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Full Length Research

Biological Control of *Erwinia Amylovora* in Apple Trees Employing Antibacterial Agents

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The northern Mexican state of Durango has two major fruit producing sectors located in the municipalities of Canatlán and Nuevo Ideal, with a height of 1859 m above sea level. Even though these fruit producing zones encompass over 12 thousand hectares, the overall fruit production has been declining over the years. Two decades ago, the annual fruit production was approximately four million crates, by 2018; the production has been reduced by up to 75%. The decline in production is attributed to diverse environmental, cultural and economic factors, being the most important; environmental temperatures above 30°C during the flowering stage, which increases the proliferation of Erwinia amylovora bacteria, the causative agent of the fire blight disease. Infected plants exhibit damaged roots, leaves and fruits, necrotic lesions, reduced vegetative and floral bud sprouting, deficient accumulation of chill hours and fertilization, and irrigation during flowering stage are only a few causative agents that promote the presence of the bacteria, making it hard to control. Local producers have employed antibiotics (such as streptomycin) and cooper sulfate to control Erwinia infections, nonetheless, recurrent use of this agents have promoted the emergence of resistance. Taking this into account, the next activities were performed with the aid of local producers: eight orchards were selected within the localities of Canatlán, Durango, over 350 fruit trees with visible fire blight symptoms were marked, georeferencing, soil and stem sampling, soil fertility analysis, bacteria isolation using King B® selective medium, and Metarhizium anisopliae, Beauveria bassiana, Estreptomyces coelicolor and Bacillus thuriengiensis bioformulation using a 1 x 10^9 spores per gram proportion. Soil analysis revealed deficient organic matter, with values below 1.5%, alkaline pH values above 7.5, sandy texture and insufficient nitrogen and phosphorous in 75% of analyzed orchards. More than 90% of analyzed orchards presented E. amylovora in both soil and tree bark after a 72-hour incubation period. Employment of bioformulations for fire blight control revealed that only the B. thuringiensis bioformulation was able to control the disease with 95% efficiency after a single administration.

Keywords: *Erwinia amylovora*, *Bacillus thuriengiensis*, apple tree, soil fertility

INTRODUCTION

The presence of *Erwinia amylovora*, causative agent of the fire blight disease in apple trees, is responsible for a 30% decrease in production during the first year, with a gradual production decrease over subsequent years (Merlin et al., 2014). Fire blight disseminates trough rain, wind, insects, poorly disinfected pruning tools and uncontrolled soil and plant fertilization (Paulin and Samson, 1973). Within the state of Durango, the municipalities of Canatlán and Nuevo Ideal encompass the

Table 1. Apple production decline in Durango´s fruit producing sectors.

Municipality	Surface area (hectares) (2018)				
	Producers	Total Surface	In development	Production	Decline
Canatlán	759	3,842	330	3,139	373
Nuevo Ideal	366	1,598	162	1,170	266
Total	1,125	5,440	492	4,309	639

major fruit producing sectors; these municipalities are located at a height of 1859 m above sea level and cover more than 12 thousand hectares. The overall production of these fruit producing sectors, which was approximately four million crates per year during the nineties, has been declining over the years, being only one million crates as of 2016 (Table 1). These declines has been attributed to diverse factors including, lack of pressurized irrigation technologies, which would allow the saving of considerable amounts of water, the scarcity of chill hours will not permit the production of certain types of apples, such as the delicious variety, late frosts, presence of hail in some localities and absence of anti-hail mesh, temperatures above 30°C during the flowering stage which increases the proliferation of *E. amylovora* which damages leaves, tree bark, fruits, and even the roots of infected trees (Ockey and Thomson, 2006).

The detection of these bacteria is achieved using the CCT medium stigma impression method, which consists of a tergitol violet crystal staining. Producers have employed various methods to try to control this disease, such as antibiotics (streptomycin, gentamycin, tetracycline and oxytetracycline), organic extracts from plants (such as oregano, thyme and cinnamon) and bactericides, which have been implicated in the development of resistance (Ramirez et al., 2003). *E. amylovora* is a rod-shaped unicellular organism with an average length of 0.5 to 1.0μm and an average width of 1.0 to 3 μm that attacks plants of the Rosaceae family (Cabrefiga, 2005). This bacterium moves using a flagellum and can use various types of sugars, organic acids and alcohols for its development; it is able to sustain growth in a temperature range from 30°C to 50°C, with an optimal growth temperature of 27°C and an optimal pH of 6.8. *E. amylovora* has a generation time of approximately 20 minutes, being able to generate over a million cells within 10 hours (Medina et al., 2006). *E. amylovora* infection starts when bacteria come in contact with susceptible trees during the flowering stage, entering trough the fruits, and buds undergoing the active phase or flowers. Afterwards, it advances through the leaves, secondary branches, the trunk and roots; infected leaves do not fall off and remain dry and connected to the tree (Ramirez et al., 2008). Other factors such as leachate and hail facilitate the entry of *E. amylovora* into susceptible trees through the roots and fruits, respectively (Merlin et al., 2014).

The presence of *E. amylovora* in fruit trees from the fruit producing sectors can be attributed to the next factors: a) Reduced chilling accumulation during dormancy over the winter months (during 1988 for example, there was a 60% reduced chilling accumulation when compared to other years); b) As a result from the previous point, long and irregular flowerings take place, with flowers remaining open for prolonged periods of time, facilitating the entry of *E. amylovora* into susceptible trees; c) The lack of resources most producers suffer from, for instance, cold compensators can help overcome the reduced chilling accumulation, nonetheless, this would increase production costs; d) The tendency of most producers to use irrigation during the flowering stage as a common practice. Diverse methodologies are employed to isolate *E. amylovora*, the procedure proposed by Lopez (2004) consists of preparing the plant sample, selecting flowers, buds, sprouts, leaves, fruits and/or subcortical tissue from branch or stem chancres, the sample is macerated in sterile water for subsequent direct isolation, the plant extracts are plated in solid medium and incubated at 26°C for 72 hours. King B agar (KB) is used as the selective media for *E. amylovora* detection and isolation (King et al., 1954). *Erwinia* is fast growing, presenting white circular colonies with a 2-5 mm diameter within 1-2 days (Pusey, 2000). Antagonism is defined as the opposite interaction between two organisms, one inhibiting the growth of the other (Luna et al., 2007). The use of entomopathogenic microorganisms to evaluate antagonism between native strains and/or *Bacillus thuringiensis*-based products has allowed the identification of native strains and products with varying degrees of toxicity against *Tecia solanivora* (Castelblanco, 2000).

B. thuringensis (*Bt*) is an entomopathogenic bacterium with toxic activity against Lepidopterans, Coleopterans and Dipterans. Bt exhibits a parasporal inclusion composed of insecticidal proteins denominated Cry and Cyt (Palacio et al., 2009). Physical, chemical and hydraulic analysis of the soil consist of pH, electric conductivity, organic matter, apparent density, soluble salts and soil texture with components such as sand, clay and silt, as well as macronutrient content like nitrogen, phosphorous, potassium, calcium, magnesium, sulfur and sodium, micronutrients like copper, iron, zinc, boron and manganese, and finally the cationic exchange capacity and sodium absorption ratio (SAR), allows to determine the factors that contribute to soil nutrient deficiency, which is a factor implicated in *E. amylovora* infection (Castelbllanco, 2000). The objectives of this work are 1) Sampling of soil and tree bark from apple trees with visible disease symptoms; 2) Isolation of the causative agent of fire blight disease *E. amylovora*, using selective media and serial dilution techniques; 3) Use of plate antibiograms employing antagonistic agents in order to inhibit bacterial growth; 4) Soil and tree bark analysis for detection of physical, chemical and fertility factors involved in bacterial growth; 5) Production, administration and field evaluation of bioformulates using commercial bactericides as controls.

Table 2. Characteristics of the selected orchards.

Estate	Marked trees	Coordinates	Orchard	Erwinia presence	Controls
1	50	23° 32, 497, N 104° 96, 260, W	Los Fresnos	30	20
1A	10	23° 32, 497, N 104° 96, 260, W	Los Fresnos	7	3
2	10	24 ° 31, 558, N 104° 42, 347, W	Tres Generaciones	5	2
3	60	24° 28, 253, N 104° 41, 982, W	KIM	40	20
3A	20	24° 28, 253, N 104° 41, 982, W	KIM	12	8
4	70	23° 30, 579, N 104° 43, 791, W	Asunción	50	20
5	70	24° 30, 854, N 104° 42, 237, W	Asención	50	20
6	50	24° 28, 024, N 104° 42, 347, W	La Esperanza	30	20

MATERIALS AND METHODS

This study was conducted from January to June, 2018; six orchards (KIM, El Fresno, La Asunción, Ascensión, La Esperanza and Tres Generaciones) were selected (Table 2). The orchards are located within the fruit sector in Canatlán Durango (Height above sea level: 1952 mamsl, latitude: 24.5228, longitude: -104.768 24° 31´). This region presents a semiarid climate with a mean annual precipitation of 535.8 mm and a mean annual temperature of 15.8°C. Trees with visible signs of fire blight disease were marked in blue, trees with low levels of *E. amylovora* presence (below 40%) were marked in white and used as controls.

The orchard selection process was carried out considering the following criteria: 1) Orchard proximity to highways and communication channels; 2) Degree of infection in trees as well as soil and tree bark sampling difficulty. Over 350 trees were marked throughout the six selected orchards, soil samples were collected by digging holes 60 cm deep, two samples were collected from orchard 1, two from orchard 2, three from orchard 3, until 14 soil samples were collected, annexing three additional orchards: El Tavo, Erick Contreras and Rodolfo Reyes. Plots were geo-referenced using a navigator (Garmin model Efrex 10®); soil fertility analysis was executed under the Official Mexican Standard 021-RECNAT-2000. Infected tissue sampling for microbiological analysis was carried out scrapping tissue with a knife disinfected with a 5% formol solution; obtained samples were placed inside a hermetically sealed plastic bag and kept at 4°C. The determination of soil microbial growth was conducted by grinding the soil samples with a wooden mallet and sieving them (No. 16, mesh size 1.19 mm), one previously filtered gram of soil was then diluted in 10 mL of distilled water (10% solute and 90% solvent), 10 milliliters were extracted from this solution and were then diluted in 90 mL of water. This procedure was repeated ten times until 1-10, 1-100 and 1-1000 dilutions were obtained. The tissue sample preparation was performed using the same serial dilution technique mentioned previously, with the difference that the tree bark sample was blended until it became a fine powder. Samples were plated using the streaking method, King B® agar was used as the selective growth media. All the procedures were conducted under aseptic conditions using a laminar flow cabinet (Heal Force Model Class II. Incubation was performed using a refrigerated incubator (Memmert® model ICP/IPP) at 32°C for 16 days. Bacterial cells were plated continuously until isolated pure colonies were obtained; *E. amylovora* possess characteristic circular mucoid white colonies. Solid media fermentation (SMF) was carried out using one gram from two strains, *Estreptomyces coelicor* SC1234 and *Estreptomyces venezolae* SC1235, both strains were kind gifts from Dr Luis Servín González (Universidad Nacional Autónoma de México). Purified colonies were grown in 250 mL Buchner flask containing a mineral salt based medium and shaken using a shaker (Thermo Scientific®) at 100 rpm for 72 hours. 10 mL of media containing bacteria were used to inoculate one kilogram of precooked rice contained in a plastic bag, 20 mL of distilled water were then added to the bag to preserve humidity. Afterwards, the plastic bags were left inside a bioclimatic chamber (model CBR-214) for 30 days at 25°C. This process was repeated six times using six commercial strains: 1) *Pisolithus tinctorius* based Ecto-Rhyza® (250 million CFU/g); 2) *Bacillus subtilis* based PHS WS® (16.5 million CFU/g); 3) *Estreptomyces griseoviridis* based PHC-F® (1 million CFU/g); 4) *Bacillus thuriengiensis* based PHC Cóndor® (15%); 5) *Paecilomyces fumosoroseus (*1×10^6 spores per gram); 6) *M. anisopliae* based PHC Metatron® (1×10^6 spores per gram). Antibiograms were performed in six petri dishes containing *E. amylovora* collected from all the selected orchards, each treatment was repeated three times.

The obtention of six purified commercial strains was conducted using serial dilution and two types of agar: nutrient agar and PDA, the same antibiogram procedure employed for

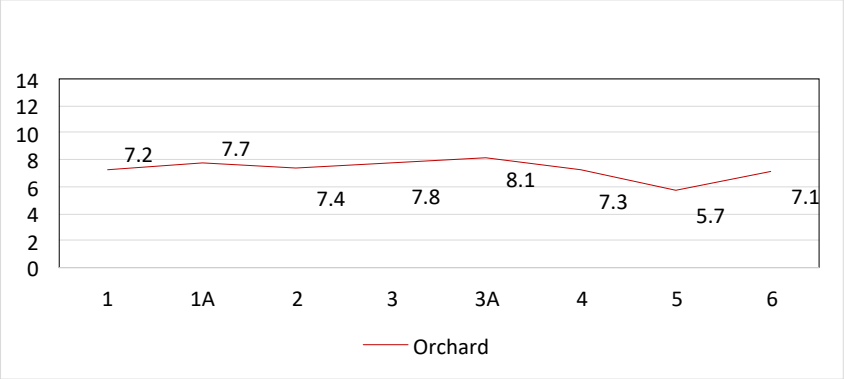

Figure 1. pH values of soil from the selected orchard.

the SC1234 and SC1235 strains was conducted. Finally, bioformulates were produced by blending the rice inoculated with each commercial strain, the spore per gram proportion was measured using a Neubauer chamber, one gram of blended powder was resuspended in 10 mL of water; 1 mL of this solution was used to measure the spore concentration. Field administration of formulations were carried out using one kilogram of each commercial strain´s powder, the formulations were sprayed throughout an area of 2500 m^2, covering approximately 60 trees of each orchard. The reduction of *E. amylovora* was evaluated by comparing the treated trees with the control treated with streptomycin (15%). Field tests were conducted following a randomized complete block design with 10 repeats, taking six 10-year-old trees per repeat. The employed treatments were as follows: T1: Administration of a mixture of strains SC1234 and SC1235 with a concentration of 1.2×10^9 conidia ml^{-1} per hectare and *B. bassiana* with a concentration of 1×10^9 conidia/g per hectare, each element constituting 33% of the solution, T2: Administration of a mixture of equal parts of *P. fumosoroseus*, *M. anisopliae* and *Bacillus thuriengiensis* (1×10^9 conidia/g and 200 liters of water per hectare), T3: Administration of a *Bacillus thuriengiensis* bactericidal strain (1×10^6 spores/g), T4: As a control, Streptomycin 15% was employed.

The experimental design was conducted as follows: $Y = \mu + B_{ij} + T_{ij} + E_{ij}$. Where: Y= Dependent variable, B_{ij}: Blocks or repeats from i to j, T_{ij}: Treatments from i to j, E_{ij}: Experimental error. Variance analysis and comparison of means by MSD was calculated using the statistical software designed by Olivares (1994).

RESULTS

Soil fertility analysis

Consistent physical analyses were performed to determinate pH, electric conductivity, organic matter, apparent density, soluble salts, sand content, clay and silt, as well as texture type. Fertilizer content in soil was determined. Macro elements such as nitrogen, phosphorous, potassium, calcium, magnesium, sulfur and sodium, as well as micro elements such as copper, iron, zinc, boron and manganese were determined. Cationic exchange capacity, SAR and fertility of each present component were also determined.

pH analysis revealed values between 7.2-8.1. *E. amylovora* requires an alkaline environment for its development, the soil from the selected orchards presents a neutral/alkaline pH (Figure 1). Electric conductivity permits to know the quantity of salts that accumulate in the soil after nitrogen, phosphorous, potassium, calcium and magnesium based fertilizers are used. Normal values for apple trees are around 1000 mmhos/cm, electric conductivity above this value can diminish production by up to 30%. Low salt quantities in the soil are an indicator of poorly conducted fertilization procedures. In order to raise the nutritive salt quantities in the soil, calcium and magnesium based salts need to be administered. Calcium sulfate allows better calcium assimilation; sulfur acidifies and solubilizes calcium (Figure 2). Organic matter is an indicator of the degree of soil fertility, the incorporation of crop residues in the fallow and the administration of bacteria capable of nitrogen, phosphorous and potassium solubilization, favor soil organic matter concentration. Optimal concentrations of organic matter for apple tree crops are between 1.6-3%, lower percentages are indicators of poor soil, which is correlated to available nitrogen for plant consumption. The soil from the selected orchards for this study had organic matter concentrations between 1.1 to 1.6 % (Figure 3). The sand, clay and silt contents of the soil allow fertilizers to reach the roots, an ideal percentage of these materials are 50% for sand, 20% for clay, and a 10-30 % of silt. The most part of analyzed soils presented a sandy loam textural class (Figure 4).

Macronutrients

Nitrogen is an integral part of proteins and permits plants to maintain their green color. The nitrogen cycle is based on fixing bacteria, ammonifiers and nitrifiers. Studied orchards exhibited deficiencies of this element; lack of nitrogen is correlated to poor fruit production, since it is responsible for conferring taste and texture. After fulfilling its nitrogen requirement, the plant quickly excretes this element. Allowable

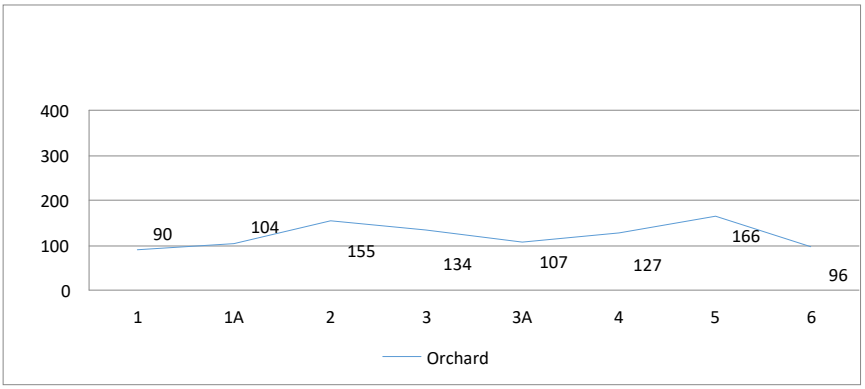

Figure 2. Electric conductivity of soil from the selected orchard (mmhos/cm)

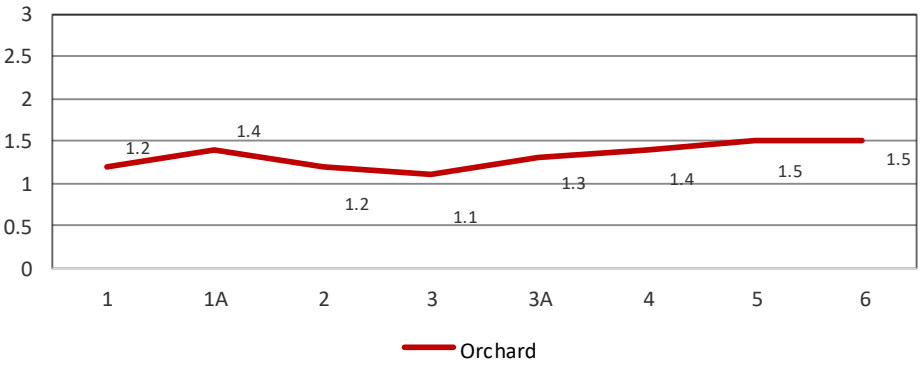

Figure 3. Organic matter of soil from the selected orchard

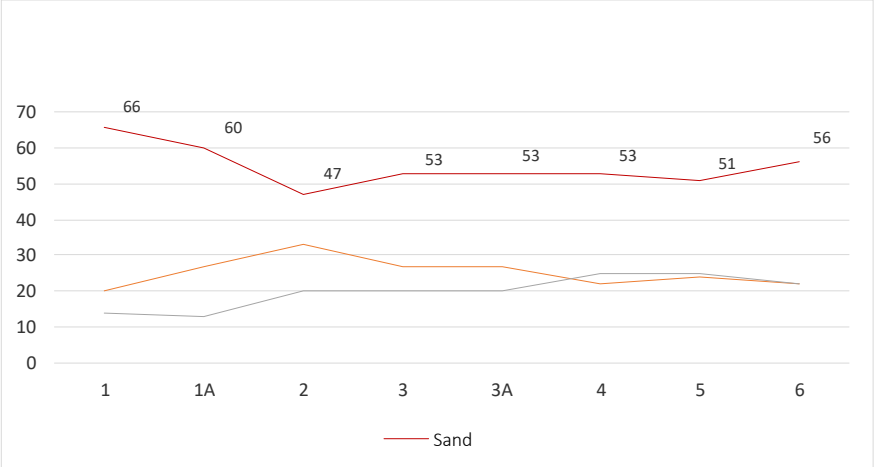

Figure 4. Contents of sand, clay and slit of soil from the selected orchard (%)

limits of fertilization with this element are 0.5 to 1.5 kilograms per tree (Figure 5). Phosphorous is absorbed by plants through the roots and is implicated in keeping the tree healthy; their movement on the ground is slow, it permits the synthesis of carbohydrates within the fruit and is responsible for ATP formation. Plants absorb this element from fertilizers, and after the phosphorous requirement is fulfilled, remnants accumulate in the soil (which can last for many years). Overall, the vast majority of analyzed orchards lacked this element, with the exception of Los Fresnos, which exhibited a proper level of phosphorous accumulation. Potassium, this element is crucial for photosynthesis and growth; it allows the opening of stomata and reduces the presence of diseases in the plant. All the orchards analyzed presented significative potassium deficiencies.

E. amylovora presence was detected in over 70% of studied orchard´s soils. *E. amylovora* requires an alkaline environment to develop; analyzed soils presented a pH value between 7.2 and 8.1, considerably more alkaline than what is considered optimal (6-6.5). Organic matter on the other hand should be in

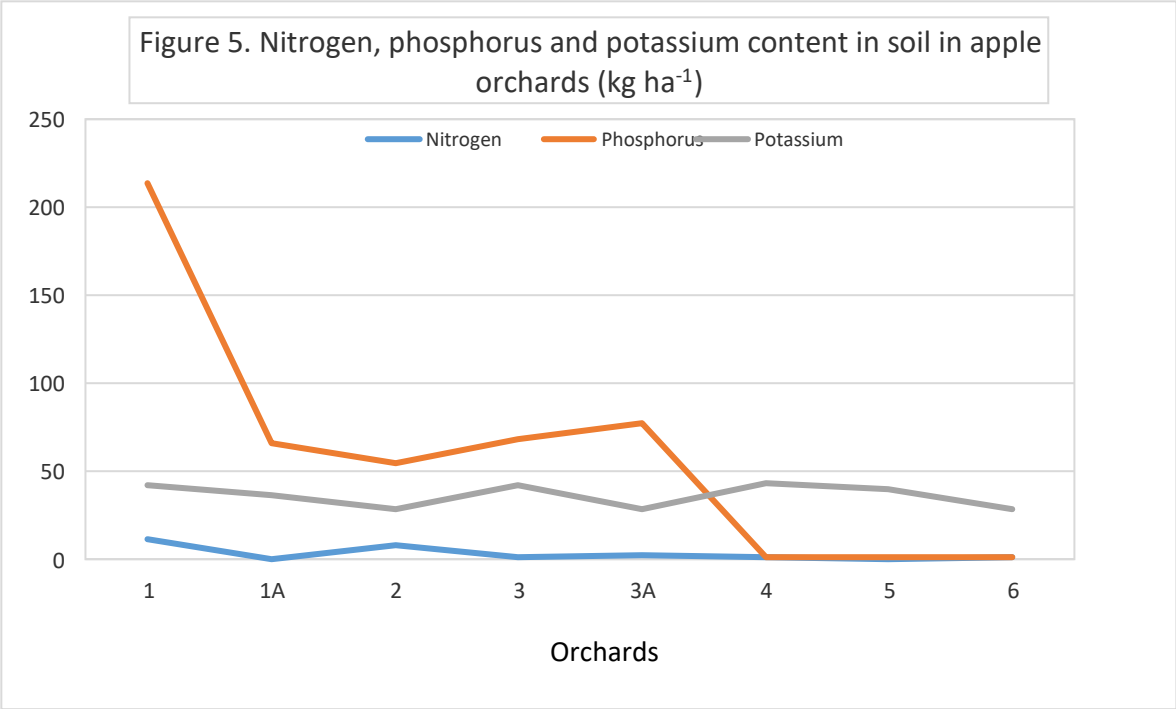

Figure 5. Nitrogen, phosphorus and potassium content in soil in apple orchards (kg ha^{-1})

the 1.6-3% range to be considered optimal; nonetheless, the soil of studied orchards exhibited values between 1.2 and 1.5 %. Nitrogen contents of the studied orchards were 4-12 kg/ha; *E. amylovora* spreads more easily in fields with a nitrogen source during the flowering and irrigation stage. Sanchez et al. (2006) and Oliver et al. (1994) report that in order to obtain good quality fruit and long postharvest preservation, strict quantities of macronutrients had to be administered: 138 nitrogen units ha^{-1}, 45 phosphorous units ha^{-1}, 50 potassium units ha-1, 110 calcium units/ha and 20 magnesium units ha^{-1} (all of these applied six times from the pre-spouting stage to fruit maturation). Producers applied a 60-30-60 fertilizer ratio, employing half of the nitrogen during the beginning of the harvest, and the other half after the floral buds begin to appear. In the case of phosphorous and potassium, they are applied during the sowing season.

Oliver et al. (1994) report that applications of calcium ammonium nitrate equal to 160 kg ha^{-1} during the flowering stage, 80 kg ha^{-1} 40 days after the flowering stage and 160 kg ha^{-1} postharvest, are enough to harvest up to 25 ton ha^{-1} of apples. Apple yields in this study range from 15 to 25 ton ha^{-1}, with no calcium administration. Casierra et al. (2003) worked with apple orchards which presented 239 ppm of iron; 24 ppm of manganese; 3.3 ppm of copper; 6.4 ppm of zinc; 0.65 ppm of boron; and sampling depth of 30-35 cm. The administration of copper sulfate as preventive measure against fungi explains the presence of copper in orchard´s soil (12 ppm of copper approximately), iron contents were lower, with 2.0 ppm of iron approximately. The studied orchards presented severe zinc and boron deficiencies. Rufat and Villar (2015) reported that applied nitrogen quantities to apple orchards should remain below 80 kg ha^{-1} in order to obtain optimal fruit yields. For this study, residual concentrations of these elements were detected below 10 kg ha^{-1}, which correspond to normal consumption quantities trees have through their life time (Figure 5).

Sanchez et al. (2006) reported that apple yields between 26 and 37 ton ha^{-1} can be achieved in sandy loam soils with pH values of 7.6 and organic matter concentrations of 1.5%. The results obtained from this research indicate soil organic matter concentrations between 1.2-1.6%, pH values between 7.2-7.6, soil textures presenting high sand and silt values with low quantities of clay. These characteristics make this soil ideal for apple tree cultivation. Soto et al. (2016) reported that in order to obtain optimal fruit color, at least 130 nitrogen units, 48 phosphorous units, 31 potassium units, 114 calcium units and 20 magnesium units were required (keeping the N-Ca ratio 1:1). To keep an adequate color percentage during postharvest, additional supplements were administered: 80-115 kg ha^{-1} of N, 35-47 kg ha^{-1} of P, 86-102 kg ha^{-1} of Ca, 37-45 kg ha^{-1} of K and 22-230 kg ha^{-1} of Mg. Therefore, postharvest necessities are greater. In the present study, macronutrients were administered during the bud sprouting, flowering and fruit filled stages.

Antibiograms

The results of the *E. amylovora* antibiograms using two different culture media are shown in Table 3. In the *In vitro* results regarding the addition of *M. anisopliae, B. thuriengiensis and B. bassiana* to *E. amylovora*, formation of inhibition zones was only observed in two media: nutrient agar and potato dextrose agar when *B. thuringiensis* was used, exhibiting an inhibition zone of 90%. Strain SC1235 exhibited an inhibition zone with a 50% reduction. SC1234 on the other hand did not present statistically significant results. The results of the antibiograms are shown in Figure 6.

Treatments 1, 2, 3 and 6 exhibited no observable inhibition, treatment 4 exhibited an inhibition of 90%, treatment 5 exhibited a 50% inhibition. Each treatment consisted of the addition of 1 ml of each strain. Correa et al. (2017) conducted assays with diverse concentrations of folic acid (0.3 µg L^{-1} to

Table 3. T1: Ecto-Rhyza, T2: PHS WS, T3: PHC-F, T4: *B. thuriengiensis*, T5: *P. fumosoroseus*, T6: *M. anisopliae*, strain SC 1234 and strain SC 1235.

TREATMENT	NUTRIENT AGAR	POTATO DEXTROSE AGAR
T1	-	-
T2	-	-
T3	-	-
T4	+	+
T5	+	-
T6	-	-
SC 1234	-	+
SC 1235	+	+

(-) no observable inhibition of *E. amylovora (+)* observable inhibition of *E. amylovora.*

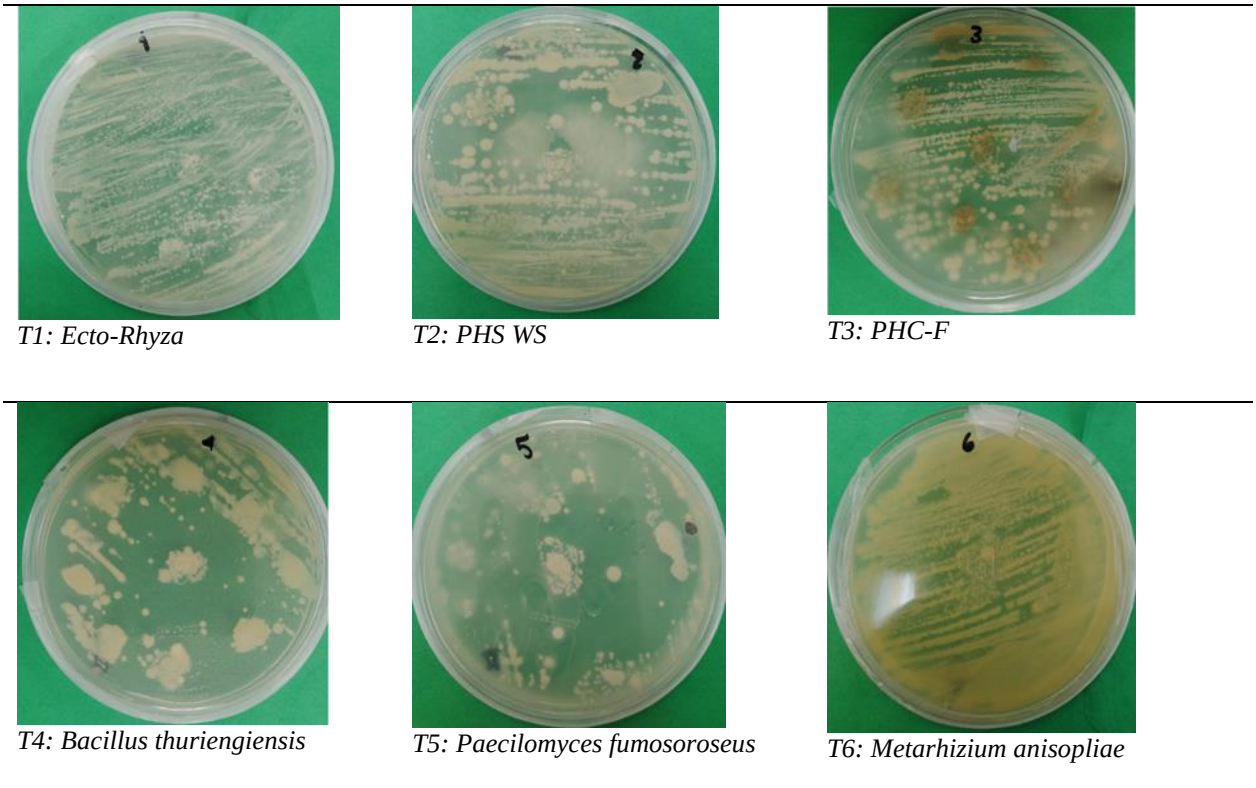

Figure 6. Growth inhibition of *E. amylovora* using six treatments

6,8 µg L^{-1}) for the biochemical characterization of *Erwinia chrysanthemi;* they reported that folic acid increments correlates with inhibition in bacterial growth. León et al. (2013) isolated *E. amylovora* strains from apple trees located in the municipalities of Guerrero and Cuauhtémoc (Chihuahua, Mexico); resistance to streptomycin was reported in 40% (Guerrero) and 24% (Cuauhtémoc) of isolated strains, levels of streptomycin administration oscillated between 200 and ≥1.000 µg ml^{-1}. Administration of streptomycin did not reduce the *in vitro* growth of *E. amylovora* in this report.

Field results

The efficiency in *E. amylovora* reduction after each field treatment is shown in Figure 7. As shown in Figure 10, treatment 3 exhibits a 42% reduction efficiency (corresponding to *B. thuringiensis*, 1×10^6 spores per milligram). Treatment 2 on the other hand, presents a 35.5% reduction efficiency (corresponding to *P. fumosoroseus*, *M. anisopliae* and *B. thuriengiensis*, 1×10^6 spores per mg). These results correspond to the first year of treatment employing direct

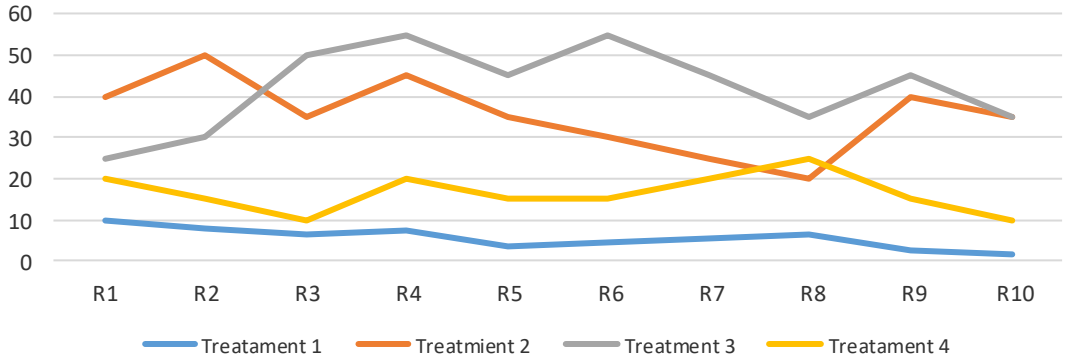

Figure 7. Field results of *E. amylovora* reduction (%) using antibacterial agents

Table 4. ANOVA results for the four treatments

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Calculated F value	P>F
Treatments	3	8410.099609	2003.366455	45.2588	0.000
Blocks	9	346.599609	38.511066	0.6217	0.769
Error	27	1672.400391	61.940754		
Total	39	10429.09960			
CV	31.67				

Table 5. Comparison of means.

Treatment	Mean
T3: Administration of a bactericidal *B. thuringiensis* strain	41.8000[a]
T2: Administration of entomopathonic fungi *P. fumosoroseus* and *M. anisopliae*, as well as *B. thuringiensis* (1×10^6 spores/mg in 240 gr)	35.5000[a]
T4: Administration of a control composed of an Agrimycin and streptomycin mixture.	16.5000[b]
T1: Administration of strains SP 40 and SP 41 (1.2×10^9 conidia ml^{-1} per ha) as well as *B. bassiana* (1×10^9 conidia g^{-1} in 240 g L^{-1} per ha	5.6000[c]
Significance level:	0.05
DMS	7.2224

Statistically, treatments 2 and 3 are similar, but numerically, treatment 3 exhibited 6.3 % higher efficiency.

spraying with a surfactant diluted in water (2 ml L^{-1}). Treatments 1 and 4 did not present any significant result.

Variance analysis

Results regarding the analysis of the four treatments are as follows (Table 4 and 5). The ANOVA revealed statistical significance ($P \leq 0.05$), indicating that at least one treatment presents statistically significant differences with the others. The high coefficient of variation value correlates with the considerable numeric differences among treatment effectiveness.

Brunetto et al. (2015) mention that nitrogen concentration of the fruit is at its peak during the first stage of fruit development (cytokinesis), afterwards, nitrogen concentrations appear to decrease during growth. Excessive nitrogen availability in the soil during the summer appears to delay fruit maturation and reduce tolerance against diseases, such as fire blight and blue mold. Interviewed producers in this study applied small nitrogen doses (below 50 kg ha^{-1}) in the first irrigation before the beginning of spring. Nischwitz (2011) reports that streptomycin is the most effective antibiotic against fire blight disease, nonetheless, in recent years streptomycin-resistant *E. amylovora* strains have been isolated in the United States.

Nischwitz also mentions that oxytetracycline is not very effective for fire blight control. Streptomycin must be administered during the flowering stage when daily mean temperatures are around 16°C. Treatment has to be conducted every 4-5 days during the flowering stage. For this study, Agrimycin, which is an oxytetracycline, streptomycin and tribasic copper sulfate based bactericide, presented no statistical significance for the control of *E. amylovora*.

Romo et al. (2011) employed molecular markers to detect streptomycin resistance in *E. amylovora*, reporting high levels of resistance in strains isolated in Chihuahua Mexico. Tsiantos et al. (2003), on the other hand, reported substantial *E. amylovora* control employing streptomycin in 0.5 g l^{-1} of water. Agrept 25 WP administration was not only preventive, but remedial as well. Kocide® 2000 (77% copper hydroxide) in 0.9 g l^{-1} of water, DM31- Dentamet® (chelated copper and zinc) in 1.5 ml l^{-1} of water, Bactosan (*Pongamia pinnata* extract) in 3.0 g l^{-1} of water and Bion® in 0.1 g/l of water also proved to be effective in the prevention of *E. amylovora*.

Thomson and Gouk (2003) mention that *E. amylovora* growth increases drastically during the first three days of flowering due to physiological and morphological changes of the stigma; bacterial growth within older stigmas is inhibited mainly due to scarcity of nutrients. For this study, no nitrogen sources were applied during the flowering stage. Pusey (2000) reported that *E. amylovora* can be controlled by limiting soil irrigation during the flowering stage and early fructification, as well as when relative humidity is below 55%. Relative humidity values above 60% were detected in the studied orchards, which indicate that producers irrigated the soil during the flowering stage. Miller and Schroth (1972) reported that *E. amylovora* can grow uncomplicatedly in potato dextrose agar. Additionally, they also reported the presence of this bacterium as an epiphyte in stems, leaves and floral organs during spring, but not during winter, where the degree of infection can reach 10^4 to 10^6 per flower. Insects from the genus *Pegamya* and *Minetta* have been associated with *E. amlovora* propagation, with reports of specimens carrying up to 10^1 to 10^5 bacteria per insect. Streptomycin concentrations up to 200 µg mL^{-1} were unable to inhibit the *in vitro* growth of *E. amylovora*.

Concluding remarks and recommendations

Presence of *E. amylovora* was detected in over 70 % of analyzed soils. *E. amylovora* requires an alkaline environment to grow and develop. The soil from analyzed orchards presented a pH value between 7.2 and 8.1, which is far more alkaline than ideal values (6.0 to 6.5). Optimal values for organic matter in apple orchards are between 1.6 and 3.0 %, values below this percentage are indicators of poor soil fertility. Detected values in studied orchards were between 1.2-1.5 %. Soil nitrogen content was calculated between 4-12 kg ha^{-1}, *E. amylovora* propagates more easily in soils with high nitrogen contents during the flowering stage. *In vitro* antibiogram bioassays against *E. amylovora* were conducted using: *M. anisopliae*, *B. thuriengiensis* and *B. bassiana*, with *B. thuringiensis* being the most effective microorganism, forming an inhibition zone between 90-100 %. *E. venezolae* on the other hand formed an inhibition zone of 50%. Regarding the application of antibacterial formulations (1.0 x 10^9 spores g^{-1}), presence of *E. amylovora* was reduced up to 42% in infested apple trees when *B. thuringiensis* was applied. Recurrent use of commercial bactericides such as Agrimicin® and oxytetracycline promote resistance in *E. amylovora* against these substances. pH control using citric acid appears to inhibit the growth of *E. amylovora*; administration of certain soil improvers (bacterial, fulvic acid and plaster based) are highly recommended in order to increase organic matter content.

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