

# Bacterial spot and early blight biocontrol by epiphytic bacteria in tomato plants

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**Abstract** – The objective of this work was to evaluate in vitro and in vivo biocontrol of bacterial spot (*Xanthomonas vesicatoria*) and early blight (*Alternaria solani*) by the epiphytic bacteria *Paenibacillus macerans* and *Bacillus pumilus*. Tomato plants were previously sprayed with epiphytic bacteria, benzalkonium chloride and PBS buffer and, after four days, they were inoculated with *A. solani* and *X. vesicatoria*. To determine the phytopathogenic bacteria population, leaflet samples were collected from each treatment every 24 hours, for seven days, and plated on semi-selective medium. The effect of epiphytic bacteria over phytopathogens was performed by the antibiosis test and antagonistic activity measured by inhibition zone diameter. The epiphytic and benzalkonium chloride drastically reduced the severity of early blight and bacterial spot in comparison to the control (PBS). In detached leaflets, the epiphytic bacteria reduced in 70% the number of phytopathogenic bacteria cells in the phylloplane. The antibiosis test showed that the epiphytic bacteria efficiently inhibit the phytopathogens growth. In all the bioassays, the epiphytic bacteria protect tomato plants against the phytopathogens.

**Index terms:** *Alternaria solani*, *Bacillus pumilus*, *Paenibacillus macerans*, *Xanthomonas vesicatoria*, biological control, epiphytic bacteria.

## Biocontrole da mancha-bacteriana e da pinta-preta por bactérias epifíticas em tomateiro

**Resumo** – O objetivo deste trabalho foi avaliar o biocontrole in vitro e in vivo dos fitopatógenos mancha-bacteriana (*Xanthomonas vesicatoria*) e pinta-preta (*Alternaria solani*) pelas bactérias epifíticas *Paenibacillus macerans* e *Bacillus pumilus*. Plantas de tomate foram previamente pulverizadas com as bactérias epifíticas, cloreto de benzalcônio e tampão PBS e, após quatro dias, receberam inoculação com *A. solani* e *X. vesicatoria*. A fim de determinar a população da bactéria fitopatogênica, amostras de folíolos foram coletadas de cada tratamento em intervalos de 24 horas, durante sete dias, e inoculadas em meio semisseletivo. O efeito das bactérias epifíticas sobre os fitopatógenos foi realizado pelo teste de antibiose e atividade antagonística avaliada pelo diâmetro da zona de inibição. As bactérias epifíticas e o cloreto de benzalcônio reduziram drasticamente a severidade da pinta-preta e da mancha-bacteriana, comparado com o controle (PBS). Em folíolos destacados, as bactérias epifíticas reduziram em até 70% o número de células da bactéria fitopatogênica no filoplano. As bactérias epifíticas inibem eficientemente o crescimento dos fitopatógenos em meio de cultura. Em todos os bioensaios, as bactérias epifíticas protegem as plantas de tomate contra os fitopatógenos.

**Termos para indexação:** *Alternaria solani*, *Bacillus pumilus*, *Paenibacillus macerans*, *Xanthomonas vesicatoria*, controle biológico, bactéria epifítica.

## Introduction

Epiphytic bacteria have been defined as populations that can survive and multiply on the surface of plants (Hirano et al., 1982). Thus, they develop survival strategies in protected positions such as the trichomes base, inside substomatal chambers, hydathodes, and, especially, in between the depressions along the junctions of adjacent epithelial cells (Beattie &

Lindow, 1999; Lindow & Brandl, 2003; Monier & Lindow, 2004, 2005a, 2005b).

Epiphytics as biological control agents are still misused, especially compared to rhizobacteria and endophytic bacteria. However, the biocontrol of diseases affecting several crops by those microorganisms have been increasingly researched (Lindow & Brandl, 2003; Halfeld-Vieira et al., 2008). Notably, the interest in the study of those microbes is related to their capacity to

occupy ecological niches on the phylloplane that could be occupied by pathogens (Monier & Lindow, 2005a), and to their broad antagonistic effect against pathogens. Biosurfactants, antibiotics, bacteriocins and volatile organic compounds (VOCs) synthesis, siderophores and competition for space and nutrients are related to the antagonistic effects of epiphytic bacteria on the phytopathogen growth (Beattie & Lindow, 1999; Lindow & Brandl, 2003). Recent surveys demonstrate that epiphytic bacteria also act as elicitors of the induced systemic resistance (ISR) in plants (Halfeld-Vieira et al., 2006).

The objective of this work was to evaluate *in vitro* and *in vivo* biocontrol of bacterial spot (*Xanthomonas vesicatoria*) and early blight (*Alternaria solani*) by the epiphytic bacteria *Paenibacillus macerans* and *Bacillus pumilus*.

### Materials and Methods

Two epiphytic bacteria were obtained from leaves of healthy tomato plants, and identified by fatty-acid analysis (FAA) (Lanna Filho, 2006) as *Paenibacillus macerans* and *Bacillus pumilus*. Afterward, bacteria were grown in medium 523 (Kado & Heskett, 1970) [10 g L<sup>-1</sup> of sucrose, 8 g L<sup>-1</sup> of casein acid hydrolysate, 4 g L<sup>-1</sup> of yeast extract, 2 g L<sup>-1</sup> of K<sub>2</sub>HPO<sub>4</sub> (anhydrous), 0,3 g L<sup>-1</sup> of MgSO<sub>4</sub>.7H<sub>2</sub>O and 18 g L<sup>-1</sup> of agar], and preserved in deep freezer at -80°C. They were also emulsified once in 30% (v/v) glycerin.

The tomato pathogens were obtained from the Plant Pathology Department collection of the Universidade Federal de Viçosa, Brazil. The fungus *Alternaria solani* (Jones & Groat) was grown in potato dextrose agar (PDA) and maintained at 4°C under mineral oil (Smith & Onions, 1994). The bacterium *Xanthomonas vesicatoria* (Doidge) Vauterin et al. was grown in medium 523 (Kado & Heskett, 1970), preserved at -80°C, and emulsified once in 30% glycerin.

The antagonistic activity was tested by the overlay diffusion method (Vidaver et al., 1972). A drop of 15 µL of cell suspension (optical density at 540 nm wavelength, OD<sub>540</sub> = 0.3) of the *P. macerans* and *B. pumilus* epiphytic bacteria was placed in the center of the solid medium 523 (Kado & Heskett, 1970), in Petri dishes, and incubated at 28°C for 24 hours. As control, a drop of 15 µL of phosphate-buffered saline (PBS)

was placed in the center of the solid medium of the same culture.

Subsequently, the colonies were killed by exposure to ultraviolet (UV) and chloroform vapors for 1 hour. Then, melted semisolid culture media [0.8% (w/v) agar, 45°C] containing propagules of the pathogens was placed over the basal layer, and incubated at 28°C for 24 hours (*X. vesicatoria*) and at 25°C for seven days (*A. solani*). After incubation, the inhibition zones were measured and antimicrobial activity was expressed by the diameter of the inhibition zone (mm). The percentage of the inhibition zone was calculated in relation to the diameter of the Petri dish (90 mm), considered 100%. The bioassay was repeated three times for each treatment (*P. macerans*, *B. pumilus* and PBS), and three zones of inhibition were measured for each epiphytic bacterium.

The epiphytics were observed on phylloplane by scanning electron microscopy. For that, seeds of Santa Cruz 'Kada' tomato were disinfected in 70% ethanol for 2 min, sodium hypochlorite solution (2% available Cl<sup>-</sup>) for 2 min, and two washes were carried out in sterilized distilled water. Then, they were transferred to 20 mL plastic cups with the epiphytic bacteria cell suspensions, adjusted to OD<sub>540</sub> = 0.3, corresponding to approximately 10<sup>8</sup> colony forming units per mL (CFU mL<sup>-1</sup>). Cell suspension volume was only sufficient to cover the seeds. After 24 hours, seeds were placed on filter paper for drying. Seeds immersed only in PBS (1 mol L<sup>-1</sup>; pH 7.0) were used as controls. After drying, seeds were transferred to three tubes containing MS medium per treatment (Murashige & Skoog, 1962), and germinated at 25°C with 12-hours photoperiod. In fifteen-day-old plants, three leaves per treatment were cut, and submitted to a fixative procedure in a modified Karnovsky solution (glutaraldehyde 2.5% and paraformaldehyde 2.5% in sodium cacodylate buffer 0.05 mol L<sup>-1</sup>, pH 7.2, CaCl<sub>2</sub> 0.001 mol L<sup>-1</sup>), for 24 hours (at 4°C), infiltrated with a cryoprotection solution (glycerol 30% in water) for 30 min, and cross-sectioned with a scalpel blade after being immersed in liquid nitrogen.

Sections were transferred to a 1% aqueous solution of osmium tetroxide for 1 hour at room temperature, and subsequently dehydrated for 10 min each in a crescent series of acetone solutions (25, 50, 75, 90 and 100%). After that, they were dried in a critical-point drier CPD 030, (Balzers, Jundiaí, SP, Brazil).

Processed materials were mounted on aluminum stubs, fractured side up, sputter coated with gold SCD 050, (Balzers, Jundiaí, SP, Brazil) and observed in a scanning electron microscopy (SEM) LEO EVO 40 XVP (K. E. Developments, Cambridge, England). Leaves of healthy tomato plants exposed to PBS were used as controls. Two images were generated and three leaflets were used for each treatment. Images of the phylloplane region were generated at random for each sample, at several magnifications, and digitally recorded. Images were processed using the software Corel Draw 12, with which comparisons among treatments were done.

The antagonistic effect of the epiphytic bacteria over the phytopathogenic one was carried out in twenty-day-old tomato plants, previously exposed to the *P. macerans*, *B. pumilus*, benzalkonium chloride and PBS treatments. After four days, the *X. vesicatoria* suspension ( $OD_{540} = 0.3$ ) was sprayed. For each treatment, four replicates were used; with three grams of leaves per pot being considered as one replicate. Leaves were collected randomly and placed in flasks containing 50 mL of sterile phosphate buffer ( $0.1 \text{ mol}^{-1}$ , pH 7.0, containing 0.05% Tween-80), and sonicated for 10 min in an ultrasonic cleaning bath (Ultrasonic Cleaner 1440D, Odontobrás, Ribeirão Preto, SP, Brazil) in order to recover bacterial cells. Bacterial populations were estimated from three grams symptomless leaflets randomly sampled from each plant pot. The obtained suspensions of washed leaves were submitted to serial dilution (factor = 1:1,000) and were inoculated in the semi-selective medium propose by Lanna Filho & Romeiro (2009), containing cycloheximide ( $50 \mu\text{g mL}^{-1}$ ), cephalixin ( $50 \mu\text{g mL}^{-1}$ ) and streptomycin sulfate ( $50 \mu\text{g mL}^{-1}$ ). Petri dishes were incubated for 48 hours at  $28^\circ\text{C}$ , and then the CFU count was made per gram of leaf tissue. The leaf samples were collected every 24 hours, for seven days. For each evaluation day, three Petri dishes were used for each dilution, with three replicates per dishes. The mean viable bacterial population size was derived from the  $\log_{10}$ -transformed bacterial population.

In greenhouse experiments, seeds of Santa Cruz 'Kada' tomato were planted in plastic pots containing non-sterilized mixture of soil, sand and manure (2:1:1), maintained in greenhouse at  $25^\circ\text{C}$  and 70% relative humidity. In each treatment, four replicates

were used, with one plant per pot considered as a replicate. Plants were sprayed with live cells of the *P. macerans* and *B. pumilus* ( $OD_{540} = 0.3$ ) 30 days after planting. For the positive control, plants were sprayed with benzalkonium chloride sanitizer ( $2.5 \text{ g L}^{-1}$  a.i.), and PBS as negative control. Four days later, plants were inoculated spraying the *A. solani* ( $1.0 \times 10^5$  conidia  $\text{mL}^{-1}$ ) and *X. vesicatoria* ( $OD_{540} = 0.3$ ) pathogen suspensions. Inoculated plants were kept in greenhouse, and after the symptoms of the disease were fully developed, the number of lesions per leaf was counted within all the leaflets. The bioassay was repeated three times.

All experiments were carried out in a completely randomized design. The results were subjected to analysis of variance (ANOVA) and means were compared by Tukey test at 5% probability, using the software Statistica, version 7.0 (Statsoft, 2005). Regression equations, coefficient of determination ( $R^2$ ) and significance levels of the curves were calculated in order to determine the bacterial population growth according to time.

## Results and Discussion

The in vitro antibiosis test showed that the epiphytic bacteria presented direct activity against the pathogens *X. vesicatoria* and *A. solani*, inhibiting their growth (Table 1). *Paenibacillus macerans* and *B. pumilus* inhibited *X. vesicatoria* growth at 10.33 and 8.67%, respectively, and *A. solani* growth at 3.11 and 4.89%. Additionally, the control treatment with PBS showed no zone of inhibition (0%) against the pathogens. These results confirmed that the antagonists produce some type of toxic substance with antimicrobial effect against the pathogens, causing the antibiosis phenomenon.

Possibly, these substances are bioactive compounds derived from lipopeptides of the surfactin, iturin and

**Table 1.** In vitro antimicrobial activity of *Paenibacillus macerans* and *Bacillus pumilus* against tomato pathogens<sup>(1)</sup>.

Pathogen	Growth inhibition (%)		
	<i>B. pumilus</i>	<i>P. macerans</i>	Control <sup>(2)</sup>
<i>Xanthomonas vesicatoria</i>	8.67a	10.33a	0.00b
<i>Alternaria solani</i>	4.89a	3.11a	0.00b

<sup>(1)</sup>Means followed by the same letter, in the lines, do not differ by Tukey's test at 5% probability. <sup>(2)</sup>Phosphate-buffered saline (PBS).

fengycin families, frequently reported as toxic to pathogens (Peypoux et al., 1999; Ongena et al., 2005). Magnet-Dana et al. (1992) reported that the iturins and fengycins exhibit a strong antifungal activity and are inhibitory for the growth of a wide range of pathogens. Additionally, the synthesis of antimicrobials by epiphytic bacteria provides higher adaptability of those microbes on the phylloplane, impeding the growth and establishment of pathogenic populations (Beattie & Lindow, 1999).

The observation of the colonization pattern of the epiphytic bacteria on the phylloplane by SEM confirmed their ability in establishing at specific sites on the foliar surface (Figure 1). This demonstrates the capacity of these bacteria to migrate from spermosphere to the aerial part, their habitat. Notably, the formation of *P. macerans* cell aggregates embedded in an exopolymeric matrix could be observed in the depressions along the junctions of adjacent epithelial cells (Figure 1 A), besides the colonization of the stomatal region (Figure 1 B). Numerous studies report the capacity of epiphytic bacteria to form aggregates on the foliar surface of different species of plants (Morris et al., 1997; Fett, 2000), which gives them protection against environmental stress and larger survival on the phylloplane, as reported by Monier & Lindow (2004). *Bacillus pumilus* also formed small aggregates between the depressions along the junctions of adjacent epithelial cells (Figure 1 C), as well as in the stomatal region (Figure 1 D). As expected, the treatment with phosphate-buffered saline did not present bacterial colonization (Figures 1 E and F). Monier & Lindow (2005a, 2005b) report the importance of the aggregate formation for the protection of epiphytic population against desiccation, UV radiation, high temperatures and environmental fluctuations, besides the higher efficiency in nutrient acquisition and migration to regions of the phylloplane more favorable for survival. This work demonstrated that bacterial cells of *P. macerans* and *B. pumilus* form large aggregates on the tomato phylloplane. This confirms the natural habitat of these bacteria, since both were exposed to previously disinfested seeds and, after 15 days of germination, they were established at specific sites on the foliar surface.

Tomato leaves previously exposed to the epiphytic bacteria and to benzalkonium chloride drastically reduced the *X. vesicatoria* population on the

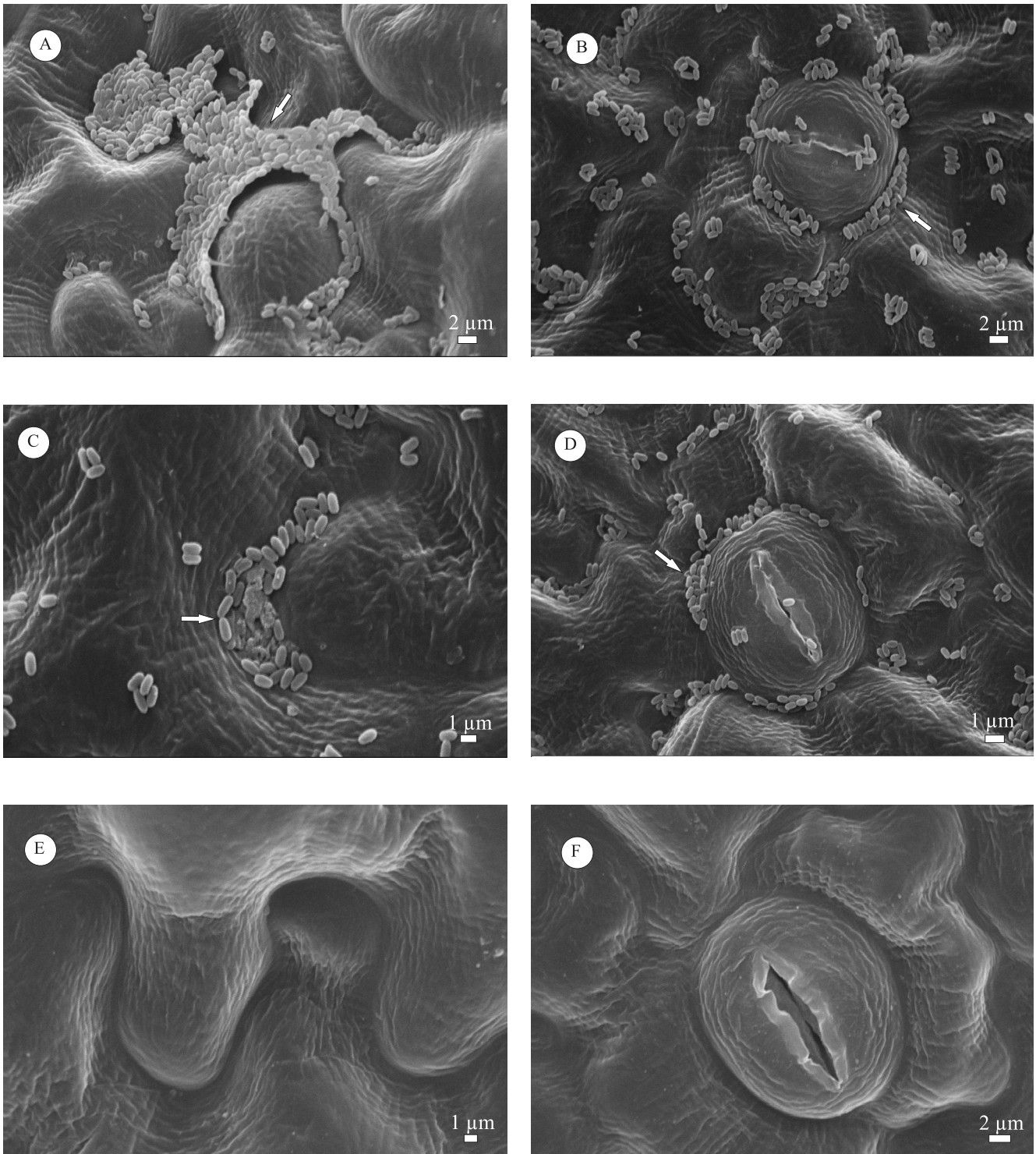
phylloplane, in comparison with the control, treated with PBS (Figure 2). The sanitizer (benzalkonium chloride) reduced the pathogen population on the phylloplane by 80.4%, while the antagonists *P. macerans* and *B. pumilus* reduced it by 68 and 70%, respectively, compared to PBS. These results confirm the antibacterial activity of the sanitizer against the phyto-bacteria, which was already expected, since quaternary ammonium based compounds possess fungicidal, bactericidal, algicidal and virucidal action (Mcbain et al., 2004; Oosterhof et al., 2006; Abreu et al., 2008). The antagonists probably acted on the reduction of the pathogen population by multiple mechanisms, such as competition for space and nutrients, antibiosis, and, possibly, resistance induction (Lindow & Brandl, 2003; Monier & Lindow, 2004, 2005ab; Halfeld-Vieira et al., 2006).

Although ISR has not been investigated in this research, Halfeld-Vieira et al. (2006) relate the phenomenon for the epiphytic *Bacillus cereus* in tomato plants, which controlled the bacterium *Pseudomonas syringae* pv. *tomato*. Besides ISR, synthesis of antimicrobial compounds and competition for niches important to pathogen establishment are reported by Lindow & Brandl (2003) as one of the most important mechanisms for antagonistic effect on the foliar surface. Leveau & Lindow (2001) reported efficient use of nutrients by epiphytics as an important mechanism for the antagonistic activity.

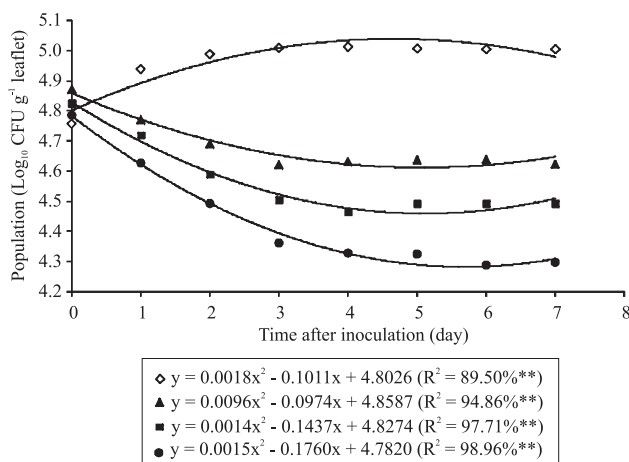
Effective control of bacterial spot and early blight was observed in the greenhouse bioassay, in the treatments using *P. macerans*, *B. pumilus* and benzalkonium chloride (Figure 3). The epiphytics and the sanitizer differed from the control treatment, reducing the severity by over 50% in some cases. These results demonstrate the use potential of those biocontrol agents against two important tomato diseases. Halfeld-Vieira et al. (2008) proved the efficiency of the epiphyte *Bacillus cereus* UFV-IEA6 against *Phytophthora infestans*.

Recent studies have shown the effectiveness of phylloplane residents in controlling several pathogens (Vasudevan et al., 2002; Velusamy & Gnanamanickam, 2003; Velusamy et al., 2004). Gnanamanickam & Immanuel (2006) report the importance of the study of these microbes in plant disease control, stressing the main species of *Bacillus* currently studied. However,

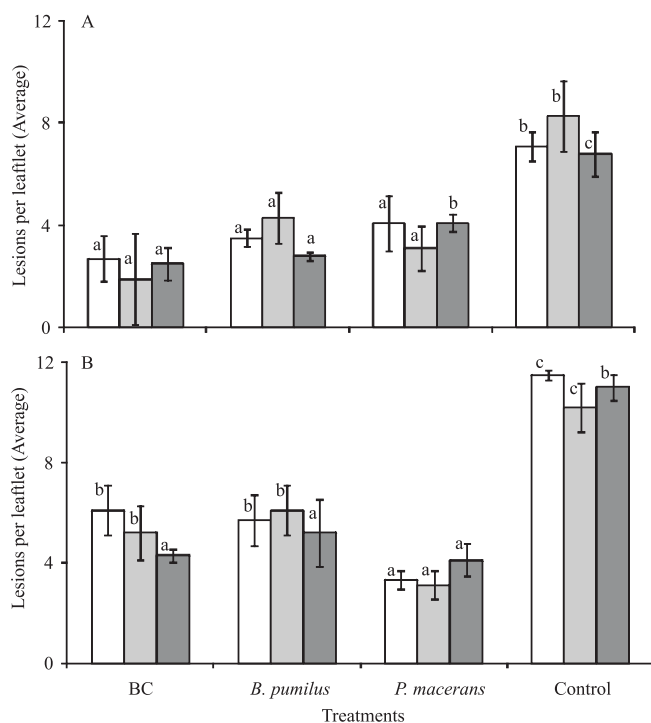




**Figure 1.** Scanning electromicrographs of tomato leaves grown from seeds treated with the epiphytic bacteria *Paenibacillus macerans* and *Bacillus pumilus*, compared with the control (phosphate-buffered saline). A, *P. macerans* aggregates in the depressions along the junctions of adjacent epithelial cells; B, *P. macerans* small aggregate formation at the stomatal region; C, *B. pumilus* aggregates in the depressions along the junctions of adjacent epithelial cells; D, *B. pumilus* aggregate formation at the estomatal region and in the depressions along the junctions of adjacent epithelial cells. E and F represent the control treatments.



**Figure 2.** Population dynamics of plant pathogenic bacterium *Xanthomonas vesicatoria* ( $\text{Log}_{10}$  CFU  $\text{g}^{-1}$  leaflet) on tomato leaves after prior exposure to treatments with *Paenibacillus macerans* ( $\blacktriangle$ ), *Bacillus pumilus* ( $\blacksquare$ ), benzalkonium chloride (BC) ( $\bullet$ ) and PBS buffer (control) ( $\diamond$ ). Each point indicates the mean of the  $\text{log}_{10}$ -transformed bacterial population.



**Figure 3.** Severity of bacterial spot (A), and early blight (B), in Santa Cruz 'Kada' tomato plants, four days after exposure to treatments with *Bacillus pumilus*, *Paenibacillus macerans*, benzalkonium chloride (BC) and PBS buffer (control). The white, light grey, and dark grey bars represent the experiment replicates. Means followed by same letter do not differ by the Tukey's test, at 5% probability.

in Brazil, few researches report the importance of epiphytic bacteria to control diseases.

## Conclusions

1. *Paenibacillus macerans* and *Bacillus pumilus* epiphytic bacteria and benzalkonium chloride reduce *Xanthomonas vesicatoria* and *Alternaria solani* disease severity in tomato plants.

2. Epiphytic bacteria are able to inhibit the growth of tested phytopathogens in vitro, and efficiently colonize the phylloplane of tomato plants.

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