

Biocontrol activity of *Bacillus* against a GFP-marked *Pseudomonas syringae* pv. *tomato* on tomato phylloplane

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Abstract We report the biocontrol activity of the endophytic bacteria *Bacillus pumilus* and *Bacillus amyloliquefacies* against the plant pathogenic bacterium *Pseudomonas syringae* pv. *tomato* strain NS4 transformed with the GFP expressing gene. *P. s.* pv. *tomato* strain NS4 was obtained from the transformation of *P. s.* pv. *tomato* wild-type strain NW with the plasmid pNKGFP containing GFP-cassette for chromosomal integration. The GFP-marked strain was tested for hypersensitivity and pathogenicity, as well as population studies on the phylloplane, to determine its epidemiology and survival. In all of the bioassays strain NS4 presented similar characteristics to the wild-type, and was hence chosen as the model strain for these studies with antagonistic endophytic bacterial strains. In the biocontrol experiments, tomato plants were pre-inoculated with the endophytic bacteria 4 days prior to inoculation with *P. s.* pv. *tomato* strains. On the tomato phylloplane the *P. s.* pv. *tomato* (strains NW and NS4) populations were drastically reduced, and tomato leaves showed reduced numbers of bacterial speck lesions, comparable to the standard chemical treatment copper oxychloride. Additionally, under epifluorescence microscopy, few GFP-tagged cells of strain NS4 were observed colonizing important niches on the tomato

phylloplane. However, leaves untreated with the antagonists presented a large number of GFP-tagged cell aggregates. Our results demonstrated that endophytic bacteria can also act efficiently on the biocontrol of bacterial speck when applied as a foliar spray on the leaves. In addition, we highlighted the use of GFP-marked strain NS4 as a model system to study biocontrol agent and pathogen interactions, and growth and development of the pathogen on the tomato leaf surface.

Keywords Bacterial speck · Biological control · Endophytic bacteria · *Bacillus pumilus* · *Bacillus amyloliquefaciens* · Green fluorescent protein

Introduction

Pseudomonas syringae pv. *tomato* (ex Okabe) Young, Dye and Wilkie is a Gram-negative bacterium that causes bacterial speck disease on tomato leaves (*Solanum lycopersicum* L.) and necrosis of stems and fruits (Jones et al. 1991). Bacterial infection in young tomato plants may result in up to 75 % yield loss, compared to only 5 % when infection occurs in older plants (Yunis et al. 1980). *P. syringae* pv. *tomato* has been found growing epiphytically on plant foliage without causing disease symptoms (Hirano and Upper 2000). Due to its importance it has been the target of numerous studies to understand the molecular mechanisms and co-evolution of pathogenesis and plant disease resistance (Shen and Keen 1993; Melotto et al. 2006).

Control of bacterial speck is usually accomplished by copper-based pesticides as well as mancozeb, zineb, and some antibiotics (Jardine and Stephens 1987; Saad and Hassan 2000). However, when environmental conditions favor disease development and inoculum levels are high, standard treatments are not always effective (Jardine and Stephens 1987). Biocontrol, therefore, may offer an ecologically viable alternative for the management of bacterial

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speck, potentially reducing the use of conventional pesticides. Currently, several researchers have demonstrated the biocontrol potential of the genus *Bacillus*, acting as phyllobacteria, rhizobacteria and endophytic bacteria (Silva et al. 2004; Halfeld-Vieira et al. 2006; Campos Silva et al. 2008; Lanna Filho et al. 2010). In the case of endophytic bacteria, numerous reports have shown that these microorganisms are also capable of inhabiting other environments, such as the phyllosphere and rhizosphere (Compant et al. 2005). This demonstrates that these microorganisms are versatile and readily able to adapt to different plant environments, expanding their possible uses in economically important crops.

In this context, here we report the transformation of *P. syringae* pv. *tomato* wild-type strain NW with the pNKGFP plasmid containing GFP-cassettes by chromosomal integration and expression of the *GFP* gene. In addition, strains of *P. syringae* pv. *tomato* expressing the *GFP* gene were subjected to population, hypersensitivity and pathogenicity studies on the tomato phylloplane. We also describe the antagonistic effect of the endophytic bacterial strains *B. pumilus* Meyer and Gottheil and *B. amyloliquefaciens* (ex Fukumoto) Priest et al. (1987) and their effectiveness as biocontrol agents against *GFP* transformed *P. syringae* pv. *tomato* strains NW, NS1, NS2, NS3 and NS4.

Materials and methods

Microorganisms, plants and growth conditions

The *P. syringae* pv. *tomato* wild-type strain NW was obtained from the collection at the Bacteriology Laboratory of the Plant Pathology Department, Federal University of Lavras. The integrative plasmid pNKGFP (Ferreira et al. 2008), which contains the Green Fluorescent Protein (*GFP*) gene in a mini-Tn10 derivative with a kanamycin (kn) resistance gene was maintained in *Escherichia coli* DH5a pir. The endophytic bacteria *Bacillus amyloliquefaciens* and *Bacillus pumilus*, previously isolated from tomato stem, were selected for this study because they had previously shown good biocontrol activity against *P. syringae* pv. *tomato* (Campos Silva et al. 2008). All bacterial isolates were maintained on 523 medium (Kado and Heskett 1970) and preserved at $-80\text{ }^{\circ}\text{C}$ in 30 % (v/v) glycerol (Gerhardt 1994).

All in vivo experiments were conducted at the Laboratory of Plant Bacteriology, Department of Plant Pathology of the Federal University of Lavras, Minas Gerais State, Brazil. Tomato plants cultivar Santa Cruz 'Kada' were selected for these experiments and grown in a non-sterilized mixture of soil, sand and cattle manure (2:1:1, v/v/v) containing 4 g/L of NPK (4:14:8) in a greenhouse at $28\text{ }^{\circ}\text{C}\pm 4\text{ }^{\circ}\text{C}$ and relative humidity of 70 %.

P. syringae pv. *tomato* strain NW transformation

P. syringae pv. *tomato* strain NW was initially grown for 18 h at $28\text{ }^{\circ}\text{C}$ in 5 mL SOB liquid medium (Sambrook et al. 1989). This starter culture was then added to 250 mL SOB liquid medium and incubated at $28\text{ }^{\circ}\text{C}$ under continuous shaking for 12 h (Final cell density was $\text{DO}_{600} = 7.0$). The cells were harvested by centrifugation ($3,000 \times g$; 10 min; $4\text{ }^{\circ}\text{C}$), re-suspended in 250 mL of cold ultrapure water and centrifuged. The supernatant was discarded and the bacterial pellet re-suspended in 10 % glycerol and centrifuged. Transformation was performed by electroporation (Gene Pulser, BioRad–2.5 kV, 25 μF , 200 Ω) in an electroporation curvette (0.2 cm) containing a mixture of 100 μL of cell suspension plus 1 μg of pNKGFP plasmid. After transformation, 1 mL of Luria-Bertani (LB) medium was added, the mixture incubated for 1 h at $28\text{ }^{\circ}\text{C}$ and plated on LB medium supplemented with kanamycin (50 mg/mL). The identification and selection of clones carrying the *GFP* gene was carried out under UV light.

Hypersensitivity and pathogenicity assays

Hypersensitivity was tested on soybean (*Glycine max*) and tobacco (*Nicotiana benthamiana*) plants. Plants were inoculated with four GFP-marked *P. s.* pv. *tomato* strains (NS1, NS2, NS3 and NS4) and the wild-type strain NW (20 μL ; $\text{OD}_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL) by infiltration into the abaxial side of intact leaves with a plastic syringe (without a needle) and the inoculation points identified by ink marks on the upper leaf surface. Water was used as a control.

Fifteen day old tomato seedlings, cultivated in polyethylene pots with 500 mL of non-sterilized mixture of soil, sand and cattle manure (2:1:1, v/v/v) containing 4 g/L of NPK (4:14:8), were used for the pathogenicity studies. Plants were inoculated (15 mL per plant) by spray (Devilbiss EGA-502) with suspensions of GFP-marked *P. s.* pv. *tomato* strains (NS1, NS2, NS3 and NS4) and the wild-type NW ($\text{OD}_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL). Inoculated plants were maintained in a greenhouse at $28\text{ }^{\circ}\text{C}$ and the number lesions counted once disease symptoms had fully developed. For each treatment, four replicates were used, with one plant per pot considered as one replicate. Each experiment was conducted three times.

Bacterial population dynamics on the phylloplane

Fifteen day old tomato seedlings were inoculated (15 mL per pot) by spray (Devilbiss EGA-502) with suspensions of the GFP-marked *P. s.* pv. *tomato* strains (NS1, NS2, NS3 and NS4) and the wild-type NW ($\text{OD}_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL). Eight leaflets were collected randomly in the upper, middle and lower third of each tomato plants

and placed in flasks containing 100 mL of sterile phosphate buffer (PBS) (0.1 M; pH 7.0; containing 0.05 % Tween-80) and sonicated for 8 min in an ultrasonic cleaning bath to recover bacterial cells. Bacterial populations were estimated from 3 g of healthy leaflets (= 8 leaflets total) randomly sampled from each plant pot. Serial dilutions (factor = $1:10^3$) of leaf washings were plated on 523 semi-selective medium containing cycloheximide (50 $\mu\text{g}/\text{mL}$) and cephalixin (50 $\mu\text{g}/\text{mL}$). The wild-type strain NW and the four GFP-marked *P. s. pv. tomato* bacteria were constitutively resistant to the cephalixin.

In parallel, *B. pumilus* and *B. amyloliquefaciens* endophytic bacteria were sprayed ($\text{OD}_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL) on tomato the plants. Eight leaflets were collected randomly in the upper, middle and lower third from seedlings grown in a greenhouse and placed in flasks containing 100 mL of sterile phosphate buffer (0.1 M; pH 7.0; containing 0.05 % Tween-80) and sonicated for 8 min in an ultrasonic cleaning bath to recover bacterial cells. Bacterial populations were estimated from 3 g of symptomless leaflets (= 8 leaflets total) randomly sampled from each plant pot. Appropriate dilutions of leaf washings were plated on 523 medium containing cycloheximide (50 $\mu\text{g}/\text{mL}$) and neomycin (50 $\mu\text{g}/\text{mL}$). The *B. pumilus* and *B. amyloliquefaciens* endophytic bacteria were neomycin resistant.

Inoculated tomato leaves were collected at 24 h intervals over a 10 days period and population sizes were estimated from plate counts made after 2 to 3 days' incubation at 28 °C. For each treatment, four replicates were used, with 3 g of leaflets (= 8 leaflets total) per pot considered as one replicate.

Phylloplane biocontrol

Fifteen day old tomato seedlings were sprayed as previously described with a suspension ($\text{OD}_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL) of live cells of *B. pumilus* and *B. amyloliquefaciens*. Four days later, plants were inoculated with *P. s. pv. tomato* strain NW and NS4 ($\text{OD}_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL). Leaves were collected and bacterial cell recovered and populations estimated as described above. For each treatment, four replicates were used, with 3 g of leaflets (= 8 leaflets total) per pot considered as one replicate.

Control of bacterial speck

Fifteen day old tomato seedlings were sprayed as previously described with a suspension ($\text{OD}_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL) of live cells of *B. pumilus* and *B. amyloliquefaciens*, copper oxychloride (CO) (2 g/L) (positive control) and water (negative control). Four days later, plants

were inoculated with *P. s. pv. tomato* strain NW and NS4 ($\text{OD}_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL). Inoculated plants were kept in the greenhouse, and after disease symptoms had fully developed, the number of lesions per leaf counted on all leaflets. Each experiment was repeated three times for each pathogen with ten replicates per trial, each consisting of one plant.

Fluorescence microscopy

Ten day old tomato plants were sprayed with a suspension of live cells of *B. pumilus* and *B. amyloliquefaciens* in water. Four days later, plants were inoculated with *P. s. pv. tomato* strain NS4. After 5 days, 1 cm diameter leaflet discs were removed and placed on a glass microscope slide in 30 % (v/v) glycerol. Fluorescence microscopy was conducted with an Axio Observer Z1 microscope (Carl Zeiss Microimaging GmbH, Göttingen, Germany), and the images taken using an AxioVision 4.6 Image Program, Zeiss. GFP-tagged bacterial cells were excited with UV light using a 488 nm filter.

Scanning electron microscopy (SEM)

Ten day old tomato plants were sprayed as previously described with a suspension of live cells of *B. pumilus* and *B. amyloliquefaciens* and water. After 4 days, three leaves per treatment were cut, and fixed using a modified Karnovsky solution (glutaraldehyde 2.5 % and paraformaldehyde 2.5 % in sodium cacodylate buffer 0.05 mol/L; pH 7.2; CaCl_2 0.001 mol/L), for 24 h 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. The sections obtained were transferred to a 1 % aqueous solution of osmium tetroxide for 1 h at room temperature, and subsequently dehydrated for 10 min each in a graded series of acetone solutions (25, 50, 75, 90 and 100 %). They were then dried in a Balzers CPD 030 critical point dryer (Balzers, Liechtenstein, Germany). The specimens obtained were mounted on aluminum stubs with double-stick carbon tape on aluminum foil, with the sectioned side in liquid nitrogen up, sputter-coated with gold in a Balzers SCD 050 sputter (Balzers, Liechtenstein, Germany) and observed with a scanning electron microscope (SEM) LEO EVO 40 XVP (Leo Electron Microscopy, Cambridge, UK). 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 randomly for each sample, at several magnifications, and digitally recorded. Images were processed using the software Corel Draw 12, with which comparisons among treatments were made.

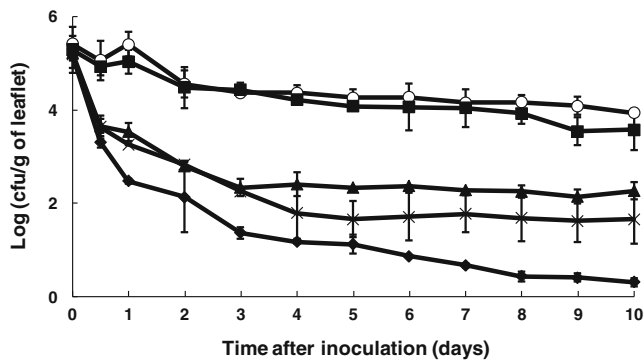


Fig. 1 Epiphytic populations over time of *P. syringae* pv. *tomato* strains on tomato inoculated by spraying ($OD_{540} = 0.2$; suspension cells $\sim 10^8$ cfu/mL): NW (○) NS1 (×), NS2 (▲), NS3 (◆) and NS4 (■). Each point represents the mean \pm standard error of the mean of four replicates

Statistical analysis

The layout for all experiments were arranged out in a completely randomized design (CRD) under plastic-greenhouse conditions, with the number of treatments and repetitions varied according to each experiment described above. The results were subjected to Analysis of Variance (ANOVA) and means compared by Tukey test ($p \leq 0.05$) using the software Statistica®, version 7.0 (Statsoft 2005).

Results

Electroporation and pathogenicity testing

The transformation of *P. s. pv. tomato* wild-type strain NW by pNKGFP plasmid containing GFP-cassettes for chromosomal integration and expression of *GFP* gene, generated four GFP-marked bacteria that under fluorescence microscopy expressed

Fig. 2 Photography, tobacco (a) and soy leaves (b) showing hypersensitive response to *P. s. pv. tomato* GFP-marked strains. The leaves were infiltrated with the 1 = water (control), 2 = strain NS1, 3 = strain NS2, 4 = strain NS3, 5 = strain NS4 and 6 = strain NW and photographed after 48 h. The bacterial cell suspensions were infiltrated at a concentration of 1.0×10^8 cfu/mL. (Bars = 2 cm)

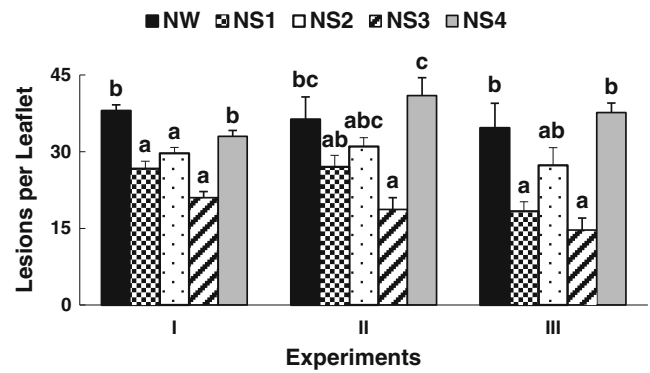
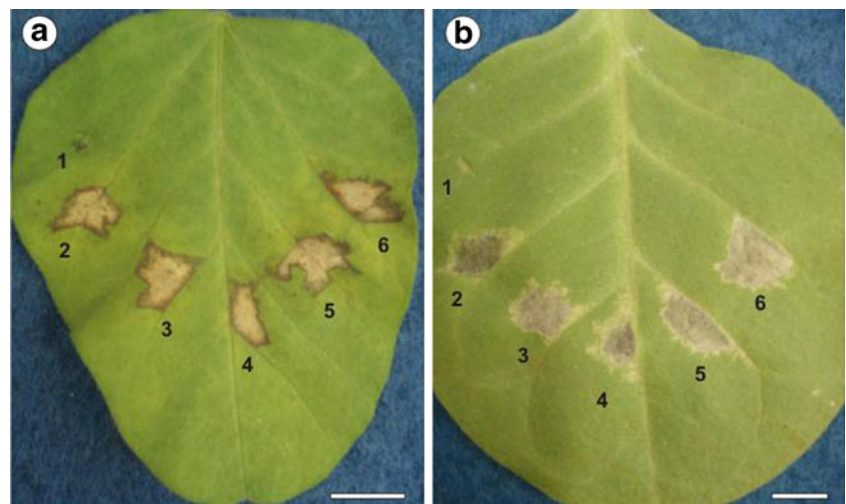


Fig. 3 Severity of *P. s. pv. tomato* GFP-marked strains and NW (control) artificially inoculated on Santa Cruz 'Kada' tomato plants. The experiments were repeated three times (I, II and III) for each strain (NW, NS1, NS2, NS3 and NS4). Each treatment was run with four repetitions and each repetition consisted of one plant per pot. The columns represent the mean and vertical bars represent the standard error of the mean. Means followed by same letter do not differ by the Tukey test ($p \leq 0.05$)

the *GFP* gene. Population behavior of the GFP-marked strains NS1, NS2, NS3 and NS4 was tested on the tomato phylloplane and, of these, strain NS4 presented population dynamics similar to that of the wild-type over the 10 days of monitoring (Fig. 1). In addition, at 10 days the population of the NW and NS4 strains on the phylloplane remained at elevated levels with 3.9×10^6 and 3.5×10^6 (cfu/g of leaves), respectively. The hypersensitivity test was positive for all the tested GFP-marked strains, showing a hypersensitive response (HR) in soybean and tobacco leaves, inducing necrotic lesions after 24 h of infiltration of the bacterial suspensions (Fig. 2). In the pathogenicity test on tomato leaves all the GFP-marked strains caused disease symptoms. Strain NS4 exhibited the highest disease severity levels, compared with the control (NW) (Fig. 3) and was subsequently chosen as the challenger pathogen against the endophytic bacteria *B. amyloliquefaciens* and *B. pumilus* for the biocontrol assays.

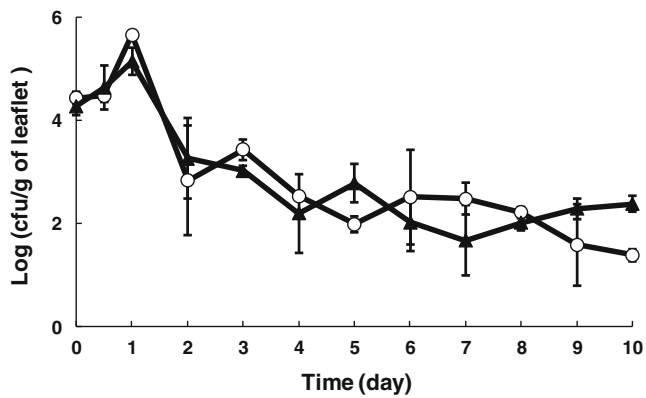


Fig. 4 Endophytic bacterial populations over time of *B. pumilus* (○) and *B. amyloliquefaciens* (▲) sprayed on tomato leaves. Each data point indicates the mean of the log (cfu/g of leaflet) bacterial population. Each point represents the mean ± standard error of the mean

Phylloplane biocontrol

The biocontrol agents *B. pumilus* and *B. amyloliquefaciens* were able to survive on the tomato phylloplane for at least 10 days resulting in population levels of 1.3×10^6 and 2.3×10^6 (cfu/g of leaves), respectively (Fig. 4). In addition, tomato leaves sprayed with the endophytic bacteria and, 4 days later, inoculated with NS4 and NW, and assessed after 10 days resulted in lower *P. s. pv. tomato* population levels (Fig. 5), compared to unsprayed leaves. Leaves pre-treated with *B. pumilus* reduced *P. s. pv. tomato* leaf populations of NW and NS4 by 75 and 84 %, respectively. However, leaves pre-treated with *B. amyloliquefaciens* reduced populations of NW and NS4 by 90 and 97 %, respectively. In addition, plants sprayed with the antagonists, and inoculated with *P. s. pv. tomato* 4 days later, developed lower bacterial speck severity than the copper oxychloride treatment (positive control). The highest disease severity

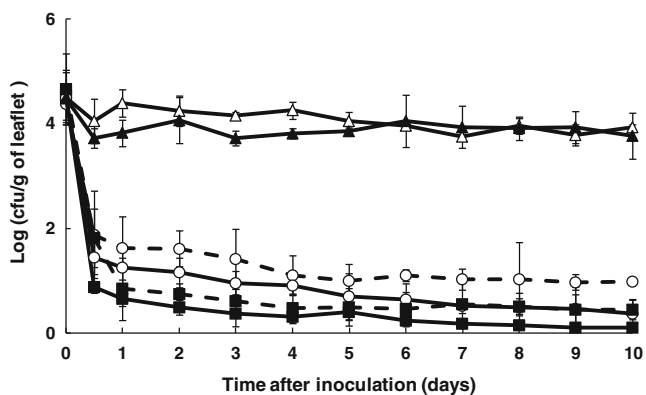


Fig. 5 Epiphytic populations over time of NW (○) and NS4 (■) on tomato leaves 4 days after exposure to treatments with *B. pumilus* (---) and *B. amyloliquefaciens* (—). For control, tomato leaves were sprayed with water and after 4 days inoculated with NW (Δ) and NS4 (▲). Each data point indicates the mean of the log-transformed bacterial population. Each point represents the mean ± standard error of the mean

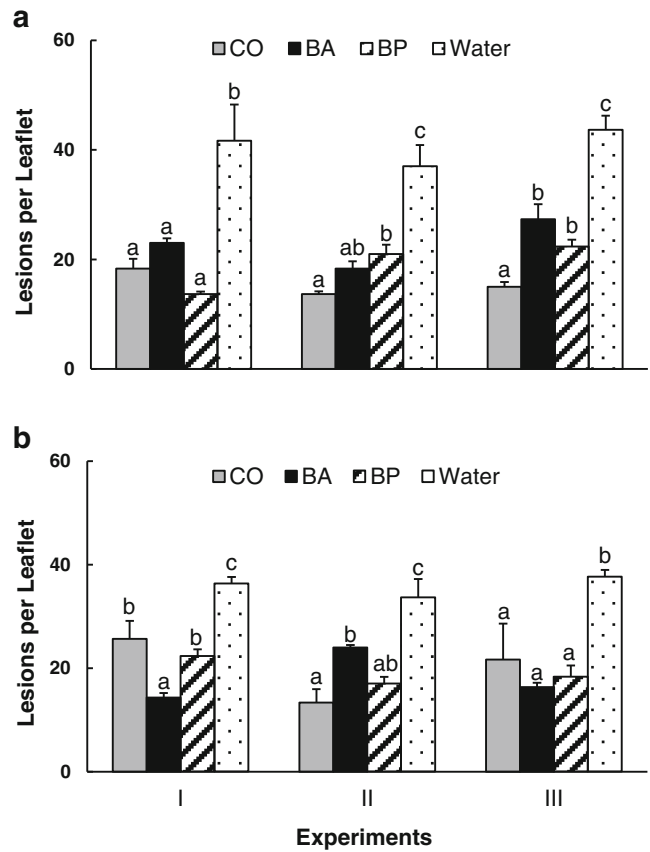


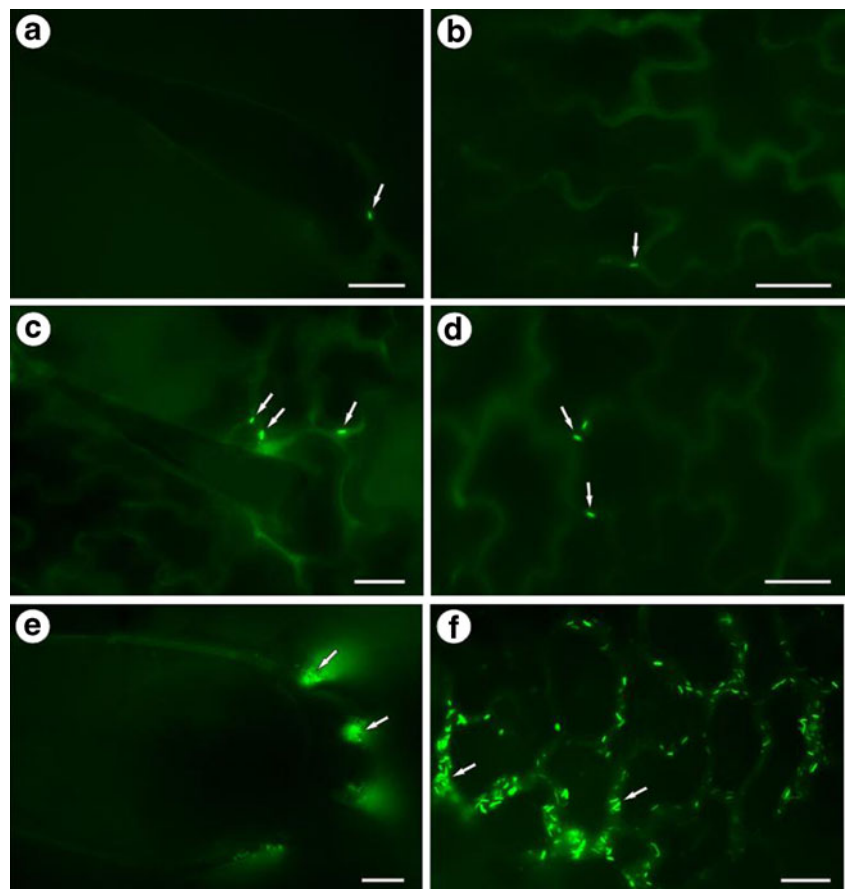
Fig. 6 Severity of bacterial speck caused by *P. s. pv. tomato* wild-type strain NW (a) and strain NS4 (b), artificially inoculated on Santa Cruz ‘Kada’ tomato plants, 4 days after exposure to treatments: copper oxychloride (CO), *B. amyloliquefaciens* (BA), *B. pumilus* (BP), and water (negative control). The experiments were repeated three times (I, II and III) for each pathogen; each treatment was run with ten repetitions and each repetition consisted of one plant per pot. Means followed by same letter do not differ by the Tukey test ($p \leq 0.05$). The columns represent the mean and vertical bars represent the standard error of the mean

levels occurred in plants sprayed with water (negative control) (Fig. 6).

Fluorescence and scanning electron microscopy

The studies under epifluorescence microscopy showed that on the tomato phylloplane treated with the endophytic bacteria, the GFP-tagged cells of *P. s. pv. tomato* strain NS4 were found in small isolated but important niches, such as at the trichome base and between the depressions along the junctions of adjacent epithelial cells (Fig. 7). However, when the phylloplane was not treated with antagonists, *P. s. pv. tomato* was found in high numbers, efficiently colonizing the foliar surface and forming large clusters at the trichome base and between the depressions along the junctions of adjacent epithelial cells (Fig. 7f). The pattern of colonization by the epiphytic bacteria on the phylloplane showed they established themselves in cell aggregates at

Fig. 7 Photomicrographs under epifluorescence microscope of living cells of *P. s. pv. tomato* strain NS4 expressing the GFP, on tomato leaf surfaces. Single bacterial cell at trichome base (a) and between the depressions along the junctions of adjacent epithelial cells (b) on leaf sprayed with *B. amyloliquefaciens*; single bacterial cells at trichome base (c) and between the depressions along the junctions of adjacent epithelial cells (d) of leaf sprayed with *B. pumilus*; large bacterial cluster formation at trichome base (e) and between the depressions along the junctions of adjacent epithelial cells (f), on tomato leaf surface not sprayed with endophytic bacteria. (Bars = 20 μ m)



specific sites on the foliar surface (Fig. 8). These were, in the depressions along the junctions of adjacent epithelial cells (Fig. 8a and b) and beside the stomatal region (Fig. 8b and c). As expected, the treatment with water did not present bacterial colonization (Fig. 8e and f).

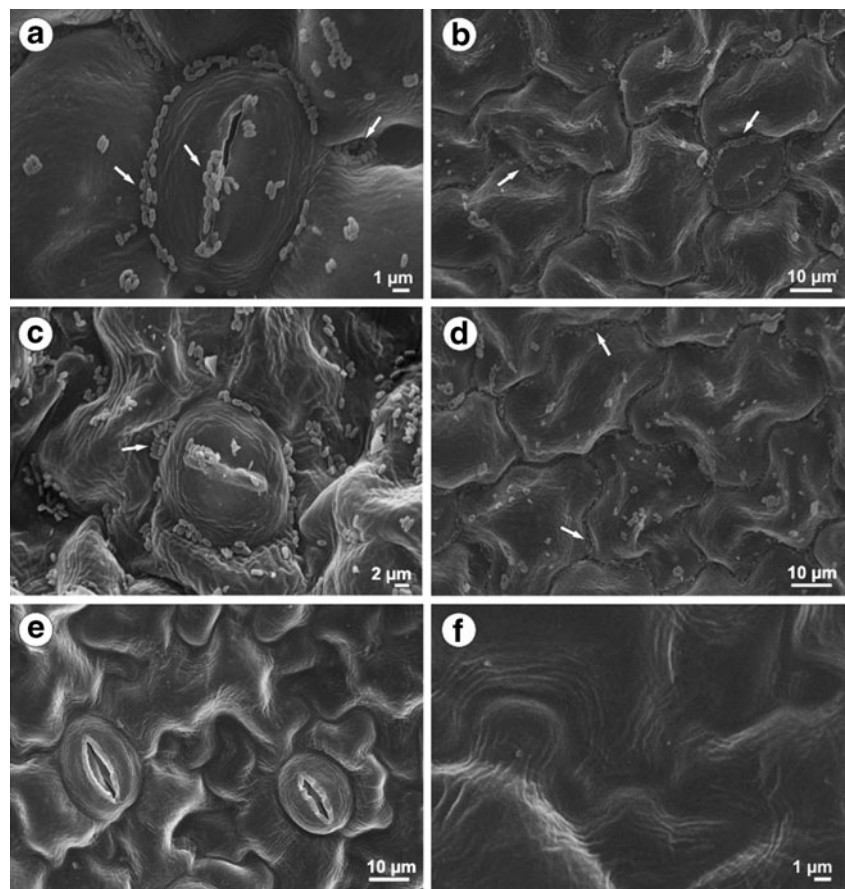
Discussion

Transformation of *P. s. pv. tomato* by electroporation using the plasmid pNKGFP produced four GFP-marked strains which showed a satisfactory *GFP* expression. However, other factors should be considered as instance the effects of *GFP* expression on the metabolism and survival of target organism, being very important studies about the mutant growth behavior (Ma et al. 2011). Some studies have reported a non-effect on the behavior of *GFP*-target bacteria (Skillman et al. 1998; Allison and Sattenstall 2007). However, other researchers have been described that *GFP* affects the bacterial growth (Bloemberg et al. 1997; Dandie et al. 2001). Our studies demonstrated that *GFP* label interfered with the growth characteristics of three host mutants under optimal growth conditions. Only one of the four *GFP*-marked strains (NS4) showed population dynamics similar to the wild-type when monitored over 10 consecutive days,

even though there is high level constitutive expression of a foreign protein (*GFP*). Monier and Lindow (2003) observed that for *P. syringae*, the constitutively expressed *GFP* marker gene of strain B728a presented a lower survival rate when compared to the wild-type strain B728a on colonizing the phylloplane of bean plants. Ma et al. (2011) also demonstrated that some strains of *Listeria monocytogenes*, had the growth negatively affected when transformed with *GFP* gene. These results should likely be due to changes on the bacterial metabolic routes that produce the *GFP* proteins. In addition, this present work suggests that also the random integration of *GFP*-cassettes in each tagged-phytobacterium may be causing differential genome disturbance and interfering in the adaptive capacity of those strains. The real causes of these changes to the bacterial behavior are not clear, and should be investigated further.

All the *GFP*-marked strains caused a hypersensitive response on tomato leaves, characterized by necrosis resulting from localized plant cell death at the infection site (Klement 1982; Dixon and Lamb 1990). In accordance with other authors who used the hypersensitivity test as a quick method to determine the pathogenicity of bacterial strains (Klement and Goodman 1967; Kiraly et al. 1970; Atkinson et al. 1985; Goodman and Novacky 1994), our studies confirmed the pathogenicity of the *GFP*-marked strains using the same

Fig. 8 Scanning electromicrographs on abaxial tomato leaves 4 days after exposure to treatments with *B. amyloliquefaciens*, *B. pumilus* and water (control). *B. amyloliquefaciens* aggregates at the stomatal region, ostiole (**a**) and between the depressions along the junctions of adjacent epithelial cells (**b**); *B. pumilus* aggregates at the stomatal region and ostiole (**c**), as well as between the depressions along the junctions of adjacent epithelial cells (**d**); **e** and **f** represent the control treatments



test. This suggests that the integration of GFP-cassettes in each tagged-phytobacterium does not prevent the ability of transformants to infect. Nevertheless, variation in the virulence of each *GFP* gene-bearing phytobacterium resulted in different disease severity levels. The difference among the disease expression of the GFP-marked strains compared to wild-type strain suggests that the random integration of GFP-cassettes interfered in regulatory activity of virulence determinants, which act co-operatively to cause disease. In studies performed by other authors with GFP-tagged plant pathogenic bacteria, virulence reduction of the strains compared to the wild-type was not observed (Newman et al. 2003; Cubero et al. 2011), although the ability to adapt to the environment was affected (Monier and Lindow 2003). Our bioassays with the GFP-marked strains showed that only strain NS4 caused similar disease expression to the wild-type strain NW making it an ideal strain to study pathogenesis of *P. s.* pv. *tomato* on tomato plants.

Biocontrol agents are known to reduce their target organism populations by a range of different mechanisms such as competition for space and nutrients, antibiosis, and induced resistance (Lindow and Brandl 2003; Monier and Lindow 2004, 2005; Lanna Filho et al. 2010). Campos Silva et al. (2008) reported the growth inhibition of *P. s.* pv. *tomato* by *B. pumilus* and *B. amyloliquefaciens* endophytic bacteria in in vitro tests.

However, the authors did not determine if the compound produced was bacteriocin, antibiotic or other antimicrobial agent. In the next step of our study we are going to identify the substance with antimicrobial activity, which may be a bacteriocin. The production of bacteriocins or bacteriocin-like substances had been described for *B. coagulans*, *B. brevis*, *B. lichniformis*, *B. cereus*, *B. subtilis*, *B. amyloliquefaciens* and other *Bacillus* species (Hyronimus et al. 1998; Hyung et al. 2001; Martirani et al. 2002; Risoen et al. 2004; Teo and Tan 2005; Lisboa et al. 2006). It is also known that *Bacillus* species synthesize large amounts of metabolites that can have both antimicrobial and induced resistance effects against other microbes (Ongena et al. 2007; Ongena and Jacques 2008). However, our study investigated competition as a mode of action and demonstrated that this can occur on the phylloplane, involving bacteria normally endophytic in nature. Similar results were demonstrated by Compant et al. (2005), where the endophytic *Burkholderia* sp. strain PsJN widely colonized the stomatal region and between depressions along the junctions of adjacent epithelial cells on leaves of *Vitis vinifera*. Therefore, endophytic bacteria appear capable of occupying niches normally occupied by phytobacteria and other pathogens, reducing the availability of nutrients necessary for the survival of these phytopathogens. Although the bacteria we investigated (*B. pumilus* and *B. amyloliquefaciens*) are endophytic in

nature, high population levels were observed on the tomato phylloplane, 10 days following application. This suggests that these microbes are able to adapt and survive under the environmental conditions present on the phylloplane. The presence of these endophytic bacteria in large numbers on the phylloplane for such an extended period was unexpected and not previously observed in other studies on endophytic bacteria (Monier and Lindow 2004, 2005). This initial colonization may provide sufficient numbers of bacteria for subsequent colonization of the whole plant through natural openings, such as stomata and hydathodes.

This study demonstrated that a foliar application of endophytic bacterial suspension to the surface of tomato leaves reduced the severity of bacterial speck. Disease levels of plants exposed to the two antagonists were similar to that of the copper-based treatment, the chemical standard for control of plant pathogenic bacteria. We suggest that the reduction of disease severity by the antagonists was related to the occupation of niches important for survival and penetration of the phytobacteria, such as: substomatal chambers, hydathodes, trichome base and cavities between the depressions along the junctions of adjacent epithelial cells.

On the phylloplane treated with the endophytic bacteria compared to non-treated plants, we observed a low density of NS4 GFP-tagged cells, suggesting a reduction in the population associated with the presence of the endophytic bacteria. Additionally, NS4 GFP-tagged cells were not observed in the substomatal chambers (data not shown), suggesting that the endophytic bacteria may be competing for space and/or nutrients as well as synthesizing antimicrobial compounds that inhibit growth of strain NS4. Campos Silva et al. (2008) have reported that the endophytic bacteria *B. pumilus* and *B. amyloliquefaciens* can produce anti-fungal compounds active against the plant pathogenic bacterium *P. s. pv. tomato*, supporting our statement that antimicrobial synthesis may be a mechanism involved in the inhibition of strain NS4 growth on the phylloplane. Our studies clearly demonstrate that endophytic strains efficiently colonize the surface of tomato leaves occupying specific niches, such as: ostiole, stomatal region and cavities between the depressions along the junctions of adjacent epithelial cells, probably interfering in the colonization of *P. s. pv. tomato* strain NS4 (Fig. 8).

Our studies presented in this work showed the importance of the endophytic bacteria *B. pumilus* and *B. amyloliquefaciens* in the biocontrol of bacterial speck, as well as the promising use of a new GFP-marked *P. s. pv. tomato* strain that may be used as a model for the elucidation of phytobacteria-antagonist-host or phytobacteria-host interactions. We also reported the ability of endophytic *Bacillus* strains to survive under the environmental conditions present on the tomato phylloplane, increasing the knowledge known of these microorganisms in association with plant hosts. Data presented in this paper provide an important contribution to better understanding how endophytic bacteria

act on tomato plants as biocontrol agents against the plant pathogenic bacterium *P. s. pv. tomato*. The next step in our research will be to develop a bio-pesticide product that consists of endophytic *Bacillus* strains, which will be sprayed on tomato plants in pre-and-post-planting for bacterial speck biocontrol. Our perspective is to commercialize the bioformulate as happens to the well-known Blighban A506[®], Serenade[®] and Sonata[®] used for other crops.

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