

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

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## Abstract

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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<sup>3+</sup> 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.**

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## Article

*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 used cultural 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.).

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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 22°C for 48 hrs. The isolated *R. solani* strains were stored at 5°C in tubes containing PDA, or at –21°C 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, innocuity 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<sub>4</sub> and subjected to ultrasound (50-55 KHz during five minutes) ([Pumarino, 1995](#)). The bacterial suspension obtained was diluted to 10<sup>-3</sup>, 10<sup>-5</sup> y 10<sup>-7</sup> 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 22°C until colony development was observed. The obtained bacteria were pre-evaluated against the isolated *R. solani* strains. 20 µL of bacterial suspensions (5 x 10<sup>9</sup> cfu mL<sup>-1</sup>) 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 22°C, 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 5°C, and in flasks containing TSB (tryptone soy broth) plus glycerol for storage at –21°C ([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<sup>9</sup> cfu mL<sup>-1</sup> 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 – 25°C 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<sup>9</sup> cfu mL<sup>-1</sup> 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 ( $5 \times 10^9$ -cfu mL<sup>-1</sup>), was placed surrounding the fungal inoculum ([Figure 1](#)). Plates were cultured for 72 hrs at 22°C 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* isolate in the presence of any of the bacterial isolates. 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 diffusible antibiotics.** PDA plates, covered with a cellophane membrane, were inoculated in the center with 100 µL of a bioantagonistic bacterial suspension ( $5 \times 10^9$  cfu mL<sup>-1</sup>). After incubation for 72 hrs at 22°C, 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 22°C 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 ± 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 ( $5 \times 10^9$  cfu mL<sup>-1</sup>) 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 22°C 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 ± 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 ( $5 \times 10^9$  cfu mL<sup>-1</sup>). Media contained either chitin or yeast glucans as the sole carbon source. Development of bacteria was assessed through colony counting after incubation at 22°C for 48 hrs of plates with solid media or through measurement of absorbance at 600 nm after incubation of tubes with liquid medium at 22°C 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<sup>+3</sup> on antagonism level.** This effect was tested according to [Pumarino, 1995](#) using FeCl<sub>3</sub>·6H<sub>2</sub>O 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 37°C.

0.5 mL of a bacterial suspension ( $5 \times 10^9$  cfu ml<sup>-1</sup>) 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<sub>4</sub>, 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 28°C, 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 glucanases 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  $\beta$ -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<sup>3+</sup> 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<sup>3+</sup> (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  $\text{Fe}^{3+}$  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  $\text{mmhocm}^{-1}$  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 10°C; *B. lentimorbus* 629 and 640 grew better at 28°C, while the best development of *B. subtilis* 639 was observed at 37°C ([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 30°C 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  $\text{Fe}^{3+}$  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<sup>3+</sup>, 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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