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RESEARCH ARTICLE

Selection of bioantagonistic bacteria to be used in biological control of *Rhizoctonia solani* in tomato

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Bacteria from the rhizoplane and surrounding soil of healthy and *Rhizoctonia solani* diseased tomato plants, cropped in greenhouse of the V Region of Chile, were collected. The best bacterial strains, based on their ability to control development of three *R. solani* isolates (identified as belonging to the anastomosis groups AG-2-1, AG-4), were identified as *B. subtilis* (one isolate) and *B. lentimorbus* (two different isolates). All bacterial isolates resulted effective for the *in vitro* control of growth of all *R. solani* isolates, where the control mechanisms used by the bacteria do not involve the secretion of fungal cell wall hydrolytic enzymes. *R. solani* AG-2-1 was more sensitive than *R. solani* AG-4. On the other hand, all bacteria grew well in conditions similar to those that can be found at the field level (considering pH, salinity, Fe^{3+} and temperature) and showed a good capacity of tomato root colonization. These results suggest that the *B. subtilis* and *B. lentimorbus* isolates studied have an excellent potential to be used as biocontrol agents of *R. solani* in tomato greenhouses at the field level.

Rhizoctonia solani is one of the most important soilborne fungal pathogens which develops both in cultured and non-cultured soils, causing diseases in different crops such as rice, bean and tomato, among others (Sneh et al. 1991).

Different methods have been used to control *R. solani,* being the most usedcultural practices, solarization, chemical and biological control. This last method has been developed successfully during the last years. It is based on the reduction of inoculum or of pathogenic activity due to the natural presence of one or more organisms, through the management of the environment, the host or antagonists (Baker and Cook, 1974).

Fungi from *Trichoderma* genus are among the biological control agents of *Rhizoctonia solani* (Hadwan and Khara, 1990; Lin et al. 1994) also bacteria belonging to *Pseudomonas* and *Bacillus* genus have been also used (Gasoni et al. 1998). Nematodes, such as *Aphelenchus avenae*, have been tested to prevent damping-off due to *R. solani* infection in cucumber seedlings (*Cucurbita pepo* L.). According to present and future regulations on the use of chemical fungicides such as methyl bromide, and considering that treatments must prevent environmental pollution, we have considered the use of biocontrol agents to control *R. solani* that affect tomato plants.

The selection of bioantagonistic microorganisms, other to take into account the direct effect on pathogen development, must consider conditions where the bioantagonist should develop, *i.e.* salinity and pH of soils and different temperature, among others. Therefore, the objective of this work is the isolation of antagonistic bacteria that could control *R. solani*, both *in* vitro and *in* vivo, and their characterization in terms of antagonistic mechanisms used to control the pathogen, and conditions for growth similar to those present in the field.

MATERIALS AND METHODS

Isolation of *Rhizoctonia solani* and determination of anastomosis groups

Pieces of tomato roots and crown obtained from tomato plants that showed symptoms of *R. solani* disease were submerged in 5% sodium hypochloride for five minutes. After this treatment, they were extensively washed with sterile distilled water and placed on Petri dishes containing potato-dextrose-agar (PDA, Difco) and incubated at 22ºC for 48 hrs. The isolated *R. solani* strains were stored at 5ºC in tubes containing PDA, or at -21ºC in flasks containing previously autoclaved and chloramfenicol (250 ppm) enriched oat seeds. The determination of the anastomosis group of the selected isolates was done using the methodology of Sneh et al. 1991.

Isolation, selection, innoquity and identification of bacteria

The potential bioantagonistic bacteria were isolated either from rhizoplane or from healthy or diseased tomato plants, as follows: a) root pieces from tomato plants were washed with tap water, placed inside tubes containing $MgSO_4$ and subjected to ultrasound (50-55 KHz during five minutes) (Pumarino, 1995). The bacterial suspension obtained was diluted to 10^{-3}, 10^{-5} y 10^{-7} for selection; b) 1 g soil was placed in a tube containing sterile distilled water, and dilutions similar as above were done. For both types of samples, 0.1 mL of each dilution were placed in B King medium and PDA, incubated at 22ºC until colony development was observed. The obtained bacteria were pre-evaluated against the isolated *R. solani* strains. 20 µL of bacterial suspensions (5×10^9 cfu mL^{-1}) 24 hrs old, obtained from different samples, were placed on different 0.5cm sterile paper disks. Disks were placed on a Petri dish containing PDA, surrounding a 10-mm diameter disk containing mycelium of a four-day culture of *R. solani*, placed in the center of the plate. It was incubated for 48 hrs at 22ºC, and inhibition of mycelium growth was checked. Bacteria that did not inhibit fungal growth were discarded. Those with bioantagonistic activity were stored in tubes containing B King medium at 5ºC, and in flasks containing TSB (tryptone soy broth) plus glycerol for storage at -21ºC (Raupach and Kloepper, 1998).

In order to test if selected bacteria were pathogenic to tomato plants, seedlings (two - three true leaves) were

treated immersing previously wounded (sterile needle) roots of tomato seedlings Cal ace variety in: a) a suspension of the antagonistic bacteria ($5x10^9$ cfu mL^{-1} in 2% methyl cellulose at pH 7.0) for 60 seconds (Raupach and Kloepper, 1998), b) sterile distilled water (control 1); c) 2% methyl cellulose at pH 7.0 (control 2). Once treated, seedlings were placed in speedlings containing a mixture of perlite : vermiculite = 1:1 (w/w). Seedlings were maintained under glasshouse at 15 - 25ºC with daily watering with the addition of fertilizers. Height, crown diameter, root dry weight and damage were checked after plants reached a development corresponding to four - five true leaves.

When pathogenicity was tested on seeds, these were immersed in bacterial solutions ($5x10^9$ cfu mL^{-1} in 2% methylcellulose at pH 7.0) for five minutes (Raupach and Kloepper, 1998). Seeds were germinated in the same conditions as for seedlings, but evaluation was done when seedlings showed two - three true leaves. This included the same parameters plus germinating days.

Experiments were repeated six times. Results correspond to mean of all experiments. Data was analyzed using ANOVA and the Duncan Multiple Test to establish significant differences.

Antagonism

Dual cultures. One 10-mm disk of a pure culture of *R. solani* was placed at the center of a Petri dish containing PDA. A circular line, made with a 6-cm diameter Petri dish dipped in a suspension of bioantagonistic bacteria (5×10^9- cfu mL^{-1}), was placed surrounding the fungal inoculum (Figure 1). Plates were cultured for 72 hrs at 22ºC and growth diameter of the pathogen (fungal growth) was measured and compared to control growth where the bacterial suspension was replaced by sterile distilled water. Each experiment considering a single *R. solani* isolate was run in triplicate and was repeated at least three times. Results are expressed as means % inhibition + S.D. of the growth of the corresponding *R. solani* isolatein the presence of any of the bacterialisolates. Percent inhibition was calculated using the following formula:

$$\% \text{ inhibition} = (1 - (\text{Fungal growth} / \text{Control growth})) \times 100$$

Student test was performed at $p < 0.05$.

Production of difusible antibiotics. PDA plates, covered with a cellophane membrane, were inoculated in the center with 100 µL of a bioantagonistic bacterial suspension (5×10^9 cfu mL^{-1}). After incubation for 72 hrs at 22ºC, the membrane with the grown bacterial isolate was removed, and the plate was inoculated in the middle with a 10-mm disk of a pure culture of R. solani. Plates were further incubated at 22ºC for 48 hrs and the growth of the pathogen was measured. Controls were run with mocked inoculated PDA containing plates on the cellophane membrane (replacing the bacterial suspension by sterile distilled water), and further inoculated with R. solani. Each experiment considering a single bacterial isolate was run in triplicates and was repeated at least three times. Results are expressed as means of % inhibition $\pm$ S.D. of growth of R. solani in the presence and absence of any bioantagonistic bacterial isolate. Student test was performed at $p < 0.05$.

Production of volatile antibiotics. 100 µL of a bioantagonistic bacterial suspension ($5x10^9$ cfu mL^{-1}) were placed at the center of one half Petri dish containing King B medium, and a 10-mm disk of a four days old pure culture of R. solani was placed at the center of another Petri dish containing PDA. Both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension, and were sealed to isolate the inside atmosphere and to prevent loss of volatiles formed. Plates were incubated at 22ºC for 48 hrs and the growth of the pathogen was measured and compared to controls developed in the absence of the bioantagonist (mocked inoculation with an 8-mm disk of PDA). Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least three times. Results are expressed as means of % inhibition $\pm$ S.D. of the growth of R. solani in the presence and absence of any bacterial isolate. Student test was performed at $p < 0.05$.

Secretion of enzymes involved in bioantagonism. Bacteria were cultured either on plates with solid medium or in tubes with liquid medium after inoculation with 100 µL of a bacterial suspension ($5*10^9$ cfu mL^{-1}). Media contained either chitin or yeast glucans as the sole carbon source. Development of bacteria was assessed through colony counting after incubation at 22ºC for 48 hrs of plates with solid media or through measurement of absorbance at 600 nm after incubation of tubes with liquid medium at 22ºC for 48 hrs. Also, concentrated supernatants from liquid cultures of each bacteria were analyzed for the presence of endochitinase, β-1,3-glucanase and protease activity, as described (Zaldívar et al. 2001; Pérez et al. 2002).

Effect of Fe^{+3} on antagonism level. This effect was tested according to Pumarino, 1995 using $FeCl_3x6H_2O$ at 0, 5, 15 and 25 ppm added to the King B medium.

For all tests, the experimental unit was one Petri dish.

Effect of different salt concentrations, pH values and temperatures on development of antagonistic bacteria

The development of bioantagonistic bacteria was tested under NaCl concentrations of 0, 50, 100, 150, 200 and 250

mM; at pH values which ranged between 5.0 and 8.0 (with intervals each 0.5 pH units); and temperatures of incubation of 10, 15, 22, 28 and 37ºC.

0.5 mL of a bacterial suspension ($5x10^9$ cfu ml^{-1}) from a 24 hrs old culture were placed in a tube containing 4.5 mL of peptone broth previously adjusted at the desired NaCl concentration and/or pH value. Tubes were incubated for 48 hrs at the temperatures mentioned above, and bacterial growth was estimated at 600 nm, after serial dilutions when necessary. The experimental unit was one tube for each experimental condition. Results correspond to the mean of all experiments, which were repeated at least four times. Data was analyzed using ANOVA and the Duncan Multiple Test was used to establish significant differences.

Bacterial ability for root colonization

Eight pre-germinated tomato seeds were placed on Petri dishes containing water-agar, and the ability of bacteria to colonize roots was established according to Misaghi, 1990. For evaluation, radicle apex were isolated and placed in tubes containing $MgSO_4$, subjected to ultrasound (50 - 55 KHz for five minutes), and the bacterial suspension obtained was diluted. 0.1 mL of these dilutions were seeded on Petri dish containing King B medium, and incubated for 24 hrs at 28ºC, and cfu were counted. The experimental unit was one Petri dish with eight pre-germinated seeds. Results correspond to the mean of all experiments, which were repeated at least four times. Data was analyzed using ANOVA and the Duncan Multiple Test was used to establish significant differences.

RESULTS AND DISCUSSION

Bacterial antagonists

Twenty bacterial isolates were initially selected from all isolates tested; and from these, only isolates 629, 639 and 640 showed antagonistic properties against the isolate 618 of *R. solani* (Table 1), which resulted to belong to the anastomosis group AG 4 according to the methodology of Sneh et al. 1991.

These three bacterial isolates were identified as *Bacillus* by CABI - Bioscience (England) as follows: Isolates 629 and 640 as *Bacillus lentimorbus* Dutky 1940 (transferred to the genus *Paenibacillus lentimorbus* comb. nov (Pettersson et al. 1999), and isolate 639 as *Bacillus subtilis* Cohn 1872).These three isolates were used in all other experiments.

Innoquity test of bacterial isolates on tomato plants

Although *Bacillus subtilis* and *B. lentimorbus* are not considered as phytopathogens, there are some reports that have related *B. subtilis* to soft rots in garlic and potato tubers (Kararah et al. 1985).

No lesions were observed in roots or in crowns of tomato plants, neither seed affected by the presence of the three selected bacteria, suggesting that they are not pathogenic both for tomato seeds and seedlings. A 100% emergence at day 8 was observed for all treatments, and no significant differences were observed related to dry weight of roots, crown diameter and seedling height. Nevertheless, it is important to mention that treatment of seeds with methylcellulose plus *B. lentimorbus* 640 resulted in a 100% emergence at day seven.

Based on these results, any negative effect of bacteria was discarded and antagonistic tested were run using these three bacterial isolates.

Antagonistic mechanisms

Direct antagonism through dual cultures. No physical contact was observed between any of the bacteria tested and *R. solani* 618; moreover, an inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. It has been described that *B. subtilis* can secrete several antifungal metabolites such as subtiline, bacitracin, bacillin and bacillomycin, which belong to the iturine family (Alippi and Mónaco, 1994). On the other hand, a change in mycelial color was observed closed to the colony end of *R. solani*, being this one of a darker brown than the one observed at the center of the colony. Microscopy observation of this zone, allowed to detect cytoplasmic leakage that could be observed up to the hyphal septum, resulting in deformation and sliming of their apex up to 1/10 of its original size (Figure 2). Similar results were obtained by Fiddaman and Rossall, 1993 (cited by Alippi and Monaco, 1994), who observed hyphal vacuolization and deformation in *R. solani* and in *Pythium ultimum* as the result of treatment with a *B. subtilis* strain which secreted a volatile metabolite with fungicide properties. The similar effect observed as the result of treatment of *R. solani* either with *B. subtilis* or *B. lentimorbus* could be also due to the secretion of antibiotics by these bacteria, because no enzymes involved in biocontrol, such as chitinases and/or glucanasas and/or proteases, could be detected (Silva et al. 2001).

When different *R. solani* isolates were tested against the three bacterial antagonists, growth of isolate 509 (AG 2) was the most inhibited by the three bacteria, whereas, the isolate 618 (AG 4) was the less inhibited (Table 2). These agree with previous results on pathogenicity levels and growth rate shown for these two *R. solani* isolates (Reyes, 2000), where the isolate 618 was shown to be the most pathogenic and with the highest growth rate. These

characteristics give to this isolate an advantage when competing for space and nutrients in the culture medium.

Difusible antibiotics. *B. lentimorbus* 640 was the antagonistic bacteria isolate that showed the best inhibitory effect on the growth of any of the four *R. solani* isolates tested, although all bacteria showed inhibitory effect on *R. solani* grow (Table 3).

Volatile antibiotics. Results, similar to those obtained when testing the effect of difusible antibiotics, were obtained when the effect of volatiles was tested (Table 4). Isolate 509 (AG2-1) of *R. solani* was the most inhibited independently of the bacterial isolate tested; while isolate 554 (AG4) was the less inhibited by volatile antibiotics as opposite to the observed for the effect of difusible metabolites on this same isolate (Table 3).

Secretion of enzymes involved in bioantagonism. None of the three bacteria was able to grow in solid or in liquid media that contained chitin or yeast glucans as the sole carbon source. Moreover, endochitinase or β-1,3-glucanase or protease activity was not detected in supernatants from liquid cultures, suggesting that enzymes, if synthesized, are not secreted, or that these enzymes are not formed at all by the bacteria.

If we consider that none of the three bacteria was able to secrete any of the enzymes involved in biocontrol, and that all had the ability to control *R. solani* through secretion of difusible and volatile metabolites, it may be concluded that they use these two latter mechanisms of biocontrol as opposite to some fungal biocontrol microorganisms that also use fungal cell wall hydrolyzing enzymes within their biocontrol mechanisms (Pérez et al. 2002).

Effect of Fe^{3+} on antagonism. Iron is a fundamental element for respiration of several aerobic and facultative anaerobic microorganisms, and therefore, its availability in soils is essential (Leong, 1986). On the other hand, siderophores are low weight compounds with high affinity for Fe^{3+} (Nielands, 1981), which are produced under limiting concentrations of iron. These compounds are able to transport this element inside the cell for metabolic functions (Press et al. 2001), and microorganisms which are able to produce siderophores show competitive advantages as compared to those that do not produce them. From this point of view, the competence for iron increases in conditions where this element is limiting, but this condition is reverted when iron is added to the culture medium (Elad and Baker, 1985).

The three antagonistic bacteria showed similar behavior on *R. solani* growth at any of the Fe^{3+} concentrations tested (Table 5). If it is considered that iron available in soils fluctuate between 1.8 and 27 ppm, it may be concluded that this element is not limiting for the antagonistic activity of these bacteria on *R. solani*.

Bacterial growth at different NaCl concentrations. Figure 3 shows the growth of the different bacterial isolates tested as antagonistics of *R. solani*, under different concentrations of NaCl. The best growth of *B. lentimorbus* 629, 640 and *B. subtilis* 639 was observed at 50, 200 y 100 mM de NaCl, respectively. Concentrations of 250 mM NaCl inhibited bacterial growth of *B. lentimorbus* 629 and *B. subtilis* 639 as compared to controls run in the absence of NaCl, while inhibition of growth of *B. lentimorbus* 640 was observed at 200 mM NaCl.

Bacillus lentimorbus 640 showed a higher growth rate, independently of the NaCl added to the medium, than the other bacterial isolates tested, although high concentrations of NaCl are not limiting for the growth of any of the bacteria. In fact, the electric conductivity of soils from where bacteria were isolated was between 1.07 and 9.52 $mmhocm^{-1}$ which correspond to NaCl concentrations lower than 50 mM; therefore, there is no possibility that NaCl could inhibit bacterial growth in soils of the V Region of Chile (Table 6).

Bacterial growth at different pH values. *B. subtilis* 639 and *B. lentimorbus* 640 grew best at pH 5.0, *B. lentimorbus* 629 showed its highest growth at pH 5.5 and the three antagonistic bacteria almost did not grow at pH 8.0 (Figure 4).

These results agree in part with those which describe the optimum growth of *B. subtilis* between 5.7 and 6.8 but only a 10% growth of *B. lentimorbus* in this pH range, being the best pH values for the growth of this latter higher than 7.0 (Claus and Berkeley, 1986). Nevertheless, our results were obtained at time periods higher than those used by Claus and Berkeley, 1986 suggesting that at longer time period there is almost no difference in growth between these two bacterial species. Therefore, in terms of use of these bacteria at the field level initial rates of growth observed at the laboratory are irrelevant.

In addition, it is important to mention that pH values where the highest growth of these bacteria were obtained, are coincident with the optimum pH for tomato development which is between pH 5.5 and 7.0 (Nuez, 1995). On the other hand, soils from where bacteria were obtained showed a pH value between 6.2 and 7.2 when suspended in water, suggesting that soil pH does not affect their growth.

Bacterial growth at different temperatures. The lowest development for all three bacteria was observed at 10ºC; *B. lentimorbus* 629 and 640 grew better at 28ºC, while the best development of *B. subtilis* 639 was observed at 37ºC (Figure 5). These results agree with those already

reported for both bacterial species (Claus and Berkeley, 1986).

When considering that soil temperatures are closed to 30ºC at the production season (González, 2001), bacterial growth will not be affected by this condition. This fact, along with results obtained for growth at different Fe^{3+} or NaCl concentrations and different pH values, suggest that development of these three bacteria should not be affected on the field conditions.

Bacterial capacity to colonize tomato roots

The use of bacteria to exert an appropriate biological control of *R. solani* and of other soil borne fungi relies on their ability to colonize roots efficiently, otherwise, their biocontrol character would be non-sense. The ability to colonize roots is highly variable between rhizobacteria, being this characteristic a reflection for their ability to compete for ecological niches in the rhizosphere (Misaghi, 1990). The analysis of results from this assay showed that no significant differences were observed among the three bacterial antagonists, although less than 10% of the initial cfu could be recovered.

From all these results it may be concluded that the biocontrol effect of antagonistic bacteria isolated from soils (*B. subtilis* 639 and *B. lentimorbus* 629 and 640) against *R. solani* (belonging to AG-2-1 and AG-4) are adequate for their use at the field level, specially *B. lentimorbus* 640. Within the mechanisms used by these bacteria are the secretion of volatile and difusible metabolites but not of fungal cell wall hydrolytic enzymes. In addition, they show growth characteristics, which are compatible to the conditions of salinity, pH, Fe^{3+}, and temperature that can be found in soils where tomato is commonly cultivated. Therefore, these bacteria could be used at the field level to biocontrol the *R. solani* disease in tomatoes.

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APPENDIX

Figures

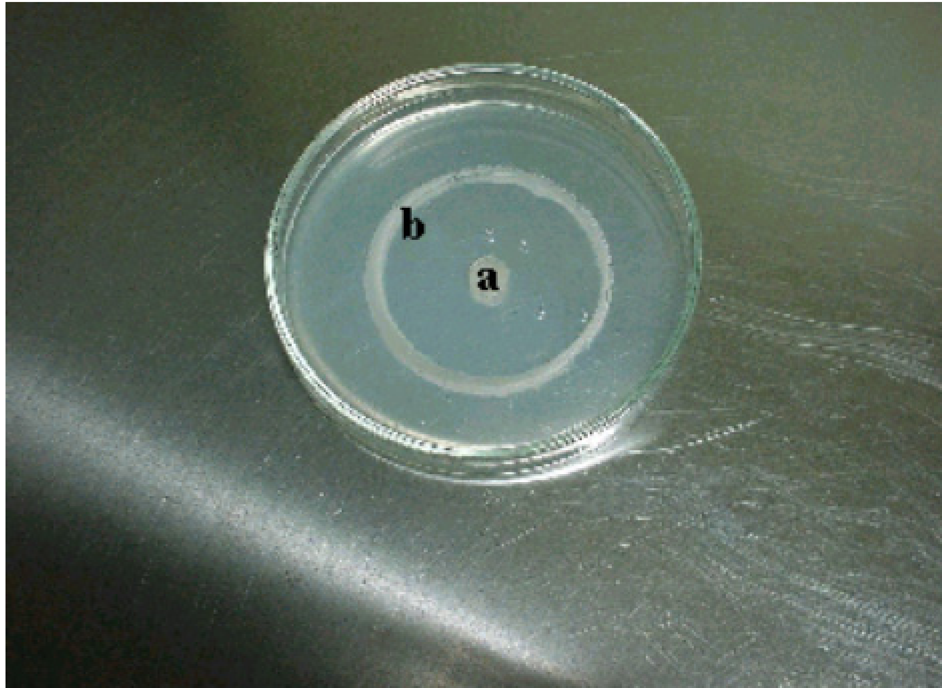

Figure 1. Dual cultures.

a) *Rhizoctonia solani*.
b) Antagonistic bacteria.

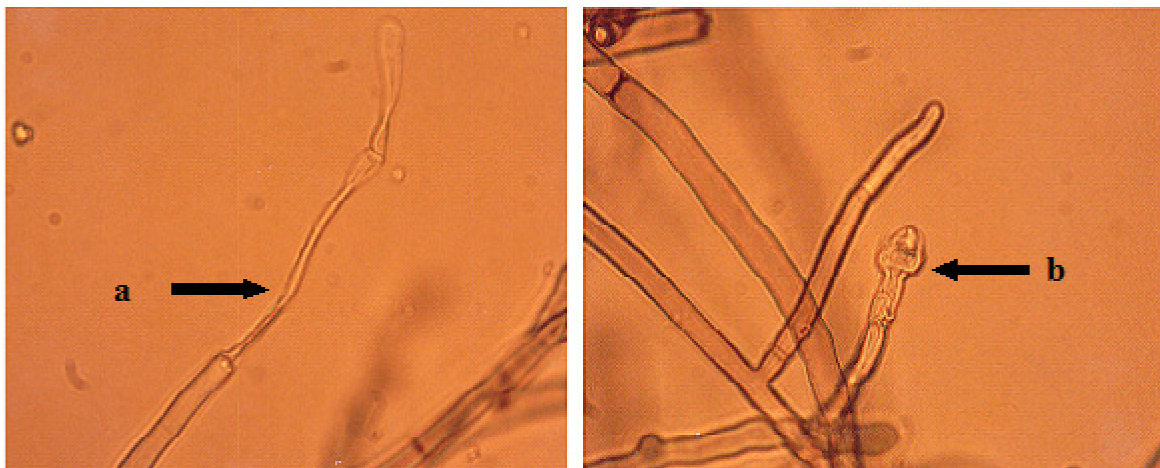

Figure 2. Hypha from *R. solani* observed in dual cultures against *B. subtilis* isolate 639.

a) Cytoplasmic leakage and further hyphal sliming.
b) Apex deformation.

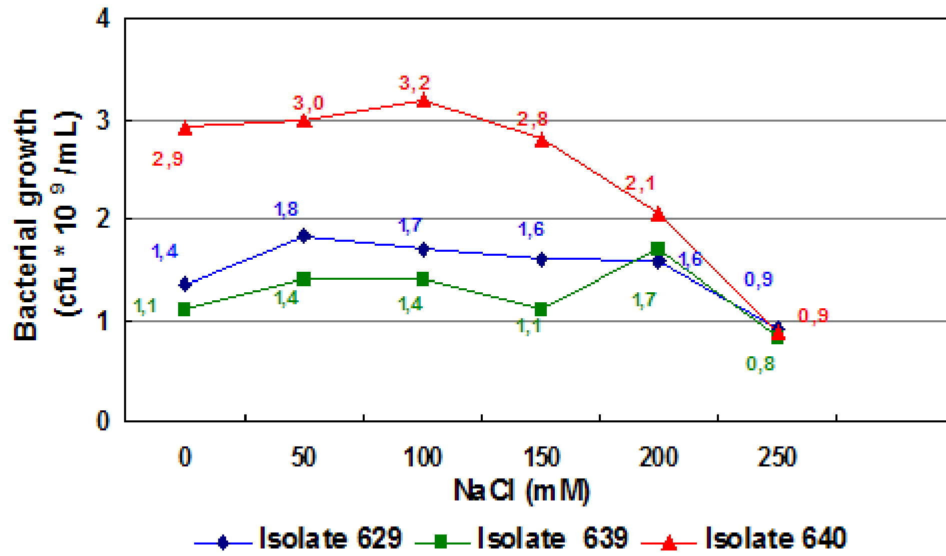

Figure 3. Growth of *B. lentimorbus* 629, of *B. lentimorbus* 640 and of *B. subtilis* 639 at different NaCl concentrations.

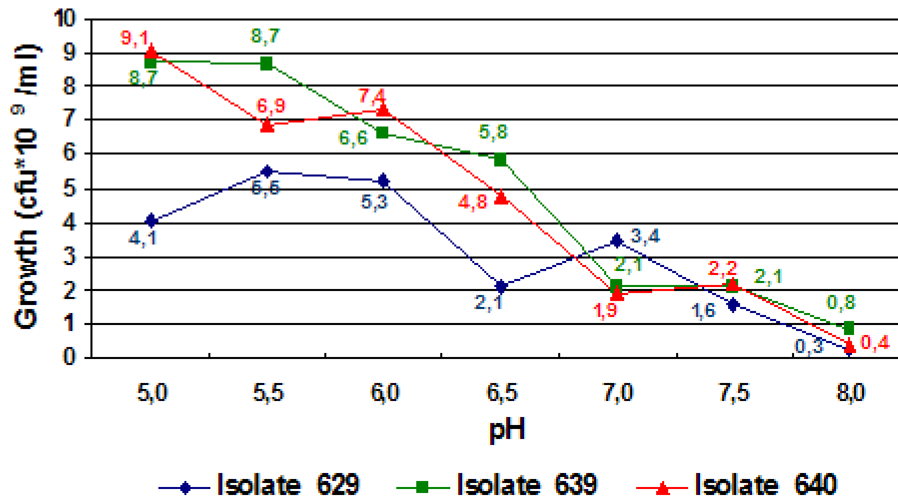

Figure 4. Growth of *B. lentimorbus* 629 and 640, and of *B. subtilis* 639 at different pH values.

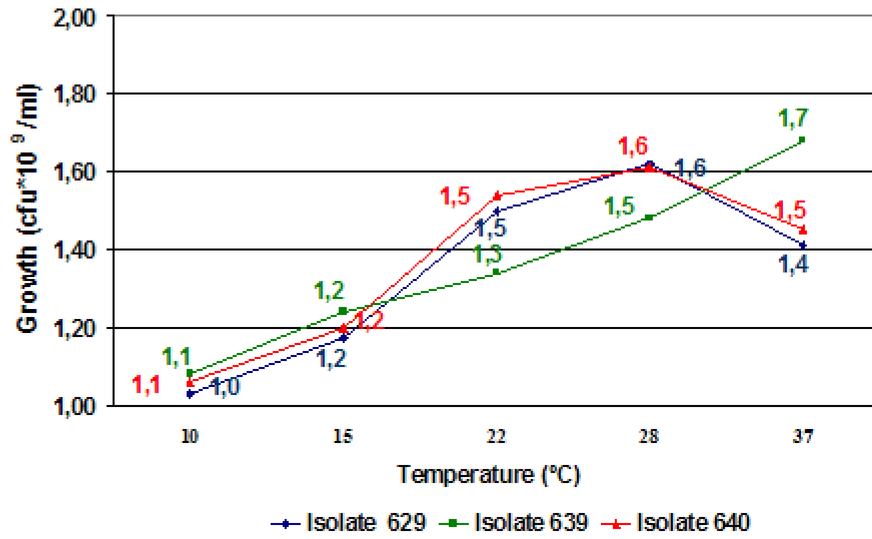

Figure 5. Growth of *B. lentimorbus* 629 and 640, and of *B. subtilis* 639 at different temperatures.

Tables

Table 1. Pre-evaluation of antagonistic properties of bacteria isolated from soil and rhizoplane of healthy and diseased tomato plants, collected at V Region - Chile, on *R. solani*, isolate 618, AG 4.

Potential antagonistic bacteria Isolate Nº	*R. solani*radial growth[1] (mm)	% Inhibition of *R. solani* radial growth
Control	30.00 a[2]	0.00
133	30.00 a	0.00
140	30.00 a	0.00
559	30.00 a	0.00
133	30.00 a	0.00
137	30.00 a	0.00
139	29.88 a	0.42
459	29.78 ab	0.75
132	29.78 ab	0.75
138	29.38 abc	2.08
137	29.18 abcd	2.75
130	29.15 abcd	2.83
136	28.80 abcd	4.00
121	28.58 bcd	5.92
135	28.18 cde	6.09
141	28.10 de	6.33
111	28.00 de	6.67
457	27.13 e	9.58
639	20.55 f	31.50
629	19.65 f	34.50
640	19.60 f	34.67

1 Growth of *R. solani* in PDA during 72 hours at 22ºC.
2 Different letters represent significant differences in the Duncan's Multiple Range Test (p£0.05).

Table 2. Growth inhibition of four isolates of *R. solani* in dual cultures against *B. lentimorbus* and *B. subtilis.*

*R. solan*Isolate Nº	Antagonistic bacteria					
	B. lentimorbus 629		*B. subtilis* 639		*B. lentimorbus* 640	
	Growth[1] of *R. solani* (mm)	% Inhibition	Growth[1]of *R. solani* (mm)	% Inhibition	Growth[1] of *R. solani* (mm)	% Inhibition
509 (AG 2-1)	16.30 c A	45.30	15.45 c B	48.15	15.58 b B	47.73
554 (AG 4)	17.23 b B	41.60	17.93 b A	39.23	18.13 a A	38.58
617 (AG 4)	17.65 b B	41.15	18.83 b A	37.28	18.43 a AB	38.60
618 (AG 4)	18.55 a B	38.58	20.40 a A	32.43	19.00 a B	37.10

[1] Growth of R. solani was evaluated after 72 hours at 22ºC in PDA medium.
Different letters in columns and different capital letters in rows represent significant differences in the Duncan's Multiple Range Test (p£0.05).

Table 3. Effect of diffusible antibiotics secreted by *B. lentimorbus* and by *B. subtilis* on radial growth of *R. solani*.

R. solaniIsolate Nº	Antagonistic bacteria					
	B. lentimorbus 629		*B. subtilis* 639		*B. lentimorbus* 640	
	Growth[1] of *R. solani*(mm)	% Inhibition	Growth[1]of *R. solani* (mm)	% Inhibition	Growth[1] of *R. solani* (mm)	% Inhibition
509 (AG 2-1)	23.25 a A	22.48	21.79 a AB	27.35	21.08 a B	29.73
554 (AG 4)	17.51 c A	41.63	16.59 a B	44.73	16.87 a AB	43.78
617 (AG 4)	21.48 b A	28.40	21.55 a A	28.18	20.85 a A	30.53
618 (AG 4)	23.04 a A	23.23	21.50 a A	30.53	20.28 a A	32.40

[1] Growth of R. solani was evaluated after 72 hours at 22ºC in PDA medium.
Different letters in columns and different capital letters in rows represent significant differences in the Duncan's Multiple Range Test (p£0.05).

Table 4. Effect of volatile antibiotics secreted by *B. lentimorbus* and by *B. subtilis* on radial growth of *R. solani*.

R. solani Isolate Nº	Antagonistic bacteria					
	B. lentimorbus 629		*B. subtilis* 639		*B. lentimorbus* 640	
	Growth[1] of *R. solani* (mm)	% Inhibition	Growth[1] of *R. solani* (mm)	% Inhibition	Growth[1] of *R. solani* (mm)	% Inhibition
509 (AG 2-1)	16.50 c A	44.93	19.29 c A	35.68	17.28 c A	42.38
554 (AG 4)	23.93 a A	20.23	23.57 a A	21.43	23.73 a A	20.90
617 (AG 4)	21.89 ab A	27.03	21.14 bc AB	29.55	20.09 ab B	33.03
618 (AG 4)	20.61 b A	31.30	22.04 ab A	26.55	19.10 bc A	36.33

[1] Growth of *R. solani* was evaluated after 72 hours at 22ºC in PDA medium.
Different letters in columns and different capital letters in rows represent significant differences in the Duncan's Multiple Range Test (p£0.05).

Table 5. Effect of different concentrations of Fe^{+3} on the radial growth of *R. solani* in the presence of *B. lentimorbus* and of *B. subtilis*.

Fe^{+3} as $FeCl_3$ (uM)	*B. lentimorbus* 629		*B. subtilis* 639		*B. lentimorbus* 640	
	Growth[1] of *R. solani*(mm)	% Inhibition	Growth[1] of *R. solani* (mm)	% Inhibition	Growth[1] of *R. solani*(mm)	% Inhibition
0	17.54 b	41.54	17.69 b	41.05	17.83 b	40.70
5	18.23 ab	39.20	18.57 ab	38.10	18.48 ab	38.41
15	18.79 a	37.38	19.09 a	36.37	18.89 a	37.02
25	19.13 a	36.25	19.38 a	35.42	19.26 a	35.80

[1] Growth of R. solani was evaluated after 72 hours at 22ºC in PDA medium.
Different letters represent significant differences in the Duncan's Multiple Range Test (p£0.05)

Table 6. Experimental design and results of the 2^2-full orthogonal design with 3 repetitions at the center point.

NaCl (mM)	Electric conductivity ($mmhoscm^{-1}$)
0	----
50	5.4
100	10.5
150	17.0
200	20.0
250	24.0

Equivalence between concentration of NaCl and electric conductivity at 25ºC.

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