Adv. Hort. Sci., 2021 35(3): 217-231 DOI: 10.36253/ahsc-10706

AHS
Advances in Horticultural Science

Punica granatum L. extract contributes to phytopathogens control and enhances *Eruca vesicaria* (L.) Cav. germination *in vitro* and *in vivo*

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Citation:
DE SOUSA SILVA S., ALVES P.C.S., COUTINHO D.F., LUZ T.R.S.A., FONTOURA G.M.G., BERRETTA A.A., GONÇALVES MACIEL M.C., 2021 - *Punica granatum L. extract contributes to phytopathogens control and enhances Eruca vesicaria (L.) Cav. germination in vitro and in vivo.* - Adv. Hort. Sci., 35(3): 217-231.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 20 March 2021
Accepted for publication 26 May 2021

Key words: antimicrobial, botanical pesticides, food security, plant diseases, pomegranate peel, punicalagin.

Abstract: The study aimed to investigate antimicrobial activity of the hydroalcoholic crude extract from the fruit peel of *Punica granatum* (Pp) and punicalagin compound (Pg) on phytopathogenic bacterial isolates and its potential use as a sustainable alternative in treatment of vegetable seeds. The antimicrobial activity *in vitro* was tested by agar well diffusion assay and through viability tests in liquid medium. *In vivo* treatment with Pp was tested on *Eruca vesicaria* seeds infected with *Xanthomonas campestris* pv. *campestris.* Pp induced the formation of large inhibition zones to the growth of the tested pathogens (35.33 mm - 6.66 mm), with dose-dependent effect. Viability tests confirmed the antimicrobial activity of the Pp on *X. campestris* pv. *campestris* and *P. carotovorum* subsp. *carotovorum* with minimum inhibitory concentration (MIC) of 125 µg/mL. Punicalagin compound presented MIC of the 31.25 µg/mL. The seed treatment with Pp indicated control of pathogen-induced symptoms in seedlings of the *E. vesicaria* and positive effect in seed germination, emergence and in stomatal functionality. The results indicate strong potential of the extract from the fruit peel of *P. granatum* and Punicalagin for formulating botanical pesticides for plant disease control.

1. Introduction

According to Food and Agriculture Organization of the United Nations estimates, by 2050 the world population should exceed 9.5 billion inhabitants, raising the demand for food by up to 60% (FAO, 2016). Plants account for 80% of food ingested in the human diet, providing affordable, safe and nutritious resources for a healthy life. However, pests and diseases pose a threat to food security, due to damage caused to crops that compromises access to food and rises product prices (FAO, 2017).

Phytopathogenic bacteria causes a large number of different plant diseases, some of which are devastating to agricultural crops (Van Der Wolf and De Boer, 2015). *Ralstonia solanacearum* (Smith) (Yabuuchi *et al*., 1995) stands out as one of most destructive pathogens due to the rapid development of wilting symptoms and death of host plants (Yuliar *et al.,* 2015). The pathogen affects a large range of host plants, comprising almost 450 species from 54 different botanical families (Allen *et al.,* 2005).

Direct losses in important crops are estimated in 0 to 91% (tomatoes) and in 33 to 90% in potatoes (Elphinstone, 2005). The bacterium *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson is a vascular (Ryan *et al.,* 2011) and seed-born (Griesbach *et al.,* 2003) pathogen which is distributed worldwide. Infested seeds may emerge in young seedlings infected by the pores on the margin of the cotyledons. This pathogen causes black rot disease, which seriously affects Brassicaceae (Cruciferous) crops (Vicente and Holub, 2013), important food items grown worldwide (Gupta *et al.,* 2013). Finally, *Pectobacterium* is widely studied soft-rot bacterial pathogen causing infections in potato crops and stored tubers, reducing the production and quality of tubers (Adeolu *et al.,* 2016). *Ralstonia solanacearum*, *X. campestris* and *P. carotovorum* have been included among the 10 most important bacterial pathogens of the plants according to their economic and scientific impact (Mansfield *et al.,* 2012).

Control of bacterial diseases in conventional agriculture often uses fast-acting synthetic pesticides and antimicrobials (Kotan *et al.,* 2014). According to national phytosanitary pesticide database (AGROFIT, 2016), substances unsafe to the environment like kasugamycin, cuprous oxide, copper hydroxide and 'extremely toxics' like benzalkonium chloride were registered for control of soft rot *P. carotovorum* subsp. *carotovorum* in potatoes. Pesticides indicated for control of bacterial wilt caused by *R. solanacearum* like Bismerthiazol and Thiodiazole copper have shown low efficacy, high phytotoxicity, harmful environmental effects and bacterial resistance development (Yang and Bao, 2017).

Cultural practices like the use of pathogen-free seeds is recommended to prevent black rot disease in crops (Chitarra *et al.,* 2002). If pathogen-free seed is not available, seed should be treated to eliminate the bacteria. However, seed treatments do not always eliminate 100% of bacteria on or in the seed, and may adversely affect seed germination and vigor (Celetti and Callow, 2002).

The need to reduce chemical pesticide use in crops, associated with demands for healthy food and development of sustainable agriculture, has driven research for natural compounds with low impact on the environment and on people health (Jiménez-Reyes *et al.,* 2019). The secondary metabolism of the plants produces many bioactive compounds that provide protection against pests and pathogens (Borges *et al.,* 2018). Unlike synthetic pesticides, natural compounds exhibit rapid biodegradation after use in the field (Soberón *et al.,* 2014), little or no phytotoxicity, abundant sources and low costs, since they come from a renewable source (Zheng *et al.,* 2016). Thus, medicinal plant uses with antimicrobial activity can be considered an effective component in the integrated management against phytopathogens (Khan *et al.,* 2020).

Punica granatum L. (Pomegranate) is a plant of the Lythraceae family, native from central Asia (northern India to Iran), nowadays cultivated in several parts of the world, including Africa and America (Viuda-Martos *et al.,* 2010; Erkan and Dogan, 2018). The fruit of Pomegranate (called balausta) is a pulp berry formed by a thick and leathery skin with variable color depending on the variety. The seeds are a reproductive structure that present a fleshy outer testa called sarcotesta where the juice is extracted (Melgarejo *et al*., 2020). The production, marketing and consumption of pomegranate fruit have increased rapidly throughout the world in recent years, mainly due to greater awareness of their health-promoting attributes (Selcuk and Erkan, 2015).

The peel of *P. granatum* represents 30 to 40% of the fruit, being usually discarded as waste during industrial processing for the production of pomegranate juice (Gullon *et al.,* 2016). However, this part of the fruit is rich in phenolic acids, tannins (such as punicalin and punicalagin) and flavonoids with vari-

ous biological functions, including activity against pathogenic microorganisms (Dey *et al.,* 2012; Türkyılmaz *et al.*, 2013). Punicalagin compound is an important bioactive agent found in pomegranate fruit peel, with antioxidant, antimicrobial, antiviral and immunosuppressive activity. The compound belongs to the ellagitannin family which includes other tannins such as punicalin and gallic acid, characterized by good water solubility (Akhtar *et al.,* 2015).

In the last years several studies have evidenced the antimicrobial activity of the pomegranate extract against many species of the plant pathogenic fungi suggesting high potential source of natural antifungal agents (Mohamad and Khalil, 2015; Balah and Nowra, 2016; Elsherbiny *et al.,* 2016; Li Destri Nicosia *et al.,* 2016; Rongai *et al.,* 2017; Karm, 2019; El Khetabi *et al.,* 2020). However, few studies have investigated the antimicrobial activity of the pomegranate extract against phytopathogenic bacteria (Quattrucci *et al.,* 2013; Farag *et al.,* 2015; Khaleel *et al.,* 2016). Khaleel *et al.* (2016) have indicated *in vitro* antimicrobial activity of the ethyl acetate pomegranate peel extract against *R. solanacearum*, *P. carotovorum* subsp. *carotovorum* and *X. gardineri* and Farag *et al.* (2015) highlighted notable *in vitro* antimicrobial activities from the methanol pomegranate peel extract against variety of temperate climate (race 3, biovar 2) of the *R. solanacearum*. Despite this, the knowledge about the antimicrobial activity of the hidroalcoholic extract of *P. granantum* and isolate compounds against these pathogens is scarce. Thus, this study aimed to investigate the *in vitro* antimicrobial activity of hydroalcoholic crude extract from the fruit peel of the *P. granatum* and Punicalagin compound on isolates of phytopathogenic bacteria. The potential of the extract for natural control of *X. campestris* pv. *campestris* as an sustainable alternative for treatment of vegetable seeds was assayed.

2. Materials and Methods

Bacterial Isolates

The bacterial isolates were provided by the collection of the São Paulo Biological Institute - São Paulo, Brazil (*X. campestris* pv. *campestris*, Isolate No. Xcc2149) and Rosa Mariano Culture Collection of the Federal Rural University of Pernambuco, Brazil (*R. solanacearum*, Isolate No. CCRMRs187, race 3, biovar 1; and *P. carotovorum* subsp. *carotovorum*, Isolate No. CCRMPcc36). Agar medium of 523 Kado & Hesket was used as a culture medium for maintenance of the microbial cultures before antimicrobial tests.

Hydroalcoholic crude extract from the fruit peel of the P. granatum and pure compound

The hydroalcoholic crude extract from the fruit peel of the *P. granatum* (Pp) was supplied by Apis Flora®. The hydroalcoholic crude extract was concentrated under low pressure, dried and the remainder was later lyophilized. For the experiments, the lyophilized dry residue was diluted in an isotonic phosphate buffered saline (PBS). The Pp was concentrated under reduced pressure. Pure Punicalagin (Pg) compound was purchased from Sigma-Aldrich Brazil Ltda (P0023, 1 mg, Batch: WXBC5016V), ≥98% (HPLC), of pomegranate, $C_{48}H_{28}O_{30}$, molecular weight of 1084.72.

Chemical characterization of the hydroalcoholic crude extract from the fruit peel of the P. granatum

Total phenolic compounds. For determination of total phenolics an analytical curve of tannic acid (Sigma-Aldrich) was carried out. Pomegranate extract was prepared in 50 mL volumetric flask using water as solvent. The samples were homogenized and, the flasks were brought to the ultrasonic bath for 30 minutes. A 0.5 mL aliquot was transferred to another 50 mL flask where 2.5 mL of Folin-Denis reagent and 5.0 mL of 29% sodium carbonate were added. The samples were protected from the light and the readings were performed after 30 minutes in a UV-Vis spectrophotometer at 760 nm (Fernandes *et al*., 2018). All samples were analyzed in triplicate.

Ellagic acid. Ellagic acid (EA) was acquired from Fluka (95.0%, Batch BCBN4398V). The High-Performance Liquid Chromatography (HPLC) grade methanol was supplied by J.T. Baker (Mexico City, Mexico), and purified water was obtained using a Milli-Q Direct Q-5 filter system (Millipore, Bedford, USA). The analytical grade acetic acid was purchased from Synth (Labsynth, Diadema, Brazil).

To determine the ellagic acid content (EAC), the extracts previously diluted in methanol were properly homogenate using a vortex and then remained for 30 minutes in ultrasound bath. The solution was filtered and subjected to HPLC analysis (Shimadzu apparatus equipped with a CBM controller, LC-20AT quaternary pump, a SPD-M 20A diode-array detector and auto sampler, Shimadzu LC solution software,

version 1.21 SP1) using a 100 mm x 2.6 mm Shim pack ODS C18 column.

The mobile phase used for ellagic acid was methanol and acetic acid aqueous solution 2% using a elution gradient (0-7 min, 20-72.5% v/v methanol, 7-7.5 min, 72.5-95% v/v methanol, 7.5-8.5 min. 95% v/v methanol, 8.5-9 min 95-20% v/v methanol, 9-10 min 20% v/v methanol) with a flow rate of 1.0 mL min^{-1}, and oven temperature of 25°C. The eluted samples were detected by UV detector at 254 nm. Calibration curve was constructed by plotting the peak area (y) against concentration in µg mL^{-1} of standard solutions (x). The standard equation obtained from the curve was used for quantification of ellagic acid as mg/g extract of sample. All assays were carried out in triplicates and the ellagic acid quantification was reported.

Determination of antimicrobial activity

The Pp was assayed for antibacterial activity at different concentrations using a standard agar-well diffusion assay (CLSI, 2012). Suspensions of bacteria strains (1.5 x 10^8 CFU/mL) were spread using swabs over the 523 Kado & Hesket agar media in sterilized Petri dishes. Then, wells with a diameter of 6 mm were punched aseptically and 25 µL of Pp at different concentrations were introduced into each well (100, 50, 25, 12.5, 6.25 and 3.125 mg/mL, solubilized in an isotonic phosphate buffered saline (PBS). All plates were incubated at 28°C for 48 hours. Measures of the zones around the wells (mm) were recorded as inhibition zone for Pp. Streptomycin sulfate (500 µg/ml, *P. carotovorum* subsp. *carotovorum*) (Pachupate and Kininge, 2013) and oxytetracyclin hydrochloride (Terramicin®) (30 µg/mL, *X. campestris pv. campestris* and *R. solanacearum*) (Santos *et al.,* 2008) were used as positive control. The isotonic phosphate buffered saline (PBS) was used as negative control. All tests were performed in six replicates.

Minimum inhibitory concentrations (MICs) were performed in 96-well micro-plates (Eloff, 1998) using serial dilutions of Pp (500; 250; 125; 62.5; 31.25 µg/mL) and Pg (250; 125; 62.5; 31.25 µg/mL). Hundred microliters of Pp or Pg diluted in liquid culture medium and the tested microorganism suspensions (1.5 x 10^5 colony-forms unity CFU/well). After incubation (28°C for 24 h), the content of each well was sown in Petri dishes with agar culture medium. The Petri dishes were incubated for 48 hours at 28°C to account for the colony-forms unity (CFU). To indicate viable bacteria cells in the microplate, 10 µL of thiazolyl blue (tetrazolium salt 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) reagent were added to the microplate wells and incubated at 28°C for 1-3 h (Mosmann, 1983). The color change produced in reaction was measured in a spectrophotometer (540 nm) and the values were correlated to the viable bacteria cells in the microplate. MIC was measured as the lowest concentration necessary to inhibit growth of the tested pathogen. Minimum Bactericidal Concentration (MBC) was considered as the minimum concentration in which no growth was visually observed in Petri dishes with solid medium, with 99.99% of eradication of the initial inoculum (De Nova *et al.,* 2019). The concentration of the Pp and Pg that inhibited the growth of half of the inoculum was estimated as the inhibitory concentration 50 (IC_{50}) (Soothill *et al.,* 1992), represented as Log (inhibitor) versus normalized absorbance (%) (dose-response inhibition model). Streptomycin sulfate (500 µg/mL) and oxytetracycline hydrochloride (30 µg/mL) were used as positive control and isotonic phosphate buffered saline (PBS). All tests were performed in four replicates.

Effect of Hydroalcoholic crude extract from the fruit peel of the P. granatum on X. campestris pv. *campestris control in seeds*

Seeds of the *Eruca vesicaria* (L.) Cav. (Feltrin®, cultivated arugula variety, germination from 7 to 10 days) were purchased from a local market. Seeds were disinfected and coated with pathogenic bacteria according standard protocol (Kotan *et al.,* 2014). Seeds coated with pathogen were directly soaked in treatments consisting in: 1 - Pp suspension (500 µg/mL or 250 µg/mL) or 2 - association between Pp (500 µg/mL or 250 µg/mL) and antibiotic (streptomycin sulfate, 500 µg/ml) for 3 hours. The seeds were left to dry on sterile Whatman filter paper sheets overnight in laminar flow hood. The seeds were sown in plastic pots containing garden soil and sand (1:1) totaling thirty seeds per treatment (ten seeds/pot). Other part of the seeds was transferred to Petri dishes with Whatman paper filter placed on the bottom (moistened with 10 mL of sd. H_2O) totaling thirty seeds per treatment (ten seeds/plate). The percentage of germination and seedling emergence was determined 10-12 days after sowing. *E. vesicaria* seedlings were assessed 18 days after emergence to determine the appearance of symptoms of disease (Vicente and Holub, 2013) and survival rate. After this, the seedlings were removed from the substrate for assessment of the effect of the extract in growth promotion. Antibiotic (streptomycin sulfate at 500

µg/ml), disinfected seeds infected with pathogen, and sterilized seeds not infected with pathogen (healthy) were used as controls. All tests were performed in triplicates.

Anatomical analysis

Eruca vesicaria (L.) Cav. seedling samples leaves were preserved in fixative solution FAA (Formaldehyde, Glacial Acetic Acid, 95% EtOH) (Johansen, 1940). To prepare the samples, leaves were sectioned in transverse and paradermic sections using disposable razors. Leaf diaphanization was performed according to the standardized technique (Kraus and Arduin, 1997) and stained with safranin and Astra blue solutions, both at 0.5%. Semi-permanent slides were analyzed with optical microscope to visualize the adaxial epidermis, abaxial epidermis, palisade parenchyma, spongy parenchyma, stomatal density and stomatal morphology. Stomatal density (D) was calculated as D= number of stomata/(40x objective ocular area) (mm^2) (Abdulrahaman *et al.*, 2009). Public domain software ImageJ 1.43a version 64 (Schneider *et al.*, 2012) was used to obtain stomatal measures of the polar diameter (PD) (µm)**,** equatorial diameter (QD) (µm) and stomatal area (A) (μm^2) to each treatment. Stomatal functionality (FUN) was calculated as FUN=polar diameter (µm)/equatorial diameter (µm) of the stomata (De Castro *et al.,* 2009).

Statistical analysis

Results were expressed as the mean ± standard deviations. To determine difference between samples, one-way ANOVA followed by Tukey post hoc test and Student's t test were performed at $p<0.05$. Inhibitory concentration 50 (IC_{50}) was performed by Nonlinear Regression analysis (dose-response inhibition model) with 95% profile likelihood. All analyses were performed in GraphPad Prism® v. 8.0 software.

3. Results

Chemical characterization of the hydroalcoholic crude extract from the fruit peel of the P. granatum

The results demonstrated that Pp possessed 6.34 mg/g of ellagic acid and 0.83 g/g of total phenolic as tannic acid. HPLC chromatogram was performed focused on ellagic acid, and the fingerprint is presented in figure 1.

In vitro antimicrobial activity of the hydroalcoholic crude extract from the fruit peel of the P. granatum

Hydroalcoholic crude extract from the fruit peel of the *P. granatum* (Pp) was tested for its antimicrobial properties against phytopathogenic bacteria. Pp produced bacterial growth inhibition zones for all three investigated isolates (Table 1 and Fig. 2). The highest mean values of inhibition zones were verified for *R. solanacearum*, followed by *X. campestris* pv. *campestris* and *P. carotovorum* subsp. *carotovorum*. There was an increase of the inhibition zone produced as the increase of the *P. granatum* extract con-

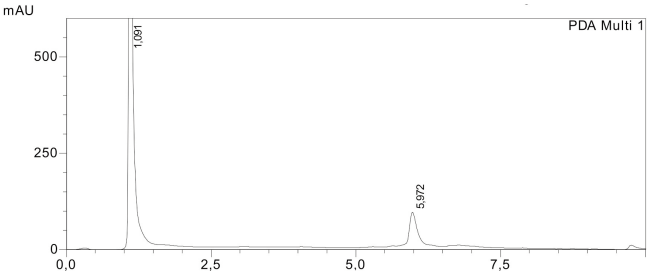

Fig. 1 - HPLC chromatogram of ellagic acid in *Punica granatum* extract (Pp). The extract was characterized considering the content of ellagic acid and the total phenolic as tannic acid by HPLC chromatography performed focused on ellagic acid. The mobile phase used for ellagic acid was methanol and acetic acid aqueous solution [2% - and elution gradient (0-7min, 20-72.5% v/v methanol, 7-7.5 min, 72.5-95% v/v methanol, 7.5-8.5 min. 95% v/v methanol, 8.5-9 min 95-20% v/v methanol, 9-10 min 20% v/v methanol)], flow rate of 1.0 mL min^{-1}, and oven temperature of 25°C -100 mm x 2.6 mm Shim pack ODS C18 column. The eluted samples were detected by UV detector at 254 nm.

Table 1 - *In vitro* inhibition zone produced by treatment with *Punica granatum* L. hydroalcoholic extract (Pp) against isolates of the phytopathogenic bacteria

Concentrations (mg mL^{-1})	Inhibition zones (mm)*		
	R. solanacearum	*X. campestris* pv. *campestris*	*P. carotovorum* subsp. *carotovorum*
Control#	42.83±1.4 a	45.03±0.6 f	35.17±0.75 j
100	35.33±1.2 b	28.83±0.7 g	22.67±0.51 k
50	31.17±2.1 b	26.33±0.8 g	20.00±0.89 k
25	25.67±1.8 c	23.0±1.0 g	16.67±1.03 l
12.5	22.17±2.6 c	19.5±0.5 g	10.17±5.11 m
6.25	16.0±2 d	15.67±1.3 h	ND
3.12	6.66±5.2 e	9.66±4.9 i	ND

Data were represented as mean ± standard deviation for six replications. ND= No detected of inhibition zone. # Control with antibiotics streptomycin sulfate for *P. carotovorum* subsp. *Carotovorum* and oxytetracyclin hydrochloride (Terramicin®) for *X. campestris* pv. *Campestris* and *R. solanacearum*.
The values followed by different letters (a-m) along each column are significantly different according to ANOVA followed by Tukey post hoc test ($p<0.05$).

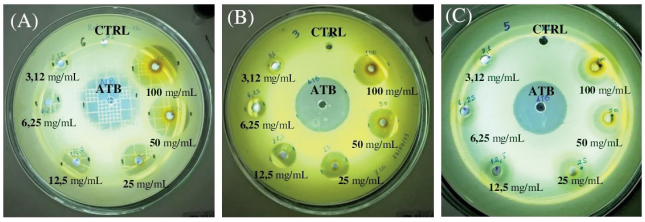

Fig. 2 - *In vitro* antimicrobial activity of *Punica granatum* L. hydroalcoholic extract (Pp) against isolates of phytopathogenic bacteria. Bacterial growth in Petri dishes with solid medium in agar-well diffusion assay. A) R. *solanacearum*; B) *X. campestris* pv. *campestris*; C) *P. carotovorum* subsp. *carotovorum*.
CTRL= negative control with PBS; ATB= positive control with antibiotic.

centration (dose-dependent effect) (Table 1).

In vitro antimicrobial activity through the microdilution test

Microdilution assays indicated susceptibility of the bacterial isolates *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris* to several concentrations of the Pp (Fig. 3 A-E and 4 A-E) and Pg (Fig. 3 B-E and 4 B-E). Were made tests with different concentrations of the Pp (500 to 31.25 µg/mL) to verify cell viability of the bacteria in comparision with negative control and antibiotic. Bacteria in the negative control remained with high celular viability. In the groups treated with Pp in the highest concentrations (500 and 125 µg/mL) there was a reduction of the cell viability of the both bacteria in relation to the negative control (Fig. 3 A-C and 4 A-C). The antimicrobial effect of the Pp in concentrations of 500 and 250 µg/mL for *X. campestris* pv. *campestris* was similar to antibiotic and different of the negative control (Fig. 4 A-B). Concerning to *P. carotovorum* subsp. *carotovorum*, the antimicrobial effect of the Pp in concentration of 500 µg/mL was similar to antibiotic and different of the negative control (Fig. 3 A).The smallest concentrations of the Pp (62.5 and 31.25 µg/mL) did not produce any antimicrobial effect in cell viability

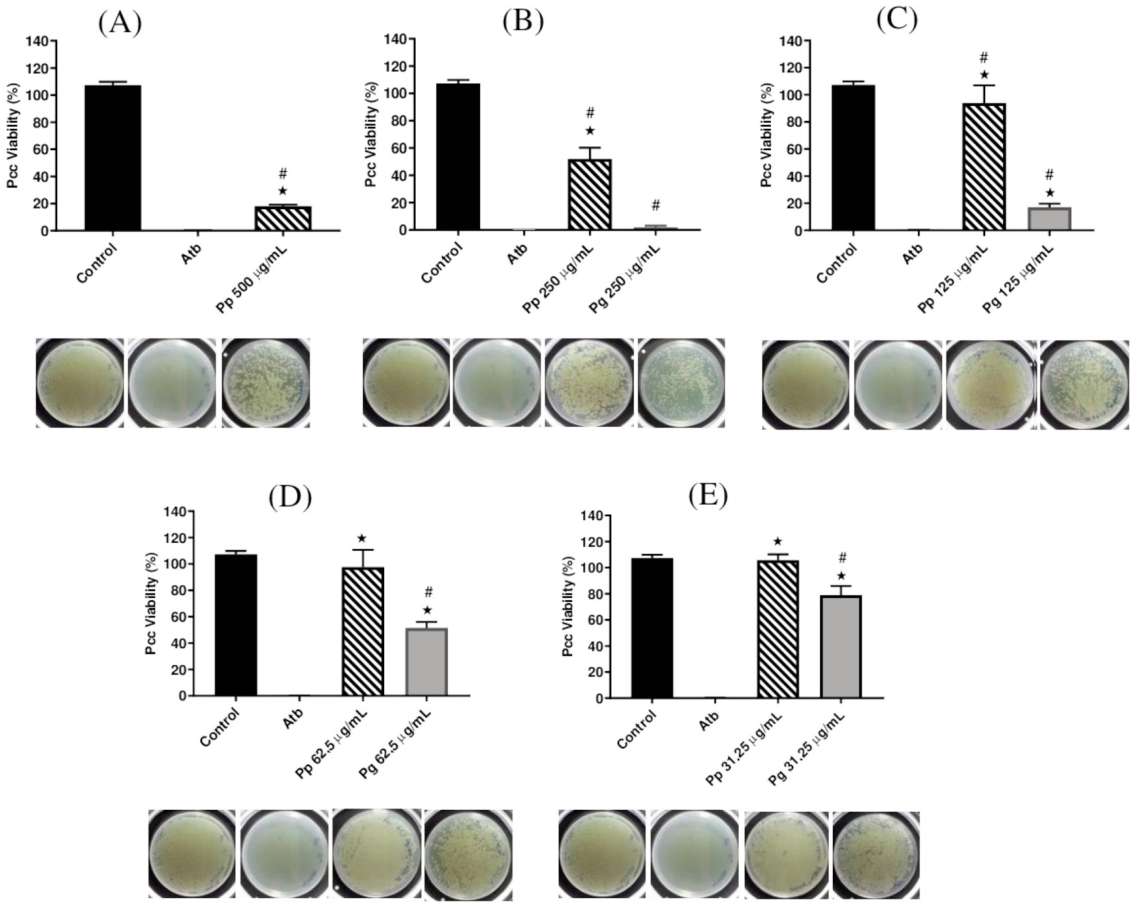

Fig. 3 - *In vitro* inhibition rates of the *P. carotovorum* subsp. *carotovorum* cell viability (%) for treatments with *Punica granatum* L. hydroalcoholic extract (Pp) and Punicalagin compound (Pg). Each graph was accompanied of bacterial growth in Petri dishes with agar solid medium. (*) significant difference between treatments (Pp or Pg) and antibiotic (Atb); (#) significant difference between treatments (Pp or Pg) and negative control (Control) ($p<0.05$, ANOVA followed by Tukey post hoc test). Each bar represents mean ± SD.

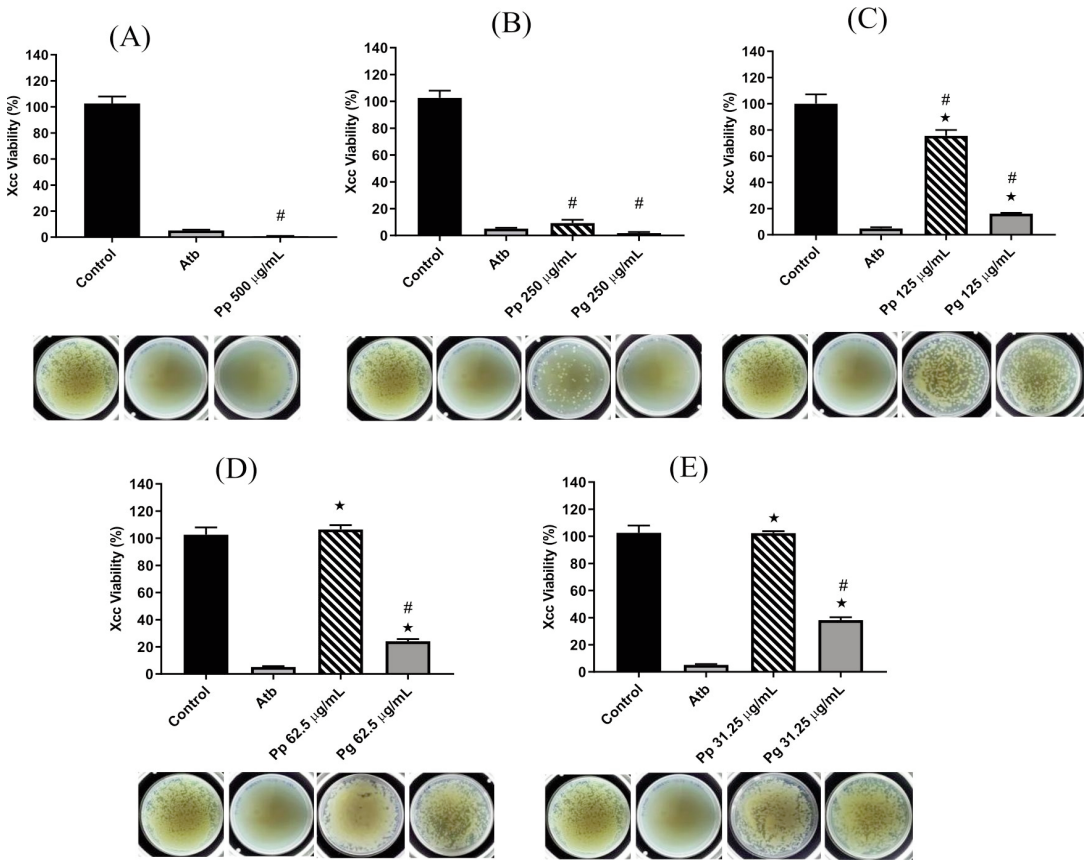

Fig. 4 - *In vitro* inhibition rates of *X. campestris* pv. *campestris* cell viability (%) for treatments with *Punica granatum* L. hydroalcoholic extract (Pp) and Punicalagin compound (Pg). Each graph was accompanied of bacterial growth in Petri dishes with agar solid medium. (*) significant difference between treatments (Pp or Pg) and antibiotic (Atb); (#) significant difference between treatments (Pp or Pg) and negative control (Control) ($p<0.05$, ANOVA followed by Tukey post hoc test). Each bar represents mean ± SD.

for these both pathogens (Fig. 3 D-E and 4 D-E).

Antimicrobial activity of the Pg against the investigated pathogens was higher than action of the Pp. The lowest concentration of the Pg that inhibits bacterial growth (MIC) to both *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris* was 31.25 µg/mL (Fig. 3E and 4E). Punicalagin compound at the highest tested concentration (250 µg/mL) showed antibiotic-like antimicrobial activity, in terms of the cellular viability of the both pathogens (Fig. 3B and 4B). The observation of bacterial growth in culture plates with agar medium indicated that Pg in this concentration (250 µg/mL) may present bacteriostatic action for *P. carotovorum* subsp. *carotovorum* (Fig. 3B) or bactericidal action to *X. campestris* pv. *campestris* (Fig. 4B).

Regarding to the concentration that inhibits 50% of bacterial inoculum (IC_{50}) Pp presented a concentration of 212 µg/mL to *P. carotovorum* subsp. *carotovorum* (Fig. 5A) and 154.6 µg/mL to *X. campestris* pv. *campestris* (Fig. 5B). The concentration of the Pg compound that inhibits 50% of bacterial inoculum (IC_{50}) was 58.96 µg/mL to *P. carotovorum* subsp.

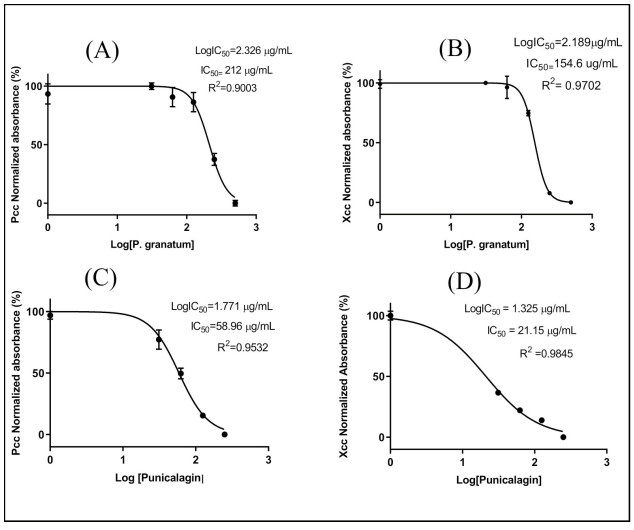

Fig. 5 - Inhibitory concentration 50 (IC50) of the treatments with *Punica granatum* L. hydroalcoholic extract (Pp) and Punicalagin compound (Pg) against isolates of the phytopathogenic bacteria. A) and B) represent inhibitory effect of the treatment with Pp on *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris*. C) and D) represent inhibitory effect of the treatment with Pg on *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris* by the Nonlinear Regression (dose-response inhibition model) with 95% profile likelihood.

carotovorum (Fig. 5C) and 21.15 µg/mL to *X. campestris* pv. *campestris* (Fig. 5D).

In vivo antimicrobial and biostimulant activity of the hydroalcoholic crude extract from the fruit peel of the P. granatum in E. vesicaria seeds infected by X. campestris pv. campestris

The most effective concentrations of the Pp in microdilution assays (500 µg/mL and 250 µg/mL) were tested for the control of the *X. campestris* pv. *campestris* in *E. vesicaria* seeds. Infected and untreated seeds (negative control) presented a lower emergence percentage compared to healthy seeds. On the other hand, treatment of infected seeds with Pp (500 µg/mL) promoted an increase of the 15% in the percentage of emergence in relation to the negative control (Table 2).

In addition, *E. vesicaria* seedlings treated with Pp did not develop main symptom of black rot disease caused by *X. campestris* pv. *campestris* (the "V" chlorotic lesion in the margin of the leaflet). This symptom was verified in seedlings of infected and untreated seeds. There was no phytotoxic effect of the Pp in seedlings development. Seedlings of the *E. vesicaria* treated with highest concentration of the Pp (500 µg/mL) showed a biggest growth length of radicle (Fig. 6). Treatment with streptomycin sulfate crude or associated with Pp resulted in seedlings with

Table 2 - *In vivo* activity of the *Punica granatum* L. hydroalcoholic extract (Pp) in germination and emergency of the *Eruca vesicaria* L. (Cav.) seeds infected with *X. campestris* pv. *Campestris*

Treatment*	Concentration (µg/mL)	Germination (%)	Emergency (%)
Health#	-	93.3±5.1 a	80±0.0 b
Ctrl-	-	93.3±5.1 a	45±5.7 c
Ctrl+	500	93.3±10.33 a	65±5.7 d
Pp	500	100±0.0 a	60±11.55 d
	250	93.3±5.1 a	45±5.7 d
Pp + Atb	500 + 500	93.3±5.1 a	60±11.55 d
	250 + 500	86.6±10.3 a	60±11.55 d

Health= seeds uninfected with pathogen;*Ctrl- = negative control (seeds infected and untreated); Ctrl+ = positive control (seeds infected treated with streptomycin sulfate); Pp= seeds infected treated with *Punica granatum* L. hydroalcoholic extract (Pp) at concentrations of 500 µg/mL and 250 µg/mL; Pp + Atb= association between Pp (500 µg/mL or 250 µg/mL) and antibiotic streptomycin sulfate (500 µg/mL). In each column, values followed by different letters (a-d) are significantly different according to ANOVA followed by Tukey post hoc test ($p<0.05$).

Fig. 6 - Growth length of radicle of the *Eruca vesicaria* L. (Cav.) seedlings treated with *Punica granatum* L. hydroalcoholic extract (Pp). (A) seedlings of healthy seeds; (B) seedlings of infected with *X. campestris* pv. campestris and untreated seeds; (C) seedlings of seeds infected and treated with streptomycin sulfate; (D) seedlings of seeds infected and treated with Pp (500 µg/mL); (E) seedlings of seeds infected and treated with Pp (250 µg/mL); (F) seedlings of seeds infected and treated with association between Pp (500 µg/mL) and streptomycin sulfate.

Fig. 7 - Appearance of *Eruca vesicaria* L. (Cav.) seedlings germinated in Petri dishes and in pots with different treatments. Healthy seeds – uninfected seeds; Seeds with antibiotic: seeds infected with *X. campestris* pv. *campestris* and treated with streptomycin sulfate; Seeds with pomegranate extract: seeds infected and treated with *Punica granatum* L. hydroalcoholic extract (Pp) (500 µg/mL).

chlorosis symptom (yellowish leaves) (Fig. 7).

The results of this study showed different seedling survival rates of the *E. vesicaria* according to each treatment (Fig. 8A-D). Infected and untreated seedlings (negative control) showed an abrupt drop in the percentage of survival at the 7th day after emergence. Seedlings treated with Pp at the highest concentration (500 µg/mL) showed slowly decrease

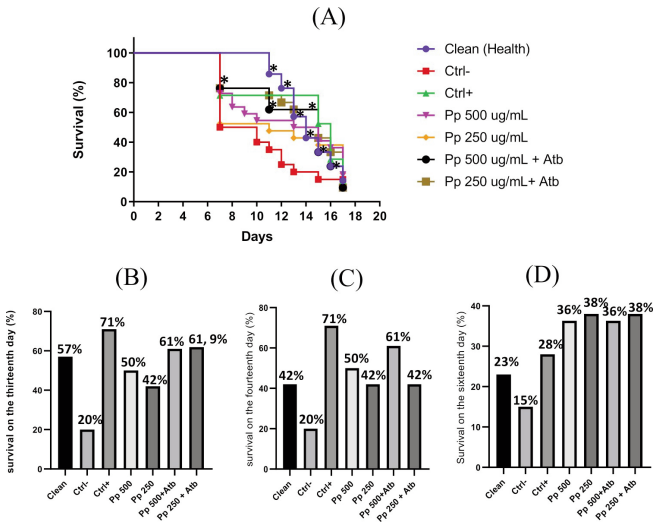

Fig. 8 - *In vivo* effect of the treatment with *Punica granatum* L. hydroalcoholic extract (Pp) in survival rates of *Eruca vesicaria* L. (Cav.) seedlings germinated in pots. Clean (health)= seeds uninfected with *X. campestris* pv. *campestris*; Ctrl- = infected and untrated seeds; Ctrl+= seeds infected and treated with streptomycin sulfate; Pp 500 µg/mL and Pp 250 µg/mL= Seeds infected and treated with *Punica granatum* L. hydroalcoholic extract (Pp); Pp 500 µg/mL + Atb or Pp 250 µg/mL + Atb= seeds infected and treated with association between *Punica granatum* L. hydroalcoholic extract (Pp) and streptomycin sulfate. A) total survival rates; B) survival rates in thirteenth day; C) survival rates in fourteenth day; D) survival rates in sixteenth day. Means with (*) are different from the negative control by the Student's t test at p<0.05.

in the percentage of survival in the same period (Fig. 8A). On the 13th day after emergence, the survival rates of the seedlings treated with Pp (500 µg/mL and 250 µg/mL) were 50% and 42%, respectively, versus 20% of the negative control (Fig. 8B). Association between Pp (500 µg/mL and 250 µg/mL) and antibiotic also promoted higher survival rates in relation to negative control in the same period (61% and 61.9%, respectively) (Fig. 8B). After 16 days of seedling emergence there were similar rates for treatment with Pp (500 µg/mL and 250 µg/mL) and treatment with association between Pp and antibiotic. These percentages remained high (36%-38%) in relation to negative control (15%) (Fig. 8D).

Anatomical analyses of *E. vesicaria* seedling leaves indicated differentiation of mesophilic structures, especially in relation to the palisade parenchyma (Fig. 9A-F). Uninfected (healthy) seedlings presented well-preserved anatomical structures (Fig. 9A). Seedlings of the infected and untreated seeds group (negative control) showed some alterations in mesophilic tissue, especially in relation to incomplete differentiation of palisade parenchyma, when compared to healthy plants (Fig. 9B). Seedlings in the group previously treated with streptomycin sulfate presented altered palisade parenchyma cells in a more rounded shape (Fig. 9C). Seedlings treated with Pp at a concentration of 500 µg/mL showed clear differentiation of mesophilic structures, with well-structured palisade parenchyma (Fig. 9D). Seedlings treated with Pp at a concentration of 250 µg/mL did not present clear differentiation of mesophilic elements (Fig. 9E).

Appearance of stomata of seedlings in different treatments with Pp was represented in figure 10 (A-F). From these images, the mean values of the fol-

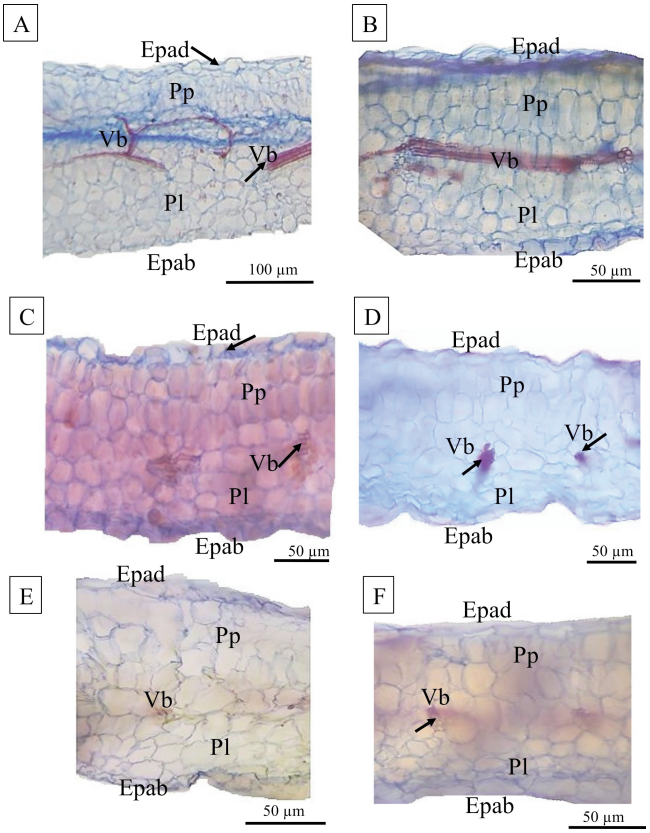

Fig. 9 - Cross sections of the mesophyl of *Eruca vesicaria* L. (Cav.) seedlings infected with the *X. campestris* pv. *campestris* submitted to different treatments. A) Uninfected seedlings; B) seedlings infected with *X. campestris* pv. *campestris* and untrated; C) seedlings infected and treated with streptomycin sulfate; D) Seedlings infected and treated with *Punica granatum* L. hydroalcoholic extract (Pp) (500 µg/mL); E) Seedlings infected and treated with *P. granatum* L. hydroalcoholic extract (Pp) (250 µg/mL); F) Seedlings infected and treated with association between *P. granatum* L. hydroalcoholic extract (Pp) (500 µg/mL) and streptomycin sulfate.
Epad= adaxial epidermis; Abed= abaxial epidermis; Lp= lacunous parenchyma; Pp= palisade parenchyma; Vb= vascular bundle.

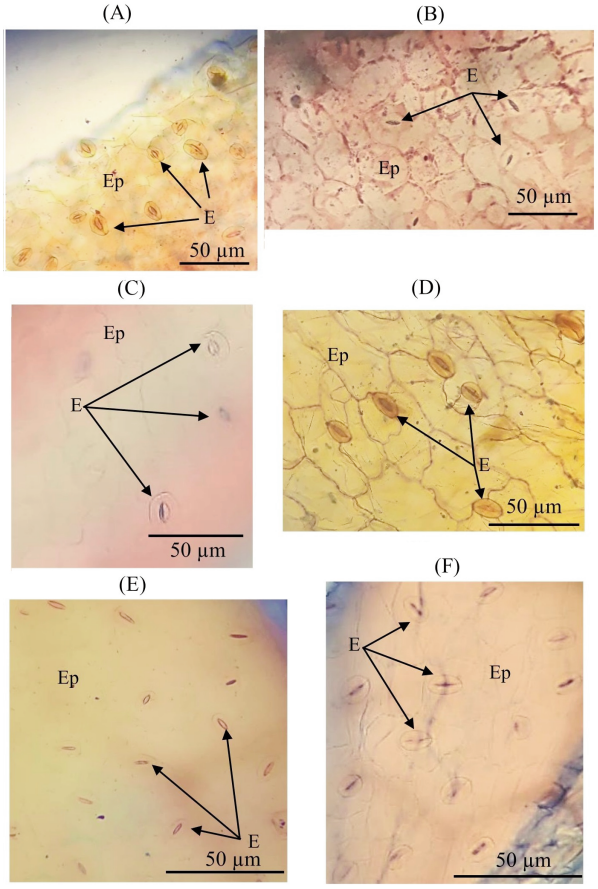

Fig. 10 - Paradermic sections of the leaf blade of *Eruca vesicaria* L. (Cav.) seedlings submitted to different treatments. A) Uninfected seeds; B) seeds infected with *X. campestris* pv. *campestris* and untrated; C) seeds infected and treated with streptomycin sulfate; D) seeds infected and treated with *Punica granatum* L. hydroalcoholic extract (Pp) at concentration of 500 µg/mL; E) seeds infected and treated with *P. granatum* L. hydroalcoholic extract (Pp) at concentration of 250 µg/mL; F) seeds infected and treated with association between *P. granatum* L. hydroalcoholic extract (Pp) (500 µg/mL) and streptomycin sulfate. St= stomata; Ep= epidermis.

lowing parameters were taken: polar diameter (PD), equatorial diameter (QD), stomatal functionality (FUN), area (A) and stomatal density (SD) (Table 3). Seedlings of the group treated with Pp (500 µg/mL) showed highest values of PD, FUN and A (µm²) in comparison to negative and positive control (Table 3). Stomatal functionality of the group treated with Pp (500 µg/mL) was similar to that of healthy seedlings. The highest mean of SD was verified for the group treated with Pp at a concentration of 250 µg/mL, being statistically similar to the clean group. Seedlings of the infected and untreated group showed smallest values of PD, FUN and SD. Small values of PD and FUN were observed too in seedlings treated with antibiotic (Table 3).

4. Discussion and Conclusions

In the present study, the effective antimicrobial action of the *P. granatum* fruit peel extract and its isolated punicalagin compound on phytopathogenic bacteria (*R. solanacearum*, *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris*), listed among the ten most important species in scientific and economic aspects worldwide (Mansfield *et al.,* 2012), was shown . The wide zones of inhibition of bacterial growth in agar-diffusion tests and marked reduction in the percentage of the cell viability in broth microdilution assays indicate high potential of pomegranate extract in the control of these phytopathogens.

Pomegranate peel has substantial amounts of phenolic compounds, such as hydrolysable tannins (punicalin, punicalagin, ellagic acid, and gallic acid), flavonoids (anthocyanins and catechins), and nutri-

Table 3 - Stomatal measures in paradermic sections of leaves of the *Eruca vesicaria* L. (Cav.) submitted to different treatments

Treatments	Stomatal measures *				
	PD	QD	FUN	A (µm²)	SD (mm²)
Health	25.2±4.5	14.9±1.07 bcdef	1.71±0.3 c	302.5±21.3 def	77.7±9.8 bcdf
Ctrl-	22.9±2.3 d	18±1.2 a	1.28±0.1 ad	348.5±36.8 df	29.3±13.7 acdef
Ctrl+	21.5±2.9 df	18.2±0.8 a	1.18±0.1 ade	270±19.6 d	46.9±5.4 abed
Pp 500 µg/ml	29.6±4 bcef	17.6±1.9 a	1.69±0.2 bcef	483±41.1 abce	49.1±7.6 ab
Pp 250 µg/ml	22.9±1.4 d	16.7±1 a	1.37±0.01 cd	335±19.7 ad	87.2±15.8 bcd
Pp 500 µg/ml +Atb	26±2.3 c	19.4±3.6 a	1.36±0.1 cd	449.8±49.2 ab	51.3±5.7 ab

Health= uninfected seedlings; Ctrl- = infected and untreated seedlings (negative control); Ctrl+ = seedlings infected and treated with streptomycin sulfate (positive control); Pp 500 µg/mL and Pp 250 µg/mL= seedlings infected and treated with *P. granatum* L. hydroalcoholic extract (Pp) at concentration of 500 µg/mL or 250 µg/mL; Pp 500 µg/mL + Atb= seedlings infected treated with *P. granatum* L. hydroalcoholic extract (Pp) at concentration of 500 µg/mL in association with antibiotic streptomycin sulfate (500 µg/mL).

* Data were represented as mean ± standard deviation for three replications. PD= polar diameter (µm); QD= equatorial diameter (µm); FUN=- stomatal functionality; A= stomatal area; SD= stomatal density (mm2). In each column, values followed by different letters (a-f) are significantly different according to Student's t test ($p<0.05$) where: a- comparison with health; b-comparison with Ctrl-; c-comparison with Ctrl+; d-comparison with Pp 500 µg/ml; e-comparison with Pp 250 µg/ml; f-comparison with Pp 500 µg/ml +ATB.

ents, which are responsible for its biological activity (Magangana *et al.,* 2020). The fruit peel has high antioxidant and antimicrobial activities and may be used as an excellent natural additive for food preservation and for quality enhancement. The health-promoting benefits of pomegranate peel have prompted the food industry to focus on pomegranate-peel-containing food preparations, which include nutraceuticals, phenolic-enriched diets, and food supplements (Opara *et al.,* 2009; Fawole *et al.,* 2012).

The Pp showed both bacteriostatic (*P. carotovorum* subsp*. carotovorum,*) and bactericidal effect (*X. campestris* pv*. campestris*). Bactericidal effect is desirable in order to inhibit the emergence of resistant bacterial strains and toxicity (Soberón *et al.,* 2014). The study results support literature data on the antimicrobial action of the ethanolic *P. granatum* fruit peel extract against phytopathogenic bacteria *Pseudomonas syringae* pv*. tomato*, the cause of bacterial spot disease in tomatoes, with bacterial growth inhibition zones of 5-26 mm and dose-dependent effect (Quattrucci *et al.,* 2013). Additionally, the results agreement with studies that reported antibacterial action of the methanolic pomegranate peel extract against *R. solanacearum*, with growth inhibition zone of the 13.9 mm (50 mg/mL), and ethyl acetate extract against *R. solanacearum*, *P. carotovorum* subsp. *carotovorum* and *X. gardneri* with inhibition zone of 8.5-22.75 mm (concentrations of 25-200 mg/mL) (Farag *et al.,* 2015; Khaleel *et al.,* 2016). Studies with extracts from other parts of the plant (leaf and seed) have shown more discrete antimicrobial activity on *R. solanacearum* and *X. campestris* (Hassan *et al.,* 2009; Uma *et al.,* 2012).

Antimicrobial activity of the *P. granatum* fruit peel extract against Gram-negative and Gram-positive bacteria has been correlated with the presence of polyphenolic compounds in it, mainly punicalagin (Gullon *et al.,* 2016). A relatively high amount of polyphenols (867 mg/g) was detected in a pomegranate peel extract preparation, especially the ellagitannin punicalagin (296 mg/g), with antimicrobial action of the extract on isolates of *S. aureus, Escherichia coli, Aspergillus niger* and *Saccharomyces cerevisiae* (Ibrahium, 2010). The antimicrobial mechanism of action of polyphenols seems to be related to the direct action of these compounds on the bacterial cell wall by formation of complexes with wall proteins, causing lysis (Akhtar *et al.,* 2015). The interaction of these compounds with sulfhydryl groups of extracellular microbial proteins results in inhibition of protein activity (Dey *et al.,* 2012).

The impressive antimicrobial action of punicalagin on *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris* verified in the present study suggests that this molecule may be an essential component in the biological activity of *P. granatum* fruit peel extract against phytopathogenic bacteria. Several studies prove antimicrobial activity of the punicalagin compound against isolates of clinical importance such as *Staphylococcus aureus* (MIC 250 µg/mL) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Xu *et al.,* 2017; Mun *et al.,* 2018). According to Xu *et al.* (2017) punicalagin compound has direct action on cell membrane disruption, increased K^+ ion flow and inhibition of biofilm formation in *S. aureus*. To the best of our knowledge, this is the first report of the antibacterial activities of the isolated compound punicalagin against plant pathogenic bacteria. Further investigations may elucidate the mechanism of action of this compound on phytopathogenic bacteria.

Data from the *in vivo* assays of this study indicated that Pp is effective in control of *X. campestris* pv. *campestris* in *E. sativa* seeds. Black rot infection causes tissue necrosis, premature leaf fall, atrophied growth and death of young plants (Vicente and Holub, 2013). In the present study, treatment with Pp in the highest concentration reduced incidence of disease symptoms and promoted a high survival rates of seedlings in comparison to infected and untreated group. Additionally, the association between Pp and antibiotic resulted in percentages of seedling survival above that observed in treatment with antibiotic alone (16^{th} day of observation), indicating probable synergistic interaction between treatments. *P. granatum* extract produced no harmful effect on germination, emergence or seedling development of the *E. sativa*. This is in agreement with a study that proved the effective action of treatment with natural plant extract (*Origanum onites*) in the control of *Clavibacter michiganensis* ssp*. michiganensis*, *Xamthomonas axonopodies* pv*. vesicatoria* and *Xanthomonas campestris pv. vitians* in seeds of tomato and lettuce, without affecting seedling germination and growth (Kotan *et al.,* 2014). Treatment of the seeds with hot water (50°C for 20-30 min) has been the most effective treatment for seedborn blackrot control. However, treatments do not always eliminate 100% of the bacteria and may adversely affect seed germination and vigor (Celetti and Callow, 2002). Natural plant extracts, like pomegranate fruit

peel extract, may represent a good alternative to control of seed born phythopatogens ensuring seed viability after treatment.

Treatment of *E. vesicaria* seeds with streptomycin sulfate, despite being effective in controlling *X. campestris* pv. *campestris*, demonstrated toxic effect for seedlings, evidenced by the yellowish aspect of the leaves (chlorosis). This result corroborates the findings of Napoles *et al.* (1991) for treatment of *Brassica oleracea* seeds with the same antibiotic (500 ppm for 1 h). The streptomycin is associated with several phytotoxic effects like blocking chlorophyll synthesis, especially in younger leaves, inhibition of methionine and phosphate absorption, production of photosynthetic process changes and enzymatic inhibition (Falkiner, 1990). In addition, treatment of seeds with streptomycin resulted in severe reduction in the ratio between the polar and equatorial diameters (stomatal functionality). However, treatment with Pp (500 µg/ml) keep values of stomatal functionality (FUN) similar to the healthy seedlings. The highest ratio of FUN indicates stomata with more elliptic morphology, a mechanism of the drought tolerant plants to keep the water present in its interior at a maximum as a response to its hydric state (Melo *et al*., 2014).

Treatments with Pp indicated antagonistic values of stomatal density (SD) and stomatal area (A). The parameters SD and A can directly affect mechanisms such as photosynthesis, transpiration and efficient water use in plants (Lawson and Blatt, 2014). The increase in SD coupled with reduction in A may result in the optimization of gas exchange (Franks *et al.,* 2009). This pattern was observed in seedlings treated with Pp at a concentration of 250 µg/ml and similarly in the health group. On the other hand, the reduction in SD may represent a more conservative water use (Bertolino *et al.,* 2019). This pattern was verified in seedlings treated with Pp at a concentration of 500 µg/ml. Since there is no damage to CO_2 fixation or plant cooling, this reduction in water loss can be advantageous in environments with low water availability (Bertolino *et al.,* 2019). Thus, the Pp can influence both pathogen control in seeds and the physiological characteristics of the plant through changes in stomatal patterns.

In conclusion, hydroalcoholic crude extract from the fruit peel of the *P. granatum* (Pp) demonstrated high potential for control of the phytopathogenic bacteria *R. solanacearum*, *X. campestris* pv. *campestris* and *P. carotovorum* subsp. *carotovorum*. Punicalagin compound potentiated antimicrobial activity on these pathogens, corroborating studies that relate the abundance of the phenolic compounds (flavonoids and tannins) with the antibacterial activity of pomegranate fruit peel. Pp extract was effective to control seedborn pathogen *X. campestris* pv. *campestris* in seeds of the *E. vesicaria* and promoted several beneficial effects to seedlings with no phytotoxic effect. Moreover, association between Pp and antibiotic indicated probable synergistic interaction between treatments (16th day of observation) potencializing the seedling survival over the observed in relation of the antibiotic utilizing in isolated way.

The investigation of antimicrobial activity of the Pp and isolate compounds like Punicalagin represents a promising path regarding the biotechnological development of botanical pesticides that ensure quality and safe of the food crop production.

Acknowledgements

The Coordination for the Improvement of Higher Education Personnel (CAPES) primarily funded this work - [Finance code 001].

This work was supported by the Foundation for Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão (FAPEMA) - Brazil.

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