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Abstract: This study evaluated the antibiotic activity and induction of resistance in plants by compounds produced by \textit{Pseudomonas aeruginosa} LV strain on the control of bacterial stem rot in tomato. Compounds were extracted from the cell-free supernatant of a bacterial culture and purified. The F4A fraction was composed of two major compounds, an antibiotic and a phenazine (PCN). The first experiment evaluated the antibiotic activity of F4A, and the second one compared the ability of F4A and PCN to elicit systemic acquired resistance (SAR). In both experiments, plants were infected with \textit{Pectobacterium carotovorum} subsp. \textit{carotovorum} (Pcc). The minimum inhibitory concentration of F4A was 7.81 $\mu$g mL\textsuperscript{-1}, and PCN did not inhibit bacterial growth. The results suggest that F4A has antibiotic activity. Scanning electron microscopy revealed changes in bacterial cells after 3 h treatment with F4A. F4A and PCN decreased symptoms of...
stem rot and increased fruit production. Plant response was estimated by determination of peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase activity. Plants treated with PCN or F4A showed greater enzyme activity than plants that were not treated or treated with Bion®, suggesting that PCN increased SAR. The compounds showed the potential to control Pcc in vitro and in vivo and to induce plant response.

**Subjects:** Crop Science; Pest Management; Horticulture

**Keywords:** SAR; Pseudomonas; biocontrol; tomato; natural compounds

### 1. Introduction

Intensive farming leads to the development of new crop diseases that are difficult to control. When the phytopathogen is a bacterium, often there is not product available for control, causing considerable crop losses (de Oliveira et al., 2016). Tomato (*Solanum lycopersicum* L.) is the most important crop in terms of yield and cultivated area (Segui-Simarro & Nuez, 2007). Brazil is one of the major tomato producers in the world with 55,592 ha (IBGE, 2013).

To control bacteria in tomatoes, there are few products available on the market, and they are not always effective (de Oliveira et al., 2016). Bacterial stem rot (BSR), affecting fruit as well, is caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) and is the most common disease of tomatoes in the world, and this disease was identified in the early 1980s in greenhouse crops in Greece (Malathrakis & Goumas, 1987).

Today, the main ways of controlling disease caused by Pcc is management of the cultivated area such as avoiding wet soils, eliminating sick plants, destroying crop residues, crop rotation and others (Yuliar & Toyota, 2015). The use of chemical pesticides such as kasugamycin hydrochloride with or without copper fungicides has shown little effect in Pcc control (Yuliar & Toyota, 2015).

Today, the challenge is to find a natural compound with high antibiotic activity, which is also not harmful to the environment and human health. Accordingly, many biocontrol agents have been tested in the control of Pcc, e.g. *Bacillus thuringiensis* (Dong, Zhang, Xu, & Zhang, 2004), *Streptomyces plicatus* (Zamanian, Shahidi, & Saadoun, 2005) and other rhizobacteria (Choudhary & Johri, 2009), using bacterial cell suspensions that contain various compounds together with cells, and they have demonstrated little effectiveness in the control of Pcc.

Another alternative to control bacteria in plants is the use of partially purified or purified compounds with antibiotic activity (de Oliveira et al., 2016), which may induce systemic resistance (ISR) and/or systemic acquired resistance (SAR) (Raaijmakers, Vlami, & de Souza, 2002). It is largely known that *Pseudomonas* spp. is the most important producer of compounds with antibiotic activity or elicitor activity triggering SAR (Choudhary, Prakash, & Johri, 2007) or ISR (Durrant & Dong, 2004).

The objective of this work was to evaluate the antibiotic activity and induction of resistance by bioactive extracellular compounds produced by *Pseudomonas aeruginosa* LV strain in the control of BSR caused by Pcc in tomato.

### 2. Materials and methods

#### 2.1. Microorganisms

The pathogenic strain of Pcc used was kindly donated by Dr Maria Jose Jimenez Pozo (CSIC, Estación Experimental del Zaidín, Spain). The strain was grown in CPG (1 g hydrolyzed casein, 10 g peptone, 5 g glucose, 17 g bacto agar pH 7.0) and cryopreserved in 30% glycerol in liquid nitrogen. The antagonistic bacterium used was *P. aeruginosa* LV strain, which was isolated from orange plants (*Citrus sinensis* cv. Valence), in the city of Astorga, Brazil (Rampazo, 2004).
The LV strain was grown on nutrient agar (NA) plus copper chloride (5.0 g peptone, 3.0 g meat extract, 0.1 g CuCl₂·2H₂O and 15 g bacto agar, pH 6.8) at 28°C, and stocks were prepared in 30% glycerol and stored at −20°C throughout the study. The bacterial strains were deposited at the Microbial Culture Collection of the Laboratory of Microbial Ecology, Londrina State University, Brazil.

2.2. Production, extraction and purification of extracellular bioactive compounds

The culture conditions were standardized to optimize LV strain growth and production of metabolites according to a patent (Andrade, 2008). The initial inoculum of LV strain was obtained from a culture stored in glycerol and cultivated as described above, when the culture was in log phase (10⁸ CFU mL⁻¹, OD 0.09, λ = 590 nm), 100 μL of cell suspension were inoculated in 1,000 mL of nutrient broth (NB) plus 100 mg L⁻¹ CuCl₂·2H₂O and cultivated on a shaker for 10 days at 28°C, 100 rpm. The culture was harvested and centrifuged at 9,000 rpm for 20 min at 4°C.

The cell-free supernatant was obtained, and the metabolites were extracted five times using two volumes of dichloromethane each time (250 mL of supernatant and 500 mL of dichloromethane). This extract was designated the dichloromethane phase (DP). DP was purified by vacuum liquid chromatography (VLC) using a vacuum at ~150 mm Hg. DP was fractionated using mobile phase (v/v) with increasing polarity. Each mobile phase (400 mL) was distributed into two aliquots of 200 mL each (A and B). The column was eluted with hexane (F1A and F1B), hexane/dichloromethane (1:1; F2A and F2B), dichloromethane (F3A and F3B), dichloromethane/ethyl acetate (1:1; F4A and F4B), ethyl acetate (F5A and F5B), ethyl acetate/methanol (1:1; F6A and F6B), methanol (F7A and F7B), methanol/water (1:1; F8A and F8B) and water (F9A and F9B). The fractions collected were monitored by thin layer chromatography (TLC), concentrated in rotatory evaporator at 40°C, lyophilized and stored at −20°C. The antibiotic activity was determined by bioautography against Pcc.

The F4A fraction (high level of antibiotic activity as shown in the bioautography test) was further purified using 0.04–0.063 mm silica gel 60 column chromatography. The column (0.8 cm diameter × 50 cm height) was coupled to a low-pressure pump and eluted with hexane, hexane/dichloromethane (1:1; v/v), dichloromethane (100%), dichloromethane/ethyl acetate (1:1, v/v), ethyl acetate (100%), ethyl acetate/methanol (1:1, v/v) and methanol (100%). Approximately 1 mL of the mobile phase was collected in tubes and monitored by TLC. Similar fractions were combined based on TLC analysis and three fractions were obtained (F4A.1 to F4A.3). One pure compound (F4A.3.6.3) named phenazine-1-carboxamide (PCN) was obtained with two more purification steps using silica-gel column chromatography using the same conditions described above. One milligram of PCN was dissolved in chloroform at room temperature and kept in a vial to slow evaporation. F4A and the pure compound were loaded on a preparative high performance liquid chromatography (prep-HPLC) column and were further subjected to chemical characterization.

2.3. TLC analysis

TLC of the fractions was carried out with silica gel plates (Merck 60 F₂₅₄). The fractions were deposited and after drying, the chromatograms were developed in hexane/ethyl acetate and/or dichloromethane/ethyl acetate. The spots were viewed under ultraviolet light at 254 and 366 nm.

2.4. Prep-HPLC analysis

F4A and PCN were further subjected to prep-HPLC using Agilent 1260 HPLC. The mobile phases used were 100% water (A) and 100% acetonitrile (B) at a flow rate 1 mL/min and injection volume was 100 μL at a column temperature of 25°C (C18 Agilent HPLC column, 5 μm, 4.6 × 250 mm). Eluates were monitored at different wavelengths (250, 264, 290, 271, 316, and 366 nm).

2.5. Chemical analysis

PCN was dissolved in CDCl₃ or CD₃OD at 1.0 mg mL⁻¹. Mass spectra were obtained with an ESI-MS Quattro LCZ (Micromass, Manchester, UK). ¹H and ¹³C nuclear magnetic resonance spectra were recorded in solution using a Bruker Avance III 400 MHz spectrometer.
2.6. Biological analysis

2.6.1. Selection of compounds with antibiotic activity by bioautography

The bioautography method (Rahalison et al., 1993) was used to determine antibiotic activity. TLC plates were developed as described above and placed in a Petri dish with 20 mL of melted NA (45°C) mixed with 1 mL of cell suspension of Pcc (10⁸ CFU mL⁻¹), which was incubated for 48 h at 28°C. The growth inhibition zone was measured and spots with antibiotic activity were identified.

2.6.2. Evaluation of antibiotic activity by disc diffusion technique

The antibiotic activity was evaluated by the disc diffusion technique. DP and F4A were assayed by agar diffusion in Petri dishes with 20 mL of NA plus 1 mL of cell suspension of Pcc (10⁸ CFU mL⁻¹) in log phase. The discs were prepared with 500 μg disc⁻¹ DP and 250 and 500 μg disc⁻¹ F4A. Plates were incubated for 48 h at 28°C and the halo formed around the disc was measured (mm). The experiment was repeated three times and the antibiotic effect was determined by measuring the growth inhibition halos formed around the disc.

2.6.3. Determination of minimum inhibitory concentration

The experimental design used twofold serial dilution of samples (500–0.97 μg mL⁻¹ F4A and 700–1.36 μg mL⁻¹ pure PCN (F4A.3.6.3), with two samples and four replications. MIC was carried out in 96-well microplates, the inoculum used was 100 μL of cell suspension of Pcc (10⁶ CFU mL⁻¹) per well. The growth control was a cell suspension of Pcc in CPG only, and the negative control was non-inoculated CPG. The antibiotic solution was mixed with CPG to check for sterility and plates were incubated for 48 h at 28°C to check for sterility. The optical density was determined at 590 nm (BioMate 3). Afterwards, 20 μL of 1% 2,3,5-triphenyltetrazolium chloride (TTC) were added to all wells and the plate incubated again at 28°C for 20 min. Afterwards, the wells that showed a pink color were considered resistant (+) and those with no color change sensitive (−).

2.6.4. Effect of F4A fraction on cell morphology of Pcc

In scanning electron microscopy (SEM), 20-μL aliquots of Pcc cell suspension, treated (3 h) and not treated with F4A at MIC, were collected and spotted on glass slides previously coated with a thin layer of poly-L-lysine. Afterwards, each slide was fixed by immersion in 1 mL of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h, following post-fixation in 1% OsO₄ for 1 h. The fixed material was dehydrated in an ethanol series (70, 80, 90 and 100%). Samples were critical-point dried with CO₂ (BALTEC CPD 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater) and analyzed by SEM (FEI Quanta 200).

2.6.5. Effect of F4A fraction and PCN on stem rot and fruit control

Two experiments were carried out using tomato seeds of cv. Santa Clara, which were sown in Plantmax™ substrate in plastic tray cells, and after 30 days of germination, seedlings were transplanted. Pathogenic bacterial infection was effected by cutting the root tip and immersing the plant root for one minute in a Pcc suspension (10⁸ CFU mL⁻¹), and the compounds were sprayed on the leaf surface.

In the first experiment, the objective was to evaluate the influence of two regimens, preventive and curative, and different concentrations of F4A fraction on the control of Pcc and plant growth. The seedlings 30 days old were transferred to 2-L plastic pots with non-sterile Rhodic Ferrasol soil (FAO, 1994), and grown for 60 days under greenhouse conditions (28/22°C; 10/14 h light/dark; 80% relative humidity) and watered when needed.

The experimental design was randomized blocks with 2 × 3 factorial arrangement [2 regimens (preventive and curative), 3 F4A concentrations (7, 70 and 700 μg mL⁻¹)] and five replicates. Positive control plants were those immersed in a Pcc bacterial cell suspension (10⁶ CFU mL⁻¹), and negative control plants were those sprayed with distilled water. The F4A concentrations were determined according to values obtained in a MIC determination. To compare the effect on resistance induction,
we used the commercial product acibenzolar-S-methyl (ASM, Bion® 50WG (0.5 g L⁻¹)), where the use and application was according to the manufacturer’s recommendations.

In preventive treatment, 30-day-old seedlings were sprayed with 1 mL of F4A at the same concentrations described above. After 24 h, seedlings were taken from sand and infected. Secondary root tips were cut and immersed for 1 min in the Pcc bacterial cell suspension with 10⁸ CFU mL⁻¹. In the curative treatment, seedlings were first immersed in the bacterial suspension for 1 min and planted in pots, and after 24 h, they were sprayed with F4A fraction. The control plant was immersed only.

The second experiment was carried out in a non-acclimatized greenhouse covered on top with polyethylene film and on the sides with screen, and there was no protection against pests. The 30-day-old seedlings were transferred to 8-L pots with the same soil described above. Plants were grown for 120 days after planting at room temperature (16–38°C) during the experiment and watered when needed.

The objective was to evaluate the effect of the F4A fraction and PCN on the control of Pcc. The experimental design was completely randomized blocks, with the two regimens preventive and curative as described above, four treatments (2 concentrations of F4A fraction, 7 and 70 μg mL⁻¹, and 2 concentrations of PCN, 3.5 and 35 μg mL⁻¹), and six replicates. Bacterial infection and application of compounds were carried out as described above, where the positive controls were plants infected with Pcc suspension (10⁸ CFU mL⁻¹) and negative controls by cutting the root tip and immersing the plant root in distilled water. Bion® (0.5 g L⁻¹) was used for comparison in the effect on resistance induction, and the application was the according to the manufacturer recommendations.

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Plant height and yield (number and weight of tomatoes produced) were evaluated after 90 days of seedling transplant. The results of both experiments were evaluated by analyses of variance (ANOVA) and Tukey test (p ≤ 0.05). We determined disease development every week, checking the number of sick and healthy plants and determining the percentage of death/survival of plants. The data were evaluated by the chi-square test.

2.6.6. Evaluation of PCN acting as elicitor in tomato plants infected with Pcc

The third experiment resistance induction was carried out in plants under the same conditions as described above. After 30 days of germination, the seedlings were sprayed with 1 mL of PCN, and after 24 h, they were planted and grown for seven days in 2-L pots with the same soil as used in the above experiments. The experimental design was completely randomized blocks with two PCN concentrations (3.5 and 35 μg mL⁻¹), Bion® (0.5 g L⁻¹), seedlings infected and non-infected with Pcc, and five replicates. Uninfected and non-treated plants were considered the negative controls, and the positive controls were plants infected and non-treated.

After 24 h, treated seedlings were removed from the trays, and the root was washed with sterile distilled water. The root tip was then cut and the root completely immersed for one minute in a Pcc bacterial cell suspension (10⁸ CFU mL⁻¹), and the seedlings were planted in pots. The use and application of Bion® was the according to the manufacturer’s recommendation.

Control plants (non-infected/non-treated) were sampled after 0, 1, 3 and 5 days of planting in soil, and infected and/or treated plants were sampled after 1, 3 and 5. Three grams of shoot were sampled and stored at −20°C (Cavalcanti, Resende, Carvalho, Silveira, & Oliveira, 2007). Stored samples were crushed with liquid nitrogen plus 150 mg poly-vinyl-pyrrolidone (PVP) with a porcelain mortar, and 200 mg were suspended in 1.5 mL of respective buffer for each enzyme as follows: 10 mM sodium phosphate plus EDTA 0.1 mM, pH 6.0 (peroxidase), 120 mM MacIlvane, pH 6.5 (polyphenol oxidase) and 0.5 M Tris-EDTA, pH 8.5 (phenylalanine ammonia lyase). The homogenate was centrifuged (12,000 rpm for 30 min at 4°C), and the supernatant was assayed for enzyme activity (Araujo & Menezes, 2009). Total protein was determined by the Bradford assay (Bradford, 1976).
Peroxidase activity was determined using guaiacol as substrate. The reaction mixture contained 0.050 mL of 0.5% (v/v) guaiacol, 2.8 mL of 10 mM phosphate buffer (pH 6.0), 0.050 mL of 3% (v/v) hydrogen peroxide and 0.100 mL of sample in a final volume of 3.0 mL, and was incubated at 50°C for 30 min. Absorbance was read at 480 nm (molar extinction coefficient of guaiacol: 25.5 mol⁻¹ cm⁻¹), and peroxidase activity was expressed as μmol oxidized guaiacol formed min/mg protein (Urbanek, Kuzniak-Gebarowska, & Herka, 1991).

Polyphenol oxidase activity (EC 1.10.3.2) (Barbosa, Dekker, & Hardy, 1996) was evaluated by adding 100 μL of extract to 600 μL of sterile distilled water, 150 μL of 120 mM MacIlvaine buffer (pH 6.5) and 150 μL of 10 mM 2,6-dimethoxyphenol (DMP, substrate) and incubating for 30 min at 45°C in a water bath. Absorbance was read at 468 nm in duplicate and enzyme activity was expressed as U/mg protein.

Phenylalanine ammonia lyase activity was assayed using L-phenylalanine as substrate. The reaction mixture contained 0.50 mL of 300 μM L-phenylalanine, 2.4 mL of 0.5 M Tris-EDTA buffer (pH 8.5), and 0.10 mL sample in a final volume of 3.0 mL, and was incubated at 40°C for 60 min. Absorbance was read at 290 nm (molar extinction coefficient of L-phenylalanine: 1.0 × 10⁴ mol⁻¹ cm⁻¹). A unit of phenylalanine ammonia lyase activity was defined as μmol of trans-cinnamic acid formed per min (Mori, Sakurai, & Sakuta, 2001). Enzyme activity was related to the amount of total protein and expressed as U/mg. All chemical reagents were supplied by Sigma.

3. Results

3.1. Production, extraction and purification of extracellular bioactive compounds

The amount of metabolites extracted with dichloromethane (DP) was 0.375 g L⁻¹ from crude supernatant, and the amounts of F4A fraction and F4A.3.6.3 pure compound obtained were 25 and 14.6 mg per 500 mg of DP.

Two major metabolites were observed in F4A fraction by prep-HPLC (Figure S1) and one was isolated and studied (de Oliveira et al., 2016). F4A.3.6.3 pure compound was analyzed by prep-HPLC (Figure S2) and identified, showing a molecular formula of C₁₃H₉N₃O (PCN) by ESI-MS and NMR (Figure S3 and S4). Both fractions F3D (de Oliveira et al., 2016) and F4A used in this study showed the same characteristics and contained two major metabolites, organocopper antibiotic compound (OAC) with high antibiotic activity and PCN.

3.2. Biological analysis

In the first purification step, the fractions were monitored by bioautography to determine antibiotic activity, and only the F4A fraction showed significant antibiotic activity against Pcc. Other fractions obtained in this purification step and PCN did not show any antibiotic activity under the experimental conditions used.

In the evaluation of in vitro antibiotic activity by the disc diffusion technique, 500 μg/disc DP showed an inhibition halo of 35 mm, while 250 and 500 μg/disc F4A fraction displayed halos of 21 and 25 mm, respectively. MIC of F4A was 7.81 μg mL⁻¹, and PCN did not show any antibiotic effect against Pcc (Table 1).

SEM demonstrated that Pcc cells treated with F4A fraction were completely lysed after 3 h when compared with non-treated culture (Figure 1).

In the first experiment, 60% of infected and non-treated plants showed bacterial soft rot symptoms and died, while plants treated with F4A fraction did not display symptoms in preventive treatment with 100% survival, being more effective than Bion® with 80% of plants surviving. On the other hand, F4A showed low antibiotic activity in the curative treatment: 20% of plants treated with 70 and 700 μg mL⁻¹ survived, and at the lowest concentration (7 μg mL⁻¹), all plants treated survived (Table 2).
When comparing preventive and curative application regimes, it was clear that plants with preventive treatment were bigger in relation to curative treatment, control plants (infected and non-infected) and Bion® treatment. The best concentration of F4A to protect plants against \textit{P. carotovorum} and to promote plant growth (total length and shoot dry weight) was 70 μg mL$^{-1}$ followed by 7 and 700 μg mL$^{-1}$, even compared with Bion® (Figure 2(A) and (B)). Root fresh weight showed the same results as obtained for shoot growth, except for Bion® (Figure 2(C)).
Figure 2. Effect of different concentrations of F4A fraction (7, 70 and 700 μg mL⁻¹) on tomato (S. lycopersicum) growth with two application regimes [preventive (pr) and curative (cu)] after 60 days of infection with P. carotovorum subsp. carotovorum under greenhouse conditions. (A) Total height; (B) shoot dry weight; (C) root dry weight. The control plants were infected (Inf) and non-infected (Ninf). Bion® was used as the reference standard for SAR induction.

Note: The treatments with the same letter did not show significant differences according to the Tukey test ($p < 0.05$).
In the second experiment, we evaluated plant growth and tomato productivity. We observed the presence of aphids and mites but decided against pest control with chemical products; plant was treated only with F4A fraction, PCN or Bion® to control Pcc.

Many treatments did not show differences in plant growth, where effects were only observed in plants treated with F4A, PCN or Bion®. The different regimes and concentrations of F4A and PCN as well influenced plant growth. No differences were observed between infected plants treated with 70 μg mL⁻¹ F4A under preventive conditions and non-infected plants treated with 35 μg mL⁻¹ PCN had the highest growth followed by curative 7 μg mL⁻¹ F4A with 40% less. Controls and other treatments were significantly lower (Figure 3(A)).

The high productivity and fruit number in plants infected with Pcc was observed in plants treated with 70 μg mL⁻¹ F4A in the preventive regime followed by 7 μg mL⁻¹ F4A in the curative regime. PCN did not show any effect on Pcc control. The non-infected plants treated with the F4A fraction (7 and 70 μg mL⁻¹) did not show any effects on yield and fruit number. Different effects were observed when plants were non-infected and treated with PCN, with increased tomato yield and fruit number, showing that PCN enhanced productivity but did not protect against Pcc. F4A was more effective in controlling bacterial spot disease caused by Pcc increasing plant productivity in infected plants, while PCN was more effective in non-infected plants, but both were more effective than Bion® (Figure 3(B) and (C)).

Peroxidase activity increased after 24 h of Pcc infection in non-treated plants followed by non-infected plant treated with Bion® or 3.5 and 35 μg mL⁻¹ PCN. All treated infected plants showed low activity after 24 h. After 3 days, infected plants treated with Bion® had three times higher peroxidase activity, and infected plants with other treatments still showed low activity. On the other hand, non-infected plants still had high activity when treated with 3.5 and 35 μg mL⁻¹ PCN. After 5 days, peroxidase activity increased in all infected plants, especially with 3.5 and 35 μg mL⁻¹ PCN, but Bion® still showed the highest activity. In the whole experiment, control plants showed low activity (Figure 4(A)).

Polyphenol oxidase activity after 24 h was increased in non-infected plants with the two PCN concentrations followed by Bion®. After 3 days, non-infected plants treated with 3.5 and 35 μg mL⁻¹ PCN and Bion® still showed high activity when compared with other treatments, except for infected plants treated with 35 μg mL⁻¹ PCN. After 5 days, non-infected plants treated with Bion® and 35 μg mL⁻¹ PCN still showed high activity. On the other hand, infected plants treated with 3.5 μg mL⁻¹ PCN showed highly increased polyphenol oxidase activity, followed by 35 μg mL⁻¹ PCN. At the three evaluation times, control plants showed low activity (Figure 4(B)).

Phenyl ammonium lyase activity was increased by 35 μg mL⁻¹ PCN in non-infected plants when compared with all treatments at the three evaluation times. After 5 days, infected plants treated with 3.5 and 35 μg mL⁻¹ PCN showed increased activity, followed by Bion® in non-infected plants. Non-treated plants and control plants showed low activity of this enzyme (Figure 4(C)). Comparing the activities of the three enzymes determined, it is clear that peroxidase activity was increased when plants were infected and treated with Bion® and PCN. Polyphenol oxidase and phenylalanine ammonium lyase were increased by PCN in plants infected and non-infected with Pcc (Figure 4).
Figure 3. Effect of F4A fraction (7 and 70 μg mL$^{-1}$) and PCN (3.5 and 35 μg mL$^{-1}$) on tomato (S. lycopersicum) yield with only application of products (F and Ph) and two application regimes [preventive (pr) and curative (cu)] with 60 days of infection with P. carotovorum subsp. carotovorum (Pcc) under greenhouse conditions. (A) Total shoot height; (B) tomato yield; (C) fruit number. The control plants were infected (Inf) and non-infected (Ninf). Bion® (Bi) was used as the reference standard for SAR induction.

Note: The treatments with the same letter did not show significant differences according to the Tukey test ($p < 0.05$).
Figure 4. Enzyme activity used as biochemical indicator of SAR in tomato leaves (*S. lycopersicum*) treated with 3.5 and 35 μg mL⁻¹ PCN after infection with *P. carotovorum* subsp. *carotovorum* (Pcc). (A) Peroxidase; (B) polyphenoloxidase; (C) phenylalanine ammonium lyase. Bion® was used as the reference standard for SAR induction.
4. Discussion

The antibiotic activity of F4A fraction against \( \text{Pcc} \) was the same as found with other Gram-negative bacteria (da Silva Vasconcellos et al., 2014; de Oliveira et al., 2011; Lopes et al., 2012; Murate et al., 2015; Spago et al., 2014). In tomato, compounds produced by \textit{Pseudomonas} sp. controlled \textit{X. campes}tral \textit{PV. vesicatoria} (Byrne et al., 2005).

The changes in the ultrastructural cell matrix observed by SEM in \( \text{Pcc} \) treated with F4A were the same as observed in \textit{Xanthomonas} spp. (da Silva Vasconcellos et al., 2014; de Oliveira et al., 2011; Lopes et al., 2012; Murate et al., 2015; Spago et al., 2014).

It is widely known that some molecules of the PCN family have antibiotic activity against many phytopathogenic fungi or bacteria (Chin-A-Woeng, Bloemberg, & Lugtenberg, 2003; Thomashow, Weller, Bonsall, & Pherson, 1990), but many of other such molecules do not show any antibiotic activity, and could act as elicitor to increase ISR and SAR (Thakur & Sohal, 2013). F4A is very similar to F3d in composition (de Oliveira et al., 2016) and has shown a low MIC against \( \text{Pcc} \). The composition of both fractions is OAC (30%), PCN (40%) and other compounds (30%). PCN in a pure state did not show any effect against \( \text{Pcc} \) growth, and the same results were obtained in other studies with \textit{Xanthomonas} spp. (de Oliveira et al., 2016).

In a greenhouse experiment, preventive 70 µg mL\(^{-1}\) F4A was the most effective on plant and root growth when compared with control and other treatments including Bion\textsuperscript{®} and plants that did not have any contact with mites or aphids and other pathogenic microorganisms, except \( \text{Pcc} \). Other authors who used F3d fraction (similar to F4A) found the same results in a preventive regime but with different concentrations (de Oliveira et al., 2016). The control of \( \text{Pcc} \) in tomato was very effective with preventive regime for all concentrations and with curative for 7 µg mL\(^{-1}\) F4A, where no plants died.

The above finding might have involved the association of PCN and OAC present in the composition of F4A acting in two ways increasing plant response and control of \( \text{Pcc} \) by antibiotic activity, respectively. PCN has not been described as an elicitor such as pyocyanine (PYO) which is well known to elicit ISR mediated by jasmonate and ethylene (Audenaert, Pattery, Cornelis, & Höfte, 2002; De Vleesschauwer, Cornelis, & Höfte, 2006; Pahm, Ran, Pieterse, & van Loon, 2003).

When tomato was cultivated in an open system in contact with the external environment, the effects of F4A fraction and treatment regimens were different. Plants infected with \( \text{Pcc} \) and treated with curative 7 µg mL\(^{-1}\) F4A showed the greatest height, followed by preventive 70 µg mL\(^{-1}\) F4A. In recent studies, the authors found the same results where the preventive regime was more effective than curative regime, suggesting that some molecules act as elicitors, probably PCN. PCN did not protect against \( \text{Pcc} \) in either regime; however, when applied to non-infected plants, growth increased with curative 7 µg mL\(^{-1}\) F4A, suggesting that PCN has some effect on plant growth, but further studies need to be done.

Tomato yield and number of fruits were increased by preventive 70 µg mL\(^{-1}\) F4A followed by curative 7 µg mL\(^{-1}\) F4A in \( \text{Pcc} \)-infected plants and by PCN in non-infected plants. To understand these effects, need to note the F4A composition, consisting of 50% PCN, which is involved in plant growth increasing tomato production but with no control of \( \text{Pcc} \). Infected plants survived and showed increased plant growth and fruit production only due to the OAC present, suggesting than this component is the responsible for the survival in infected plants.

The increased of enzyme activity reinforced the idea that PCN could act as elicitor, where different results were obtained in infected and non-infected plants for different enzymes. However, further studies evaluating gene expression in tomato need to be carried out to determine how PCN influences tomato response, since in orange plants, it was observed that gene expression increased in plants treated with F4A (Pistori, 2014).
The results suggested that F4A fraction could be an important tool in the control of Pcc in tomato, with disease control based on antibiotic activity and the action of PCN in plant defenses. In addition, the mechanisms involved need to be elucidated.

Abbreviations

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| ASM          | acibenzolar-S-methyl                             |
| BSR          | bacterial stem rot                               |
| DMP          | 2,6-dimethoxyphenol                              |
| DP           | dichloromethane phase                            |
| ISR          | induced systemic response                        |
| MIC          | minimum inhibitory concentration                |
| OAC          | organocopper antibiotic compound                 |
| Pcc          | Pectobacterium carotovorum subsp carotovorum     |
| PCN          | phenazine-1-carboxamide                          |
| PYO          | aspiocianine                                      |
| SAR          | systemic acquired resistance                     |
| SEM          | scanning electron microscopy                     |
| TTC          | triphenyltetrazolium chloride                    |

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Competing Interest

The authors declare no competing interests.

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