CP008712.1
DFs2247	<i>Bacillus amyloliquefaciens</i> FZB42 strain FZB42 16S ribosomal RNA, CS	99%	NR_075005.1
DFs2251	<i>Bacillus cereus</i> strain KCd1 16S ribosomal RNA gene, PS	99%	FJ613630.1

^a (PS) Partial sequence.

^b (CS) Complete sequence.

^c (CG) Complete genome.

M. fructicola, which is used as an indicator of antibiotic production because *M. xenoplax* is an obligate pathogen and thus cannot be cultured in vitro. Therefore, it cannot be affirmed that the antibiotic activity is the same for the nematode.

In addition, 44% of the isolates produced ammonia, which is a compound that has previously been associated with pathogen control^{26,37} and has the additional advantage of being volatile and the ability to expand through soil pores, reaching a higher volume around the bacterial colonies producing the compound. Furthermore, ammonia is a compound that acts during organic matter decomposition, which improves soil structure and plant nutrition, resulting in higher productivity by the crop and higher tolerance to phytoparasites.³⁸ The phosphate solubilization observed among the isolates may improve plant nutrition. This aspect is especially important, as the damage to peach trees by *M. xenoplax* is aggravated under conditions of low soil fertility.³⁹

A high percentage (85%) of bacteria selected in vitro exhibited high mortality in mobile forms of the ringed nematode. The potency of this ability may be related to different mechanisms of action, which is indicated by the number of bacteria-produced CRBPGs. Other studies have associated the individual production of some compounds with the control of different nematodes.^{26,40,41} However, none of these studies examined the diversity in compounds produced for the selection of biocontrol isolates.

Sequencing of 16S rRNA allows the accurate identification of bacterial genera from various plant species. *Bacillus* is an important bacteria genus because of the synthesis of a wide range of metabolites with diverse properties.^{42,43} However, for

the precise definition of species and subspecies analysis of other genes is needed, for example *gyrA*.⁴⁴

It is very important to develop fast and efficient methods to select biocontrol microorganisms, especially when evaluating a large number of candidates. In vitro evaluations allow for a reduction in the number of isolates, which makes further in vivo tests viable. This aspect is crucial when working with perennial plants and long-cycle, obligate pathogens, such as the peach tree-*M. xenoplax* pathosystem.

Thus, the results obtained in the present study indicate the efficacy of the selection strategy and validate the use of the CRBPG production profile in reducing the number of isolates for in vivo evaluation.

Conflicts of interest

The authors declare no conflicts of interest.

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REFERENCES

1. Hyakumachi M. Research on biological control of plant diseases: present state and perspectives. *J Gen Plant Pathol*. 2013;79(6):435–440.
2. Gray EJ, Smith DL. Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. *Soil Biol Biochem*. 2005;37:395–412.
3. Kloepper JW, Schroth MN. Plant growth promoting rhizobacteria on radishes. In: *Proceedings of the 4th International Conference on Plant Pathogenic Bacteria*. Angers: INRA; 1978:879–882.
4. Van Loon LC. Plant responses to plant growth promoting bacteria. *Plant Pathol*. 2007;119:243–254.
5. Lugtenberg B, Kamilova F. Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol*. 2009;63:541–556.
6. Glick BR. *Plant Growth-Promoting Bacteria: Mechanisms and Applications*. Hindawi Publishing Corporation, Scientifica; 2012:15.
7. Lindow SE, Leveau JHJ. Phyllosphere microbiology. *Curr Opin Biotechnol*. 2002;13:238–243.
8. Kishore K, Pande S, Podile AR. Phylloplane bacteria increase seedling emergence, growth and yield of field-grown groundnut (*Arachis hypogaea* L.). *Appl Microbiol*. 2005;40:260–268.
9. Schisler DA, Slininger PJ. Microbial selection strategies that enhance the likelihood of developing commercial biological control products. *J Ind Microbiol Biotechnol*. 1997;19:172–179.
10. Validov S, Kamilova F, Qi S, et al. Selection of bacteria able to control *Fusarium oxysporum* f. sp. *Radices lycopersici* in stonewool substrate. *J Appl Microbiol*. 2007;102:461–471.
11. Köhl J, Postma J, Nicot P, Ruocco M, Blum B. Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Bio Control*. 2011;57:1–12.
12. Kado CI, Heskett MG. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. *Phytopathology*. 1970;60:969–976.
13. Cappuccino JG, Sherman N. *Microbiology – A Laboratory Guide*. Menlo Park: Benjamin Cummings Science Publishing; 1998:477.
14. Fahy PC, Persle GJ. *Plant Bacterial Diseases: A Diagnostic Guide*. Sydney: Academic Press; 1983:393.
15. Schaad NW. *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. 2nd ed. St. Paul: The American Phytopathology Society; 1988:158.
16. Renwick A, Campbell R, COE S. Assessment of in vitro screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. *Plant Pathol*. 1991;40:524–532.
17. Nautiyal CS. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol Lett*. 1999;170:265–270.
18. Chen SY, Dickson DW. A technique for determining live second-stage juveniles of *Heterodera glycines*. *J Nematol*. 2000;32(1):117–121.
19. Scott AJ, Knott M. A cluster analysis method for grouping means in the analysis of variance. *Biometrics*. 1974;30(2):507–512.
20. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow MN, eds. *Nucleic Acid Techniques in Bacterial Systematics*. Chichester: Wiley; 1991:115–147.
21. Fabry CFS, Freitas LG, Neves WS, et al. Obtenção de bactérias para o biocontrole de *Meloidogyne javanica* por meio de aquecimento de solo e tratamento com filtrado de raízes de plantas antagonistas a fitonematóides. *Fitopatol Bras*. 2007;32:079–082.
22. Kloepper JW, Rodríguez-Kábana R, McInroy JA, Collins DJ. Analysis of populations and physiological characterization of microorganisms. *Plant Soil*. 1991;136:95–102.
23. Kloepper JW, Rodríguez-Kábana R, McInroy JA, Young RW. Rhizosphere bacteria antagonistic to soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne incognita*) nematodes: identification by fatty acid analysis and frequency of biological control activity. *Plant Soil*. 1992;139:74–84.
24. McInnis T, Kluepfel D, Zehr E. Suppression of *Criconebella xenoplax* on peach by rhizosphere bacteria. In: *Second International Nematology Congress, Abstracts of Papers* 106. 1990.
25. Kluepfel DA, McInnis TM, Zehr EI. Involvement of root-colonizing bacteria in peach orchard soils suppressive of the nematode *Criconebella xenoplax*. *Phytopathology*. 1993;83(11):1240–1245.
26. Westcott SW, Kluepfel D. Inhibition of *Criconebella xenoplax* egg hatch by *Pseudomonas aureofaciens*. *Phytopathology*. 1993;83:1245–1249.
27. Zavaleta-Mejia E, Van Gundy SD. Effects of rhizobacteria on *Meloidogyne* infection. *J Nematol*. 1982;14(4):475–476.
28. Duffy B, Schouten A, Raaijmakers JM. Pathogen self-defense: mechanisms to counteract microbial antagonism. *Annu Rev Phytopathol*. 2003;41:501–538.
29. Aalten PM, Vitour D, Blanvillain SR, Gowen SR, Sutra L. Effect of rhizosphere fluorescent *Pseudomonas* strains on plant parasitic nematodes *Radopholus similis* and *Meloidogyne* spp. *Lett Appl Microbiol*. 1998;27:357–361.
30. Dunne C, Moenne-Loccoz Y, de Bruijn FJ, O’Gara F. Overproduction of an inducible extracellular serine protease improves biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* strain W81. *Microbiology*. 2000;146:2069–2078.
31. Feifilova EP. The fungal cell wall: modern concepts of its composition and biological function. *Microbiology*. 2010;79(6):711–750.
32. Hallmann J, Rodríguez-Kábana R, Kloepper JW. Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control. *Soil Biol Biochem*. 1999;31:551–560.
33. Slimene IB, Tabbene O, Gharbi D, et al. Isolation of a chitinolytic *Bacillus licheniformis* S213 strain exerting a biological control against *Phoma medicaginis* infection. *Appl Biochem Biotechnol*. 2015;175(7):3494–3506.
34. Brzezinska SM, Jankiewicz U, Burkowska A, Walczak M. Chitinolytic microorganisms and their possible application in environmental protection. *Curr Microbiol*. 2014;68:71–81.
35. Hsu SC, Lockwood JL. Powdered chitin agar as a selective medium to enumeration of actinomycetes in water and soil. *Appl Microbiol*. 1975;29:422–426.
36. Siddiqui IA, Haas D, Heeb S. Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode *Meloidogyne incognita*. *Appl Environ Microbiol*. 2005;71(9):5646–5649.
37. Fravel DR. Role of antibiotics in the biocontrol of plant diseases. *Annu Rev phytopathol*. 1988;26:75–91.
38. McSorley R. Alternative practices for managing plant-parasitic nematodes. *Am J Altern Agric*. 1998;13:98–104.
39. Ritchie DF, Clayton CN. Peach tree short life: a complex of interacting factors. *Plant Dis*. 1981;65(6):462–469.
40. Araújo FF, Silva JFV, Araújo ASF. Influência de *Bacillus subtilis* na eclosão, orientação e infecção de *Heterodera glycines* em soja. *Ciência Rural*. 2002;32(2):197–202.

41. Dawar SM, Tariq M, Zaki MJ. Application of *Bacillus* species in control of *Meloidogyne javanica* (Treb) Chitwood on cowpea and mash bean. *Pakistan J Bot.* 2008;40(1):439–444.
42. Jacobsen BJ, Zidack NK, Larson BJ. The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases. *Phytopathology.* 2004;94: 1272–1275.
43. Velmurugan N, Choi MS, Han SS, Lee YS. Evaluation of antagonistic activities of *Bacillus subtilis* and *Bacillus licheniformis* against wood-staining fungi: in vitro and in vivo experiments. *J Microbiol.* 2009;47:385–392.
44. Chun J, Bae KS. Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences. *Anton Van Leeuw J Microbiol.* 2000;78:123–127.