Induced defense responses in tomato against bacterial spot by proteins synthesized by endophytic bacteria

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ABSTRACT

Some endophytes can synthesize molecules that elicit the induction of plant resistance to infection by pathogens. The objective of this study was to demonstrate that protein fractions 42 and 75 from *Bacillus amyloliquefaciens* and *Bacillus pumilus* were capable of acting as elicitors of induced resistance in tomato plants against *Xanthomonas vesicatoria*, following partial resolution by gel-filtration chromatography. Tomato plants sprayed with protein fractions 42 and 75 reduced, respectively, 63.5 and 56.6% of bacterial spot, compared with control plants. Additionally, these fractions promoted the increase of peroxidase (POX) and polyphenol oxidase (PPO) enzyme activities in treated plants. In SDS-PAGE stained with silver nitrate, protein fractions 42 and 75 appeared as simple bands with estimated molecular mass of 28 and 43 kDa, respectively. We report the partial characterization of two macromolecules synthesized by endophytic bacteria that act as elicitors of systemic resistance in tomato against *X. vesicatoria.*

Key words: *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Solanum lycopersicum*, *Xanthomonas vesicatoria*, biological control.

INTRODUCTION

Endophytic bacteria are common inhabitants of the internal tissues of various plant species (Strobel et al., 2004). The vast majority survive internally without causing harmful effects to the plant and some can provide beneficial effects, favoring phytohormone synthesis, resistance induction and biological control of pathogens (Ryan et al., 2008). Many endophytic bacteria are common members of the genus *Bacillus* (Lodewyckx et al., 2002). This genus is known to synthesize low-molecular-weight secondary metabolites, including antibiotics, volatile organic compounds, siderophores and surfactants. However, very little is known about macromolecules synthesized by these microbes that can promote disease control in plants, specially by proteins that act as elicitors of defense responses.

Recent studies demonstrated that macromolecules of proteic nature synthesized by rhizobacteria of the genus *Bacillus* may have antifungal activity (Kavitha et al., 2005) and induce resistance against plant pathogens (Romeiro et al., 2005, 2010). However, there are no studies with endophytic bacteria from the genus *Bacillus* that synthesize and release resistance-inducing proteins in the environment.

Defense responses in plants elicited by macromolecules of proteic nature are usually related to systemic acquired resistance (SAR) (Wei et al., 1992a, 1995; Tanaka et al., 2001). However, Romeiro et al. (2005, 2010) showed that there is induced systemic resistance (ISR) in

tomato by a protein synthesized by the rhizobacterium *Bacillus cereus*. These studies may encourage research lines that eventually lead to bioproduct development. This was the case with the harpin protein synthesized by bacteria of the genera *Erwinia, Ralstonia* and *Pseudomonas* (Wei et al., 1992a, 1992b, 1993, 1995, 2000; Tanaka et al., 2001; Dayan et al., 2009) that generated the commercial products Messenger® and ProAct® with broad-spectrum activity against phytopathogens of various crops (Dayan et al., 2009).

This study reports the synthesis of macromolecules of proteic nature by the endophytic bacteria *Bacillus amyloliquefaciens* and *Bacillus pumilus*, which induce resistance in tomato plants (*Solanum lycopersicum*) against bacterial spot caused by *Xanthomonas vesicatoria*.

MATERIALS AND METHODS

Microorganisms

Endophytic bacteria *Bacillus amyloliquefaciens* (BA) and *Bacillus pumilus* (BP) were previously isolated from tomato stem and acted as efficient biocontrol agents (Campos Silva et al., 2008). They were grown in 523 medium (Kado & Heskett, 1970) and preserved at -80ºC in 30% (v/v) glycerin (Gerhardt, 1994). *Xanthomonas vesicatoria* (Xv) was obtained from the collection of the Plant Bacteriology Lab and was grown and stored as described above. Santa Cruz 'Kada' tomato plants were

grown in a non-sterilized mixture of soil, sand and manure (2:1:1, v/v/v) in a greenhouse at 28±4ºC and relative humidity of 70%.

Preparation of bacterial supernatants

The endophytic BA and BP were grown in a modified Simmons minimal liquid medium (SM) (Simmons, 1926) in which citrate was replaced by 0.1 % (w/v) glucose as sole carbon source (Gijsegem et al., 1995, 2000). The turbidity of liquid cultures in side-arm flasks under continuous shaking, at room temperature (28±1ºC), was recorded at different time intervals to determine the inflection point in the exponential growth phase of the growth curve.

At the inflection point of the exponential growth phase, cells were removed from suspension by centrifuging once at $10,000 \times g$ for 15 min in a RC-5C Sorvall refrigerated centrifuge. The supernatant was filtered under sterile conditions through a cellulose membrane of 0.22 µm pore size, transferred to dialysis bags (cut-off = 12 kDa) and dialyzed with continuous stirring against 400 volumes of phosphate-buffered saline (PBS) (0.1 M; adjusted to pH 7.0) at 4ºC. The PBS was changed several times during the 48 h of dialysis at 4ºC. Dialysates were lyophilized and stored at -20ºC for later usage.

***In vitro* bioassays**

The putative toxic activity of supernatants against tomato pathogenic bacteria was verified by the overlay diffusion method (Vidaver et al., 1972). Molten semi-solid culture medium [0.8% (w/v) agar, 48ºC] containing 10^8 CFU mL^{-1} of *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas corrugata*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Ralstonia solanacearum* and *Xanthomonas vesicatoria* was poured into Petri dishes containing a layer of solid water-agar [2% (v/v)], in an amount that was enough to provide a 1 mm-thick overlay. After polymerization, a cork borer was used to produce a 0.5 cm² well in the central part of the overlay and 30 µL of supernatant (2 mg mL^{-1}) was placed in the cavity. PBS buffer (0.1 M, pH = 7.0) and SM medium were used as negative controls. Bioassay plates were incubated at 28ºC and checked daily for one week for inhibition halos. For each bacterial pathogen the bioassay was repeated three times.

Exposure of tomato plants to the supernatants

Fifteen days after sowing, tomato plants cv. Santa Cruz 'Kada' were sprayed with a suspension cell of BA and BP (OD_{540} = 0.3, corresponding to approximately 10^8 CFU mL^{-1}), supernatants of *B. amyloliquefaciens* (SBA) and *B. pumilus* (SBP) (2 mg mL^{-1}), Acibenzolar-S-Methyl (ASM) (0.05 g L^{-1} water) and controls of SM medium and PBS buffer (0.1 M; pH 7.0). Four days later, plants were inoculated with Xv by spraying with an inoculum suspension (OD_{540} = 0.2, corresponding to approximately 10^8 CFU mL^{-1}). In all cases, plants were kept in a moist chamber for 24 h before and after inoculation. Inoculated plants were maintained in a greenhouse and lesions counted after disease symptoms had fully developed. All experiments were performed in a completely randomized design with five replicates per treatment. The results were subjected to analysis of variance and means were compared by Tukey test ($p \leq 0.05$) using the software Statistica®, version 7.0 (Statsoft, 2005).

Partial resolution of supernatants by gel filtration

The purification system with a peristaltic pump (Pharmacia Biotech) was established at 4ºC. The Sephacryl S-300 HR column, 60 cm long and 2 cm in diameter, was equilibrated with PBS buffer (0.1 M; pH 7.0). The flow rate was set at 15 drops min^{-1}, [equivalent to 0.37 mL min^{-1} (0.22 mL h^{-1})]. The column void volume was calculated with Blue Dextran (2 mg mL^{-1}). Successive 2 mL aliquots (2 mg mL^{-1}) of the lyophilized supernatants of each endophyte were applied into the column, from which 2 mL fractions were collected (BioRad model 2110 fraction collector) and proteins monitored by the absorbance at 280 nm. Proteins in the supernatants were quantified according to Bradford (1976) with a Microplate Spectrophotometer (PowerWave™ XS) and the results were expressed as bovine serum albumin (BSA) equivalents. Purification of the supernatants was repeated seven times.

Exposure of plants to purified protein fractions

Purified fractions BA1, BA2 and BA3 from *B. amyloliquefaciens* and, BP1 and BP2 from *B. pumilus,* obtained by gel filtration, were sprayed (1.0 mg mL^{-1}) on tomato plants, cv. Santa Cruz 'Kada', 10 days after germination. At the same time endophyte cells (10^8 CFU mL^{-1}), ASM (0.05 g L^{-1} water), and PBS (0.1 M; pH 7.0) were also sprayed. Four days later, plants were inoculated with Xv cell suspension and evaluated by counting the lesions as described above. The experiment was performed in a completely randomized design with four replicates per treatment and means were compared by the Tukey test.

***In planta* enzyme activity**

To evaluate the activity of guaiacol peroxidase (POX - EC 1.11.1.7) and polyphenol oxidase (PPO - EC 1.10.3.1), tomato seeds were sown in 500 mL pots containing a non-sterilized mixture of soil, sand and manure, maintained in a greenhouse under the conditions described above. Ten-day-old tomato plants were sprayed with BA, BP, BA2, BP2, ASM and controls with PBS buffer and water. Treated and control plants were harvested at 0.5, 1, 2, 4, 5, 6 and 7 days after spraying. In another trial, 10-day-old plants were sprayed with the test treatments and 4 days later they were inoculated with 100 mL Xv cell suspension (10^8 CFU mL^{-1}). Leaves were harvested at 4, 5, 6 and 7 days after spraying. The leaves were excised and frozen in liquid nitrogen and immediately utilized. Fresh leaf material (2.0 g) from treated and control tomato plants was homogenized

with a pestle and mortar in 3 mL of ice-cold 50 mM sodium acetate buffer, pH 5.2. After filtration through cheesecloth, the homogenate was centrifuged at $13,000 \times g$ for 15 min and the supernatant (crude extract) used as the source of enzymes. All the steps were carried out at 0-4°C. Protein content of the crude extracts was determined using the Bradford (1976) protein assay, using BSA as a standard.

The activity of POX was determined according to Urbanek et al. (1991) by adding 25 µL of the crude extract to 2 mL of a solution containing 50 mM sodium acetate buffer, pH 5.2, 20 mM guaiacol, and 20 mM hydrogen peroxide (H_2O_2). After incubation at 30°C for 10 min, the absorbance was read at 480 nm. One POX unit of activity (UA) was expressed as the variation of 1U of absorbance at 480 nm mg^{-1} of soluble protein per minute (UA mg P^{-1} min^{-1}). PPO was determined by adding 50 µL of the crude extract to 3 mL of a solution containing 100 mM potassium phosphate buffer, pH 6.5, and 25 mM pyrocatechol. The increase of absorbance at 410 nm, for 10 min at 30°C, was measured (Gauillard et al., 1993). One PPO unit was expressed as the variation of absorbance at 410 nm.mg^{-1} of soluble protein per min and expressed as UA per milligram protein per min (UA mgP^{-1} min^{-1}). All experiments were arranged in a randomized block design with three blocks, and one experimental unit (plot) consisted of a 500 mL pot containing a single plant.

Determination of molecular weight by SDS-PAGE

Fractions 42 and 75 corresponding to BA2 and BP2 obtained by gel filtration were electrophoresed in a discontinuous buffer system using 5% stacking gel (pH 6.8) and 10% separating gel (pH 8.8) in Tris-glycine buffer (pH 8.3) according to Laemmli (1970). Prior to SDS-electrophoresis, the protein (10 µg in 30 µL) was mixed with an equal volume of sample buffer (100 mM Tris-HCl buffer, 4% SDS, pH 6.8) containing 5% β-mercaptoethanol. A mixture of standard marker proteins (Genei Pvt. Ltd.)

was used. The electrophoresis was conducted at a constant current of 90 V per sample in a slab gel measuring 170 x 150 x 1.5 mm. The gel was silver stained and photodocumented (Vilber Lourmat).

RESULTS

Supernatants of *B. pumilus* and *B. amyloliquefaciens*

The growth of the endophytic bacteria *B. amyloliquefaciens* and *B. pumilus* in minimal medium reached the stationary phase in approximately 14 hours. In the exponential growth phase, the inflection point was reached at 0.45 of absorbance after 10 hours of growth (Figure 1). The inflection point was chosen as ideal because the bacterial cells secrete the highest amounts of proteins into the medium. After the inflection point or near the stationary phase, target protein production decreases and bacterial cells use these proteins as carbon and nitrogen sources (Madigan et al., 2003). Additionally, before the inflection point or near the lag-phase growth the concentration of target proteins synthesized are very low. In our study the inflection point was estimated according to the literature (Gijsegem et al., 1995, 2000).

Bioassays with supernatants

The concentrated supernatant (2 mg mL^{-1}) of each endophytic bacterium submitted to the *in vitro* bioassay by the overlay diffusion method (Vidaver et al., 1972) did not show toxic activity against the phytobacteria *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas corrugata*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Ralstonia solanacearum* and *Xanthomonas vesicatoria* (Xv). Additionally, neither PBS buffer nor Simmons medium (SM) used as controls presented toxic activity against the tested phytobacteria. Experiments repeated three times in a greenhouse demonstrated that
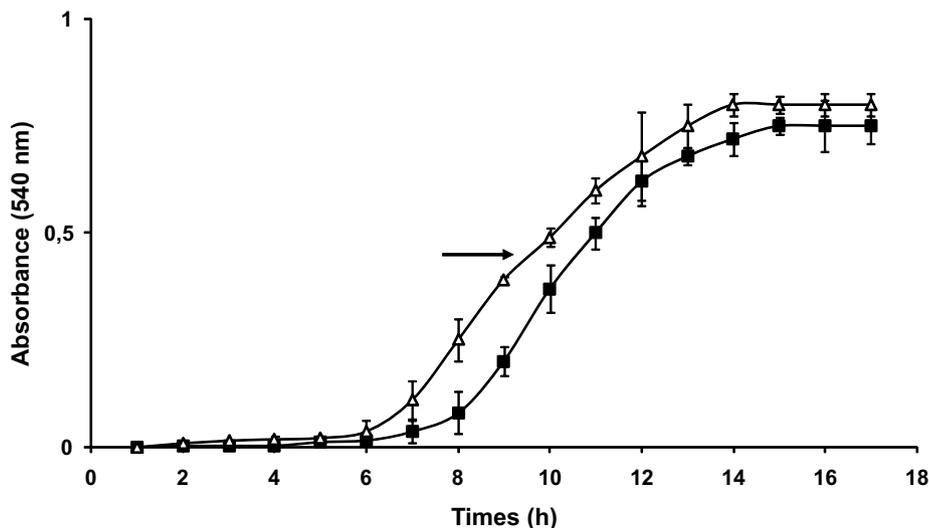

FIGURE 1 - Growth curve of *B. pumilus* (Δ) and *B. amyloliquefaciens* (■) on modified liquid Simmons medium at 28°C. The inflection point in the exponential growth phase was reached at 0.45 absorbance, as shown by the arrow. The inflection point was estimated according to the literature. Each data point indicates the mean growth of endophytic bacteria in three replications, and vertical bars represent the standard error of the means.

tomato plants of cv. Santa Cruz 'Kada' sprayed with 2.0 mg mL^{-1} supernatant of *B. pumilus* (SBP) and supernatant of *B. amyloliquefaciens* (SBA) reduced the severity of bacterial spot by 56.6 and 58%, compared to SM medium and PBS buffer (controls) (Figure 2). The highest reduction in severity of *X. vesicatoria* was conferred by a *B. amyloliquefaciens* cell suspension (BA) [67.2%] followed by the suspension of *B. pumilus* (BP) and Acibenzolar-S-Methyl (ASM) with reductions of 63.4 and 62.2%, respectively. ASM is a well-known inducer of resistance in plants and was used in this study as a standard for comparison purposes. Control treatments with SM medium and PBS buffer showed the highest, but similar disease severity levels in all experiments (Figure 2).

Bioassays with purified fractions

Gel filtration revealed purified fractions BA1 BA2 and BA3 in supernatants of *B. amyloliquefaciens* (SBA), while supernatants of *B. pumilus* (SBP) showed purified fractions BP1 and BP2 (Figure 3A), which were sprayed on tomato plants in greenhouse experiments. BA2 and BP2 corresponding to purified fractions 42 and 75 presented activity against Xv (Figures 4A and B). In SDS-PAGE the purified fraction 42 (BA2) and 75 (BP2) (Figure 3A)
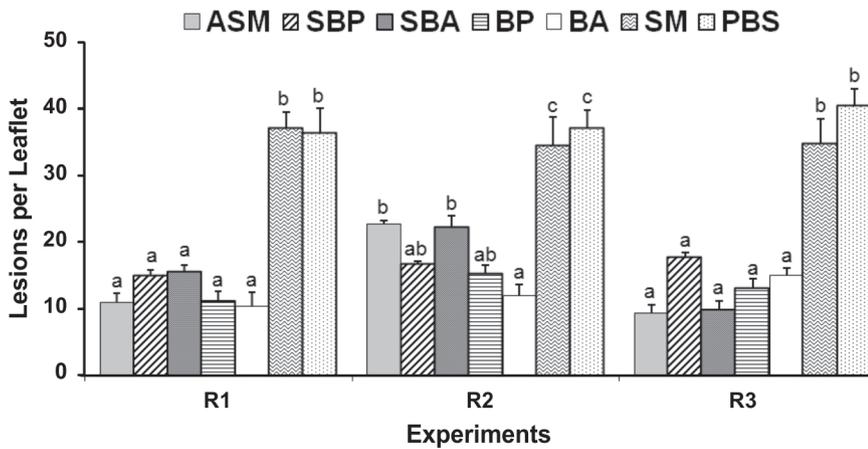

FIGURE 2 - Bacterial spot severity on Santa Cruz 'Kada' tomato plants, four days after exposure to Acibenzolar-S-Methyl (ASM), supernatant of *B. pumilus* (SBP), supernatant of *B. amyloliquefaciens* (SBA), *B. pumilus* cell suspension (BP), *B. amyloliquefaciens* cell suspension (BA), Simmons medium (SM) and phosphate-buffered saline (PBS). The bioassay was repeated three times (R1, R2 and R3). Means followed by the same letter do not differ by Tukey's test ($p \leq 0.05$). The columns represent the means and vertical bars the standard error of the means.
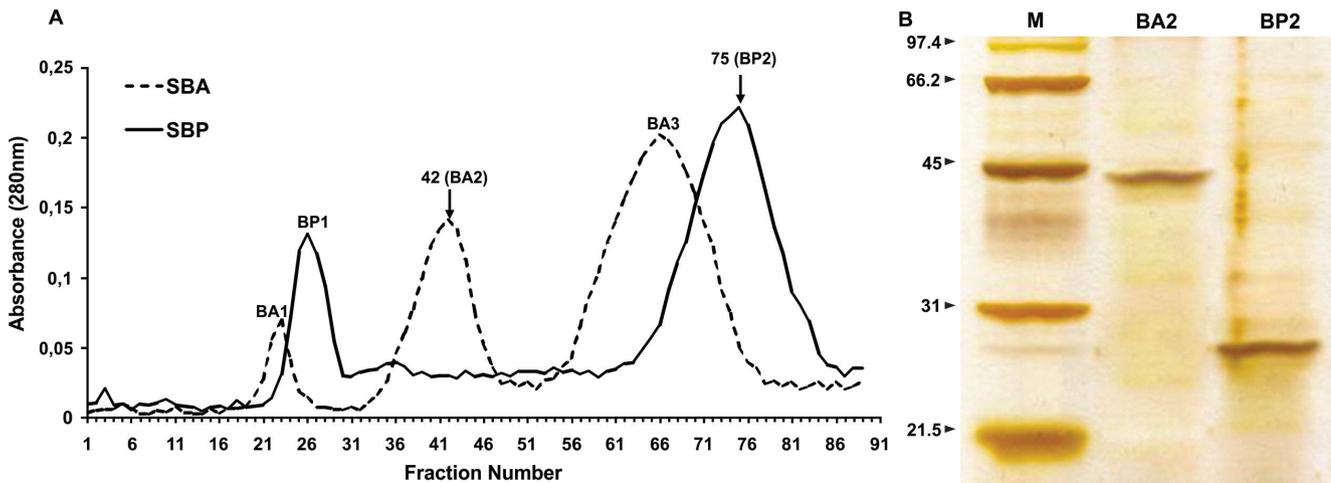

FIGURE 3 - Purification and determination of the molecular weight of proteins produced by endophytic bacteria. **A.** Purified fractions BA1, BA2 and BA3 in supernatant of *B. amyloliquefaciens* (SBA) and purified fractions BP1 and BP2 in supernatant of *B. pumilus* (SBP) by gel filtration in a Sephacryl S-300 HR column. Fractions 42 (BA2) and 75 (BP2) from *B. amyloliquefaciens* and *B. pumilus*, respectively, indicated by the arrows were selected for eliciting resistance against *X. vesicatoria*; **B.** SDS-PAGE of protein fractions 42 (BA2) and 75 (BP2). Line M, broad range standard protein markers (phosphorylase, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa); lines BA2 (43 kDa) and BP2 (28 kDa), correspond to purified fractions. The gel was stained for proteins with silver nitrate.
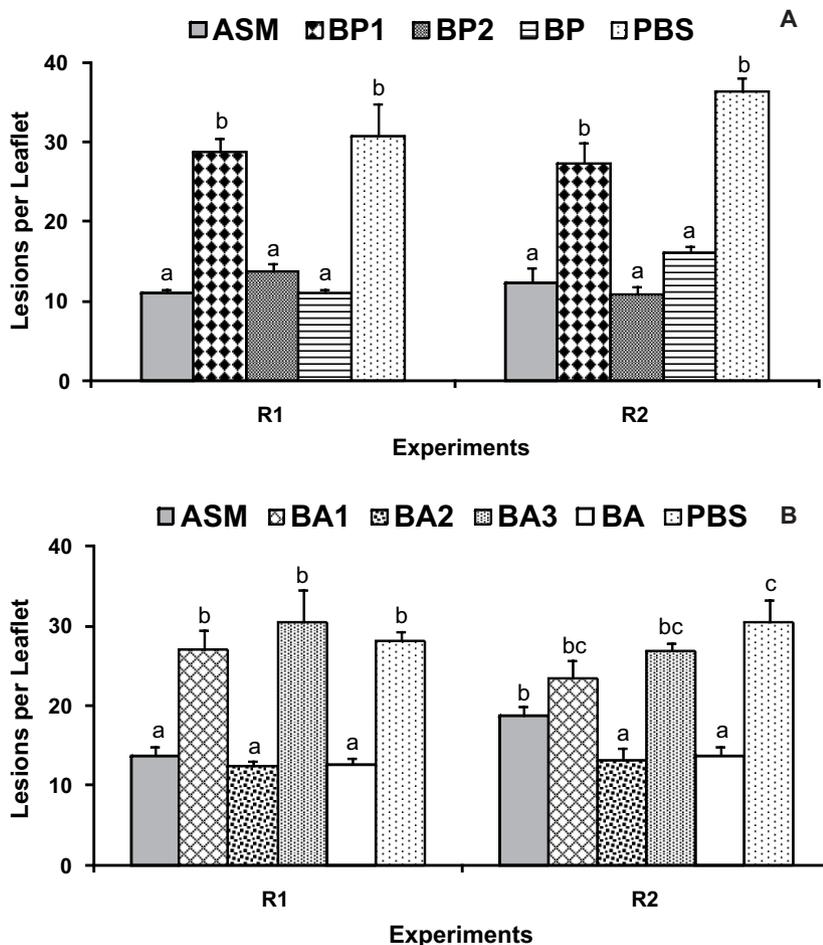
FIGURE 4 - Bacterial spot severity on Santa Cruz 'Kada' tomato plants, four days after exposure to treatments: **A.** Acibenzolar-S-Methyl (ASM), first (BP1) and second (BP2) purified fractions from *B. pumilus*, *B. pumilus* cell suspension (BP) and phosphate-buffered saline (PBS) (control); **B.** Acibenzolar-S-Methyl (ASM), first (BA1), second (BA2) and third (BA3) purified fractions from *B. amyloliquefaciens*, *B. amyloliquefaciens* cell suspension (BA) and PBS (control). Fifteen-day-old tomato plants were sprayed with the challenging pathogen (*Xanthomonas vesicatoria*) and lesions were counted when the symptoms were well developed. The bioassay was repeated twice (R1 and R2) with five replicates per treatment. Means followed by the same letter do not differ by the Tukey's test ($p \leq 0.05$). The columns represent the means and vertical bars the standard error of the means.

appeared as single protein bands in gels stained with silver nitrate. The proteins presented an estimated molecular mass of 28 and 43 kDa, respectively (Figure 3B). Figure 4A shows that ASM controlled 65.2% of the bacterial spot, but did not differ from treatments with BP2 and BP with mean control values of 63.5 and 59.6%, respectively. Figure 4B shows that the best mean control values against bacterial spot were provided by BA2 and BA, with protection of 56.6 and 54.7%, respectively followed by ASM with 44.4%. Purified fractions BP1, BP3 and BA1 did not protect tomato plants against the bacterial disease.

Determination of enzyme activity

Purified fractions 42 (BA2) and 75 (BP2) induced an increase in POX and PPO enzymes, but ASM induced the highest accumulation of these enzymes (Figures 5A and B). PBS buffer and water did not increase POX and PPO activity after spraying. An increase in activity of the enzymes was observed in plants sprayed with BP2, BA2 and ASM, and 4 days later inoculated with Xv (Figures 5A and B). No increase in enzyme activity was observed in plants sprayed with PBS buffer or water and later inoculated with Xv. The activity of PPO in plants inoculated with Xv increased after

48 h in all treatments (Figure 5A). The highest accumulation of PPO was observed in plants previously sprayed with BP2, BA2 and ASM, when compared to PBS buffer and water.

DISCUSSION

In this study, we demonstrated that cell suspensions, supernatants and purified protein fractions of two endophytic *Bacillus* isolates were able to induce systemic resistance in tomato against *X. vesicatoria*, the casual agent of bacterial spot. These macromolecules, with molecular mass of approximately 28 and 43 kDa were secreted in a nutrient-poor medium. The Simmons medium (SM) was selected to be used in this study because other authors reported its suitability for the detection of secreted macromolecules (Gijsegem et al., 1995, 2000).

Systemic resistance was first indicated in our studies when the supernatants did not present toxic activities against the tested pathogens, but reduced bacterial spot *in planta* when the supernatants were sprayed before pathogen inoculation. This phenomenon can be evidenced by two criteria presented by Steiner and Schönbeck (1995): a) absence of toxic effects of the inducer agent on the defiant
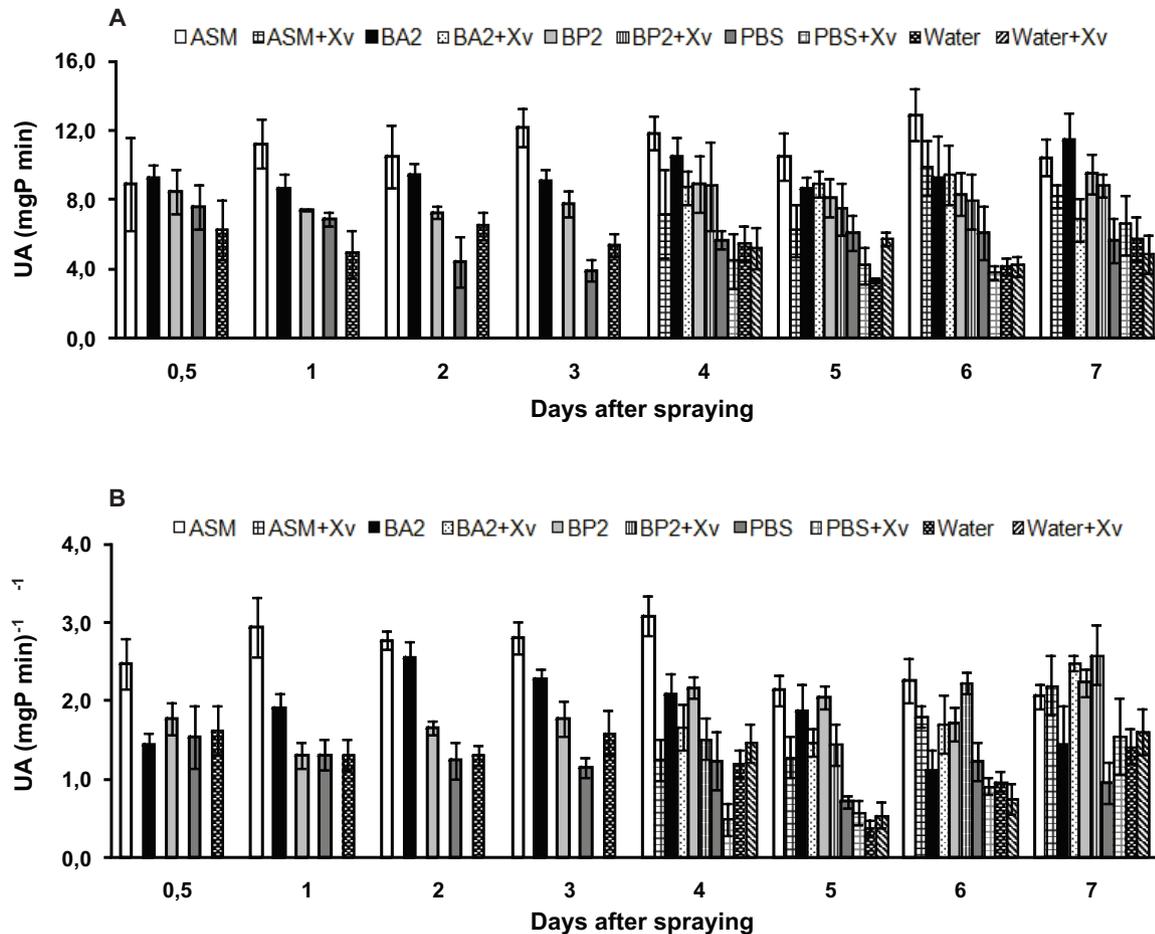
FIGURE 5 - Effects of treatments on **A.** peroxidase (POX) and **B.** polyphenol oxidase (PPO) activity in leaves of tomato cv. Santa Cruz 'Kada'. The treatments purified fraction from *B. pumilus* (BP2), purified fraction from *B. amyloliquefaciens* (BA2), Acibenzolar-S-Methyl (ASM), PBS buffer (PBS) and Water were sprayed on tomato leaves 10 days after sowing and plantlets were inoculated with virulent *X. vesicatoria* (Xv) strain 4 days later. Data are the average of three replications, and bars indicate standard error of the means.

pathogen and b) necessity of a time interval before the plant expresses resistance. These two criteria were met in our studies, strongly indicating a role for induction of systemic resistance. Although Romeiro et al. (2005) showed that the rhizobacterium *B. cereus* strain UFV-101 produces a protein that is responsible for eliciting defense responses in tomato against *Corynespora cassiicola,* its molecular mass was not determined with certainty. Our study is the first to show that endophytic bacteria from tomato produce elicitors of proteic nature able to induce resistance against *X. vesicatoria* and the first to determine the molecular weight of these macromolecules.

Our study also reports the biocontrol activity of the endophytic bacteria against bacterial spot on the tomato phylloplane. Although endophytic bacteria use the interior of plants as a habitat, they can easily adapt to the foliar surface as reported by Lanna-Filho (2011), surviving for a certain amount of time (Compant et al., 2005). This adaptative capacity lead us to speculate that endophytic *B. amyloliquefaciens* and *B. pumilus*, in contact with the plant surface, can continuously synthesize and release proteins with defense response-eliciting activity, and at the same time exercise biocontrol by mechanisms such as competition for space and nutrients.

Our studies with ASM only confirm its protective effect on tomato plants against *X. vesicatoria*, as reported by other authors (Silva et al., 2003; Cavalcanti et al., 2006a, 2006b). In comparison with studies reported by Cavalcanti et al. (2006c), we also observed an abrupt increase in the values of POX and PPO enzymes in tomato plants sprayed with ASM and after inoculation with Xv. The increase of these enzymes is associated with cell wall reinforcement, local and systemic resistance (Anterola & Lewis, 2002), which certainly resulted in a reduction of bacterial spot on tomato plants (Figures 2 and 4).

The molecular weights of the proteins purified in our work are in agreement with most of the proteins synthesized by antagonistic bacteria that have effect against pathogens (Gaudriault et al., 1998; Kavitha et al., 2005; Wong et al., 2008). However, this is the first report on the

synthesis of proteins by endophytic Gram-positive bacteria with the capacity to induce resistance in tomato plants. Our data demonstrate the discovery of two novel proteins with resistance-eliciting activity synthesized by endophytic bacteria of the genus *Bacillus* with control efficacy that compares to ASM, the main resistance inducer used in tomatoes.

The highest accumulation of POX and PPO enzymes in tomato plants after spraying with BA2, BP2 and ASM treatments indicates the occurrence of the SAR phenomenon in our system (Hammond-Kosack & Jones, 2002). SAR is quickly triggered after the plant tissues come in contact with the elicitor, and is characterized by the increased expression of a large number of pathogenesis-related genes (*PR* genes), in a local and systemic manner (Loon, 1997; Durrant & Dong, 2004). Unlike it, ISR occurs in response to non-pathogenic inducers and biochemical changes characteristic of ISR-expressing plants become obvious only in response to a further infection particularly in plant parts where effective resistance is required. This phenomenon has been described as 'priming', 'conditioning' or 'sensitization' (Sticher et al., 1997; Conrath et al., 2001).

Studies similar to ours are not common for Gram-positive bacteria, but were done for Gram-negative bacteria, such is the case of the harpins (Wei et al., 1992a, 1992b; Gaudriault et al., 1998; Wei et al., 2000). Harpin proteins were isolated on the basis of their capacity to induce resistance in tobacco. Named HrpN (44 kDa) in *Ewinia amylovora* (Wei et al., 1992a; 1993), HrpZ (45 kDa) in *Pseudomonas syringae* (He et al., 1993) and PopA (38 kDa) in *Ralstonia solanacearum* (Arlat et al., 1994) these elicitor proteins are heat stable, hydrophilic, glycine-rich and lack cysteine. The next step in our research will be to identify these newly discovered proteins by mass spectrometry (CID-MS/MS). This will then allow further studies to establish the DNA regions responsible for their synthesis.

ACKNOWLEDGMENTS

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