



Pomegranate peel extract as a sustainable plant protection agent against *Xanthomonas campestris* pv. *campestris*: mechanisms and applications

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Abstract

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), poses a significant threat to crucifer crops worldwide. This study investigates pomegranate peel extract (PGPE) as a sustainable and eco-friendly alternative to chemical control for managing Xcc. PGPE, rich in bioactive polyphenols, demonstrated strong antimicrobial activity, inhibiting bacterial growth, motility, and biofilm formation under in vitro conditions. In vivo applications of PGPE, both as a foliar spray and seed coating, effectively reduced black rot symptoms and controlled seed-borne infections, with higher concentrations showing efficacy comparable to copper-based and hot-water treatments, respectively. Importantly, PGPE exhibited no phytotoxicity, maintaining plant health and seed germination. The use of PGPE not only provides an environmentally safe alternative to synthetic bactericides but also promotes the valorisation of agro-industrial waste, aligning with sustainable principles. These findings highlight PGPE as a promising tool for effective black rot management, paving the way for broader applications in plant protection.

Keywords Plant-based antimicrobial agent · Black rot · Anti virulence therapy

Introduction

Xanthomonas campestris pv. *campestris* (Xcc) is a rod shaped, gram-negative plant pathogenic bacteria that causes black rot, a major disease of crucifer crops. Black rot was first reported in late 1800s and studied in detail by Russell (1898) and is now causing severe crop losses worldwide (Vicente and Holub 2013). The ability of Xcc to infect wide range of hosts along with its efficient infection,

transmission and persistence mechanisms make it one of the most notorious elements for crucifer industry (Vicente and Holub 2013). The primary symptoms are V-shaped chlorotic lesions which start from leaf margins and extend towards midrib and can lead to systemic infection causing serious yield losses (Shigaki et al. 2000). In severe cases, the entire crops may render unmarketable and significantly impact economy.

The transmission of Xcc is primarily seed-borne but may occur through contaminated water, wind-driven rain splashes, infected plant debris and carried-over by wild plant hosts (Vicente and Holub 2013). Once introduced into a field, Xcc may persist in plant-debris and subsequently in soil even after the complete decomposition of plant material and hence make it difficult to completely eradicate pathogen for subsequent cropping (Köhl et al. 2011). The sophisticated virulence mechanism of Xcc makes it an inexorable pathogen. The bacterial cells are equipped with one polar flagellum and the flagellar motility enables it to navigate plant surface, locate entry points and invade internal tissues (Malvino 2024). The infection is facilitated by type III

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secretion system which functions like a molecular syringe, injecting effector proteins into host cells (Büttner and Bonas 2010). Once inside the plant either through seed-borne or foliar infection, Xcc may explore internal microenvironments to reach optimal infection sites through swimming and can develop complex, matrix-enclosed bacterial communities through biofilm production and adhere to plant surfaces and internal tissues (Crossman and Dow 2004; Kearns 2010). Biofilm protects Xcc cells from environmental stresses, antibiotics and defensive mechanisms by host tissues which contributes to persistent infection and disease progression (Dow et al. 2003). Production of certain extracellular polysaccharides, for instance xanthan gum, by Xcc enhances biofilm production and cause the clogging of vascular system of plant which exacerbate disease symptoms (Sabuquillo and Cubero 2021). Furthermore, Xcc employs quorum sensing i.e., cell-to-cell communication to coordinate virulence mechanism like flagellar associated chemotactic motility and biofilm formation (Dow et al. 2003). Disrupting these processes or important virulence factors could significantly reduce the pathogenicity of bacteria and provide a strategic target for disease control (Martínez et al. 2019).

The management of black rot in brassicas has conventionally relied on chemical treatments, crop rotation, and the use of resistant varieties and disease-free seeds. However, these methods have limitations. Chemical control methods specifically using copper-based bactericides, while initially effective, present significant drawbacks. Environmental pollution from chemical bactericides is a growing concern, as these substances can contaminate soil and water, posing risks to non-target organisms and human health (Sundin et al. 2016; Ali et al. 2021). Moreover, the continued use of these chemicals and antibiotics leads to the gradual development of pathogen resistance, rendering treatments less effective over time (Gnanadhas et al. 2013). Likewise, breeding programs aimed at developing resistant varieties also face substantial challenges. The genetic complexity of resistance traits and the time-consuming nature of breeding cycles make it difficult to produce durable resistant cultivars. Additionally, the ever-evolving virulence of Xcc strains further complicates these efforts, as newly emerging strains can quickly overcome existing resistance, potentially leading to significant crop losses (Vicente and Holub 2013). These issues underscore the urgent need for alternative approaches that are both effective and environmentally friendly.

In response to these challenges, biocontrol solutions and plant-based extracts have emerged as vital components of sustainable black rot management. Biocontrol methods involve the use of natural antagonists to suppress pathogen populations, reducing the reliance on chemical bactericides.

Plant-based extracts represent another promising avenue. Compounds derived from plants often possess antimicrobial properties that can effectively control a wide range of pathogens and can pave a way to reduce the use of copper in bacterial disease management practices (Schiavi et al. 2022a). These extracts, such as essential oils and plant secondary metabolites, provide a biodegradable and less toxic alternative to synthetic chemicals (Bajpai et al. 2012). Studies have demonstrated that plant extracts can disrupt the cell membranes of Xcc, inhibit its biofilm formation, and interfere with its quorum sensing mechanisms, thereby reducing its virulence and ability to cause disease (Adonizio et al. 2006). Specially, the plant-based substances which are known to be rich in phenolic compounds have demonstrated significant potential for managing bacterial plant pathogens (Rossetti et al. 2017). Therefore, integration of plant-based substances and extracts into black rot management not only aligns with the principles of sustainable agriculture but also enhances the resilience of cropping systems against pathogen pressures.

Among various plant-based bacterial control solutions, pomegranate peel extract (PGPE) has shown promising results due to its rich content of bioactive compounds, including ellagitannins, phenolic acids and flavonoids (Singh et al. 2019; Belgacem et al. 2021). PGPE's mode of action is multifaceted, offering both direct antimicrobial effects against a broad spectrum of pathogens and inducing systemic acquired resistance in plants, thereby, enhancing overall plant health and resilience to infections (Belgacem et al. 2021). Studies have indicated that PGPE is effective against vascular plant pathogenic bacteria under both in vitro and in planta conditions (Quattrucci et al. 2013; Rongai et al. 2023). Moreover, the use of pomegranate peel extracts as a source of polyphenols with promising antimicrobial properties demonstrates how agro-industrial waste can be turned into plant protection solutions, reflecting the goals of green chemistry and circular bioeconomy (Bernini et al. 2024). This approach not only addresses the ecological impact of agriculture but also adds economic value to otherwise discarded materials and hence contribute toward sustainability (Belgacem et al. 2021). The broader adoption of PGPE in agricultural practices is also supported by growing consumer demand for products derived from natural sources and regulatory pressures for produced through environmentally friendly methods. In view of increased regulatory pressures, market demand for sustainable agricultural inputs and consumer concerns regarding chemical residual levels in agricultural commodities, PGPE stands out as an effective, eco-friendly, and socially responsible alternative to chemical pesticides (Carvalho et al. 2019).

This study aims to evaluate the efficacy of PGPE as a sustainable plant protection agent against Xcc through a

series of in vitro and in vivo experiments. Specifically, the research focuses on assessing the effects of PGPE on bacterial growth, motility, and biofilm formation under laboratory conditions, determining the biocompatibility of PGPE with *Brassica rapa* plants, and evaluating the protective efficacy of PGPE as a foliar treatment and seed coating agent against black rot disease.

Materials and methods

Bacterial strain, pomegranate peel extract, pure compounds and plant material

A virulent strain of *Xanthomonas campestris* pv. *campestris* (Xcc) – NCPPB 528 (type strain representing race 3), obtained from National Collection of Plant Pathogenic Bacteria, UK, was utilized for both in vitro and in vivo experiments. Xcc was cultured on Nutrient Agar (NA) medium (Duchefa Biochemie) and incubated at 28 °C and regularly sub-cultured every 48 h to maintain viability and virulence. Pomegranate peel extract (PGPE) was obtained according to the procedure (with few modifications) described by Rongai et al. (2017, 2023). Pomegranate peels were cut into small pieces and added to a hydroalcoholic solvent: 85% water (double distillation from a Milli-Q-System, Millipore, Bedford, UK) and 15% ethanol (RPE analytical grade, Carlo Erba Reagents, Milan, Italy). The mixture was first sonicated at 80% amplitude for 15 min (3 s On and 7 s Off) and then stirred for 16 h at 50 °C. Subsequently the material was centrifuged at 15,000 (rpm) for 10 min, then the solvent was evaporated under vacuum in a rotary evaporator. Finally, the extract was spray dried and the powder obtained has been stored in a freezer at -20 °C until further use. Analytical grade ellagic acid (EA) and gallic acid (GA) were chosen as references for comparing their antimicrobial properties against ethanol+water extracted PGPE and were procured from Sigma-Aldrich (USA). For positive control treatments, Cu(OH)₂ was procured from Thermo Fisher Scientific Inc. (USA). For in vivo evaluation of PGPE, disease free plants and seeds of Rapini Broccoli Rabe cv. Leccese San Martino (*Brassica rapa*) were obtained from a certified nursery in Viterbo, Lazio.

Total phenolic and flavonoids content, acidity and pH analysis

Total phenolic content (TPC) of PGPE was determined by Folin-Ciocalteu reagent according to Gregoriou et al. (2021). The measurement was conducted on a UV-VIS spectrophotometer T70+ (PG Instruments Ltd) at $\lambda = 760$ nm against blank. Gallic acid was used as standard phenolic

compound for making the calibration curve that ranges from 2.8 to 22 µg/mL (LOD=0.43 mg/mL, LOQ=0.79 mg/mL, $R^2=0.9998$). The results were expressed as mg of gallic acid equivalent (GAE) for g of dry weight (DW) of lyophilized plant extracts. Acidity was determined by titration method with a 0.01 N alkaline sodium hydroxide solution using phenolphthalein (1%) as indicator and was expressed in g/L citric acid. The pH value of each extract was determined with a Hamilton pH electrodes sensor.

Polyphenols content analysis by using liquid chromatography–tandem mass spectrometry

Liquid chromatographic separation analysis was performed on a UHPLC–HRMS system consisting of an Agilent 1290 Infinity II combined with the Agilent 6560 mass spectrometer (Agilent Technologies Inc., Santa Clara, California, United States). The chromatographic separation was done by a ZORBAX RRHD Eclipse Plus C18 column (50 mm×2.1 mm, 1.8 µm, 95 Å, Agilent Technologies Inc.). HPLC eluent A was water (LC-MS grade, LiChrosolv, Supelco) with 0.1% formic acid (LC-MS grade, LiChropur, Supelco) while eluent B was methanol (LC-MS grade, LiChrosolv, Supelco) with 0.1% formic acid (LC-MS grade, LiChropur, Supelco). The gradient was initiated with 5% eluent B for 1 min, continued with a linear increase up to 20% B in 5 min; then 97% B in 4 min and this condition was maintained for 2 min. Finally, the system returned to 5% B in 1 min and was re-equilibrated for 1 min. The column temperature was kept at 30 °C and the flow rate was 0.45 mL min⁻¹. The injection volume was 5 µL. UV Detection was mainly performed at 280 nm with 1290 DAD (G7117B, Agilent Technologies Inc). Detection mass was based on electrospray ionization (ESI) in negative polarity using an MS detector Agilent 6550UHD Accurate Mass Q-TOF. An aliquot of PGPE (1 mg) was solubilized in methanol (1 mL) and sonicated for 20 min. The sample was then filtered (0.45 µm) and analyzed by UHPLC–HRMS. Gallic acid, ellagic acid and punicalagin (A+B) containing punicalin were used as standards.

Antimicrobial activity under in vitro conditions

Effect of EA, GA and PGPE on Xcc growth

The impact of PGPE, EA, and GA on bacterial growth was assessed using the method described by Carvalho et al. (2019). Freshly harvested bacterial colonies, adjusted to a concentration of 10⁴ colony forming units per millilitre (CFU/ml), were incubated in glass tubes containing varying concentrations of PGPE, EA, and GA prepared by making suspensions in sterile distilled water (0.01%, 0.025%,

0.05%, 0.1% and 0.5% w/v). Sterile distilled water and 0.05% w/v $\text{Cu}(\text{OH})_2$ were used as controls. The tubes were incubated at 28 °C for 24 h under oscillation (150 revolutions per minute) to ensure uniform exposure. Following incubation, 100 µl from each tube were plated on NA. The plates were then incubated for 48 h at 28 °C, allowing visible bacterial colonies to grow. Counting was performed manually. Results have been expressed as $\text{Log}_{10}(\text{CFU/ml})$. For each treatment four replicates were performed.

Effect of EA, GA and PGPE on swimming motility of Xcc

To evaluate the effect of PGPE, EA, and GA on the swimming motility of Xcc, NA plates at 0.3% (w/v) agar were prepared with incorporating different concentrations (0.01%, 0.025%, 0.05%, 0.1% and 0.5% w/v) of the tested compounds (Silva et al. 2019). NA plates without any amendments were included as untreated control. 5 µl of a 10^7 CFU/ml of a fresh bacterial suspension were placed at the centre of each Petri plate. The plates were statically incubated at 28 °C for 48 h. After incubation, the spread diameter of the bacterial colony (i.e., the motility “halo”) was measured using a centimetre scale. The area of each halo was calculated and analysed to evaluate the influence of PGPE, EA, and GA on the swimming motility of Xcc. For each thesis four replicates were performed.

Effect of EA, GA and PGPE on biofilm production of Xcc

The influence of PGPE, EA, and GA on biofilm production by Xcc was evaluated using a 96-well microtiter plate assay (O’Toole 2011; Schiavi et al. 2022b). The minimal XVM2 broth, known to simulate the apoplastic conditions and to promote biofilm formation (Sabuquillo and Cubero 2021), was supplemented with PGPE, EA, and GA at concentrations 0.01%, 0.025%, 0.05%, 0.1% and 0.5% w/v. XVM2 alone was used as control. 20 µl of a 10^7 CFU/ml of a fresh bacterial suspension were inoculated into 180 µl of previously amended broth and statically incubated at 28 °C. Two parallel plates were prepared for 12- and 24-hours experiment. Following incubation, the wells were gently washed with phosphate-buffered saline to remove non-adherent cells. To visualize biofilm formation, the wells were stained with 0.1% crystal violet for 15 min, followed by rinsing with distilled water to remove excess stain. The bound dye was solubilized using 30% acetic acid, and absorbance was measured at wavelength (λ) of 550 nm with a microplate reader to quantify biofilm production. The absorbance values were adjusted by subtracting the average absorbance of the blank wells (containing only acetic acid) from all readings. The data were statistically analysed to assess the effectiveness of

PGPE, EA, and GA on biofilm formation in Xcc. For each thesis six replicates were performed.

Efficacy of PGPE against Xcc under in vivo conditions

All in vivo experiments were performed under growth chamber conditions maintained at temperature of 24 °C with relative humidity of 60% and photoperiod for 16/8 hours.

Biocompatibility of PGPE with *Brassica rapa* plants and seeds

To assess the biocompatibility of PGPE two parallel experiments were performed. For the first one, healthy seedlings were sprayed with different concentrations of PGPE (0.5%, 1%, 2% and 3% w/v) by making suspension in sterile distilled water along with 0.01% Tween-80 while control treatment was sprayed only with water. Four plants for each treatment were used as replicates. Chlorophyll contents for two leaves (top-most and lower-most) on each plant were recorded at 1 day post treatments (dpt) and 10 dpt using handheld chlorophyll content measuring device (SPAD-502Plus, Konica Minolta Inc. Japan) in terms of SPAD unit values which are directly related to leaf chlorophyll concentration (Uddling et al. 2007). For the second experiment, *Brassica rapa* seeds were coated with 0.25% of Xanthan gum along with different concentrations of PGPE (0.5%, 1%, 2% and 3% w/v) to see the effect of PGPE on seed germination. The seeds were sown in seed trays in soil media and seed germination was observed for evaluation of any toxic effects (Luo et al. 2018). Three replicates of 50 seed each were sown, and germination was observed for two weeks and then germination percentage was calculated.

Effect of protective treatment of PGPE against foliar infection of Xcc on *Brassica rapa* plants

The efficacy of PGPE against Xcc was evaluated through protective treatments followed by spray infection and measured in terms of Area Under Disease Progress Curve (AUDPC) for symptoms of black rot. The young and healthy seedling of *Brassica rapa* at 4-leaf stage were sprayed with five different concentrations of PGPE (0.025%, 0.05%, 0.1%, 0.25%, and 0.5% w/v) suspended in sterile distilled water along with 0.01% Tween 80 to ensure adherence. The treatments were applied by thoroughly spraying the plants, ensuring complete coverage of both the upper and lower leaf surfaces. After 24 h, the plants were inoculated with a bacterial suspension of Xcc adjusted to 10^8 CFU/ml. Inoculation was performed by thorough, uniform and abundant spraying of the bacterial suspension on the plants, which were then covered with a transparent polyethylene bag for 24 h

to facilitate the bacterial penetration into plant tissues. After that plants were maintained under growth room conditions. The progression of black rot symptoms was monitored visually on alternative days from 3 to 27 days post-inoculation (dpi) using a severity 6 levels scale from 0 to 9 values corresponding to healthy to severe disease symptoms based on the method described by Sheng et al. 2020 (Fig. 1). On each data taking time point plants of each replicate were observed and a number was assigned to disease progression for comparative disease severity on symptomatic leaves. The AUDPC value was calculated for each treatment using the following formula as described by Shaner and Finney (1977):

$$\text{AUDPC} = \sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \cdot (t_{i+1} - t_i)$$

Where, y_i represents disease severity at the i^{th} observation, t_i denotes time of the i^{th} observation and n is the total number of observations.

Effect of *Brassica rapa* seed coating with PGPE against Xcc infection

The impact of film coating of *Brassica rapa* seeds with PGPE was evaluated against artificial infection of Xcc in terms of AUDPC for black rot symptoms in mature plants. Seeds were coated using xanthan gum as biopolymer to form a film around seed coat as carrier for PGPE at different concentrations (Hassanisaadi et al. 2025). First, xanthan gum was dissolved to form slurry @ 0.25% w/v in sterile distilled water and then varying concentrations of PGPE were mixed in 10 ml for each treatment to reach 0.25%, 0.5%, 1%, 2%, and 3% w/v. For each treatment, 1 gram of seeds were thoroughly mixed with 3 ml of slurry in 10 ml falcon tubes using vortex mixer to ensure uniform coating. The seeds were then spread in sterile Petri plates under laminar flow cabinet for overnight to dry. Controls were included in experiment, non-coated seeds as negative

control and hot water treated (suspension of seeds in sterile distilled water at 50 °C for 15 min) as positive control (Kim et al. 2022). Also, seeds coated with only xanthan gum were included. The seeds were sprayed with bacterial suspension (10^8 CFU/ml) prepared from freshly harvested Xcc colonies (grown on NA media at 28 °C for 48 h) and left to dry on sterile Petri dishes. After drying, the seeds were thoroughly rotated by shaking, and the infection procedure was repeated to ensure significant adherence of bacterial cells and then dried again under laminar flow cabinet.

The coated and infected seeds were sown in seed tray in sterile commercial soil media. Germinated seedlings were transplanted to bigger pots containing sterile soil media for evaluation of symptoms development for calculation of AUDPC. For each treatment, 12 plants in three replicates were studied and maintained under controlled environment of 24 °C, 60% relative humidity and 16/8 hours of photoperiod. Symptoms were observed at 5 days interval to record the AUDPC as previously described.

Statistical analysis

Data from all the experiments was analysed through analysis of variance (ANOVA) as complete randomized design. All pairwise comparison of means was performed using Tukey's HSD test at significance level of $p < 0.05$ to assess the significance of results. For each mean value standard deviation ($\pm$ SD) was calculated manually in Microsoft Excel worksheets. Statistical analysis was performed using DSAATSAT macro for Microsoft Excel software (Onofri and Pannacci 2014).

Results

Chemical characterization and analysis of PGPE

The identification of phenolic and other compounds in various natural products is typically conducted by using liquid chromatography analyses. To this end, the chemical

Fig. 1 Scale developed and followed for visual observation of *Brassica rapa* plants infected with Xcc to observed disease progression showing leaf pictures with increasing disease severity assigned numbers from 0 to 9 based on symptoms development

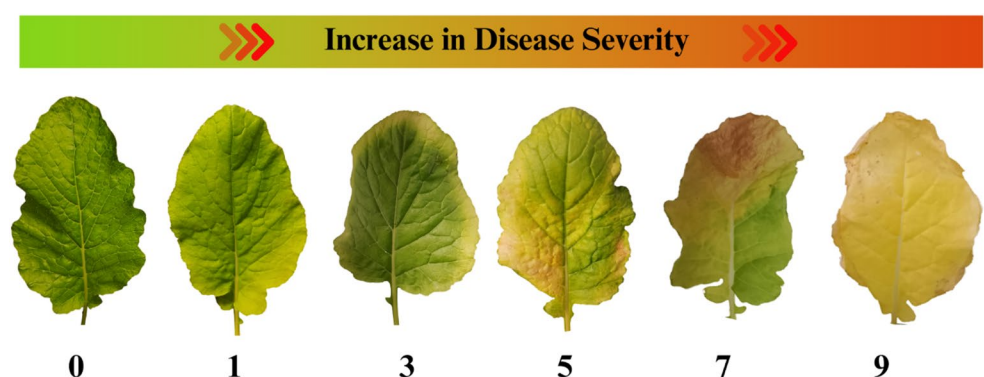
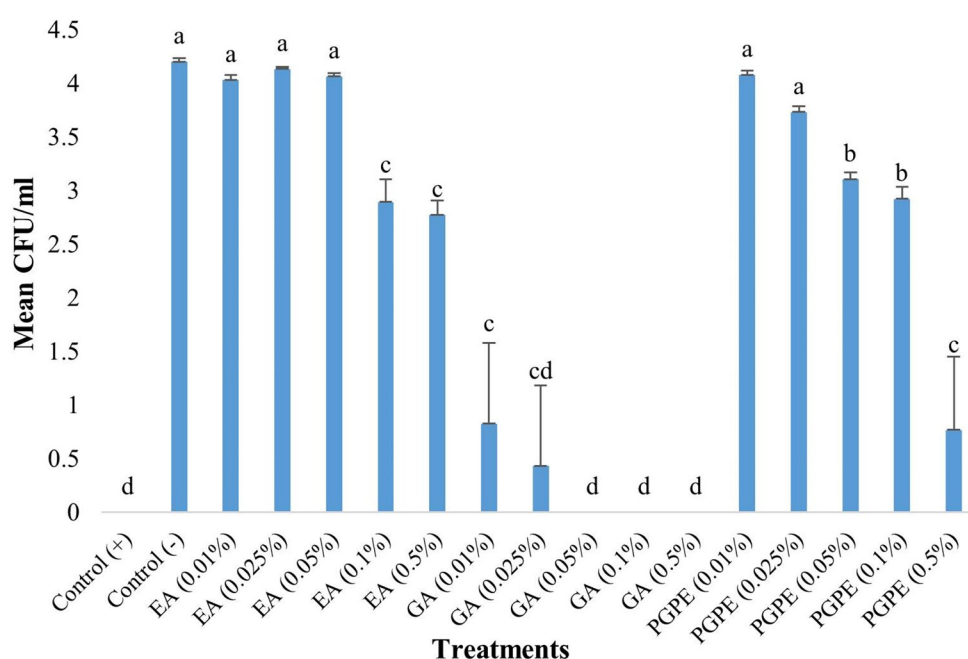


Table 1 Compounds identified in PGPE according to their retention time (RT), molecular formula, high resolution mass found (m/z) and mass accuracy (Δ ppm, part per million error). Qualitative score was estimated with the information obtained from the mass spectra: “+” present, “++” and “+++” highly present

	RT	Compound	Mol. Formula	HRMS, m/z	Δ ppm	Score
1	0.43	5-Hydroxymethylfurfural (5-HMF)	C ₆ H ₆ O ₃	(M-H) ⁻ 125.0244	-0.03	+
2	0.45	Ascorbic acid	C ₆ H ₈ O ₆	(M-H) ⁻ 175.0246	2.85	+
3	0.45	Tartaric acid	C ₄ H ₆ O ₆	(M-H) ⁻ 149.0090	-1.49	+
4	0.50	2,3- <i>O</i> -hexahydroxydiphenoyl- <i>D</i> -glucose (HHDP-glucose) ($\alpha + \beta$)	C ₂₀ H ₁₈ O ₁₄	(M-H) ⁻ 481.0629	0.93	+++
	0.60			(M-H) ⁻ 481.0631	1.26	
5	0.60	Citric acid	C ₆ H ₈ O ₇	(M-H) ⁻ 191.0200	1.40	+++
6	1.08	Gallic acid	C ₆ H ₈ O ₇	(M-H) ⁻ 169.0142	-0.38	++
7	1.36	Punicalin	C ₃₄ H ₂₂ O ₂₂	(M-H) ⁻ 781.0534	0.45	++
8	2.60	Punicalagin ($\alpha + \beta$)	C ₄₈ H ₂₈ O ₃₀	(M-H) ⁻ 1083.0574	0.01	+
	3.98			(M-H) ⁻ 1083.0576	0.25	
9	5.98	Urolithin A	C ₁₃ H ₈ O ₄	(M-HCOO) ⁻ 273.0462	-0.61	+
10	8.20	Ellagic acid	C ₁₄ H ₆ O ₈	(M-H) ⁻ 300.9990	0.01	+

Fig. 2 Mean values for Log₁₀ CFU/ml representing in vitro growth of Xcc under varying concentrations of EA, GA, and PGPE along against copper treated (+) and non-treated (-) controls. Error bars reflect $\pm$ SD and labels assigned representing all pairwise comparison with confidence level of $p < 0.05$



composition of the PGPE was analyzed through a UHPLC–HRMS method, which indicated the presence of ellagitannins, gallotannins, and organic and phenolic acids (Table 1). Among the biologically active class of ellagitannins, the main compound with molecular formula C₃₄H₂₂O₂₂ was assigned, and confirmed by the standard, as punicalin following by the two punicalagin anomers and its derived metabolites: ellagic acid, HHDP-glucose and urolithin A. The presence of organic and phenolic acids, mainly the citric acid, contribute to the acidic pH of aqueous PGPE solutions. Indeed, the value of total acidity was 1.38 meq NaOH/g while the pH was about 4.08.

The well-known antioxidant property of such extracts was estimated through their total phenolic content (TPC) which turned out to be about 270 mg GAE/g DW. This high

polyphenol content helps to explain their interesting biological activity.

Antimicrobial effects of EA, GA, and PGPE under in vitro condition

Effect of EA, GA, and PGPE on in vitro growth of Xcc

Under in vitro conditions, all tested substances influenced bacterial growth differently depending on concentration (Fig. 2). Among all substances, GA was the most effective, significantly reducing bacterial colonies at all tested levels. At higher concentrations (0.05%, 0.1%, and 0.5%), GA completely inhibited bacterial growth, similar to the effect of the copper control treatment. Conversely, EA had a milder impact, with lower concentrations (0.01%, 0.025%,

and 0.05%) being the least effective and statistically similar to the control (-). Even at higher concentrations, bacterial growth was only inhibited by up to two log units. Unlike GA and EA, PGPE demonstrated a dose-dependent reduction in bacterial growth. The highest tested concentration of PGPE (0.5%) resulted in up to a four-log unit decrease in CFU counts, with bacterial colony numbers on NA media inversely correlated with increasing PGPE concentrations.

Effect of EA, GA, and PGPE on swimming motility of Xcc

The areas of swimming halos of Xcc showed a significant reduction of motility under all the treatments in comparison to negative control (Fig. 3). Only the lower concentrations (0.01% and 0.025% for EA and 0.01% for GA and PGPE) incorporated into agar media allowed some swimming of Xcc while all the higher concentration effectively halted the expansion of bacterial colonies. These findings suggest that all the tested substances may severely hinder the bacterial motility.

Effect of EA, GA, and PGPE on biofilm production by Xcc

Biofilm production is an important stress responsive physiological process by Xcc. All tested treatments (including control) resulted in production of biofilm, however, the level of biofilm varied uniquely against different concentrations of tested substances (Fig. 4). The patterns of biofilm production, observed in terms of absorbance at λ of 550 nm reflected a significant difference among EA, GA and PGPE at 12 and 24 h. For instance, EA significantly halted biofilm production on all concentrations (0.01–0.5%) compared to control at both the 12- and 24- hours incubation periods. The GA showed an increasing trend for biofilm production

from 0.01 to 0.1% which was below the control for 12 h incubation period while higher than the control for 24 h incubation period. However, the highest tested concentration of GA showed significantly less biofilm production than control at both 12- and 24- hours incubation periods. On the other hand, PGPE showed an increasing trend of biofilm production from 0.01 to 0.1% and then a significant decline at 0.5% while at all concentration and both incubation periods the biofilm production in presence of PGPE was lower than control.

Biocompatibility and efficacy of PGPE as foliar treatment and seed coating against black rot in *Brassica rapa*

Biocompatibility

For both tested methods to assess the biocompatibility of PGPE with *Brassica rapa* plants and seeds, no significant difference was observed in terms of SPAD values for chlorophyll pigment against foliar treatments (Table 2) and germination percentages for seed coating treatments (Table 3). This reflects that the tested concentrations of PGPE were not phytotoxic by different approaches and therefore could be used for subsequent in vivo experiments.

Efficacy of protective treatment of PGPE on *Brassica rapa* plants against foliar Xcc infection

Protective treatment of PGPE on *Brassica rapa* plants resulted in dose dependent control of black rot symptoms reflecting the suitability to contain Xcc (Fig. 5). The results of this assay showed a decline of AUDPC value with increase in concentration of PGPE as a protective treatment.

Fig. 3 Effect of different concentrations of EA, GA, and PGPE on Swimming motility of Xcc in terms of area (cm^2) of bacterial swimming halo in comparison with a negative control. Error bars reflect $\pm$ SD and labels assigned representing all pairwise comparison with confidence level of $p < 0.05$

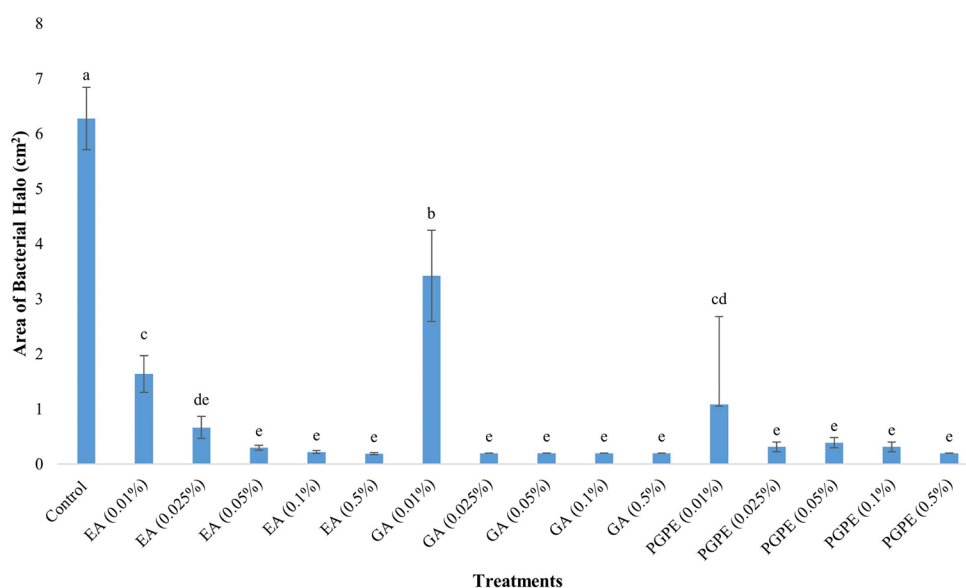


Fig. 4 Effect of different concentrations of EA, GA, and PGPE on biofilm production by Xcc 12- and 24-hours incubation intervals measured in terms of absorbance recorded at wavelength of 550 nm. Error bars reflect $\pm$ SD and labels assigned representing all pairwise comparison with confidence level of $p < 0.05$

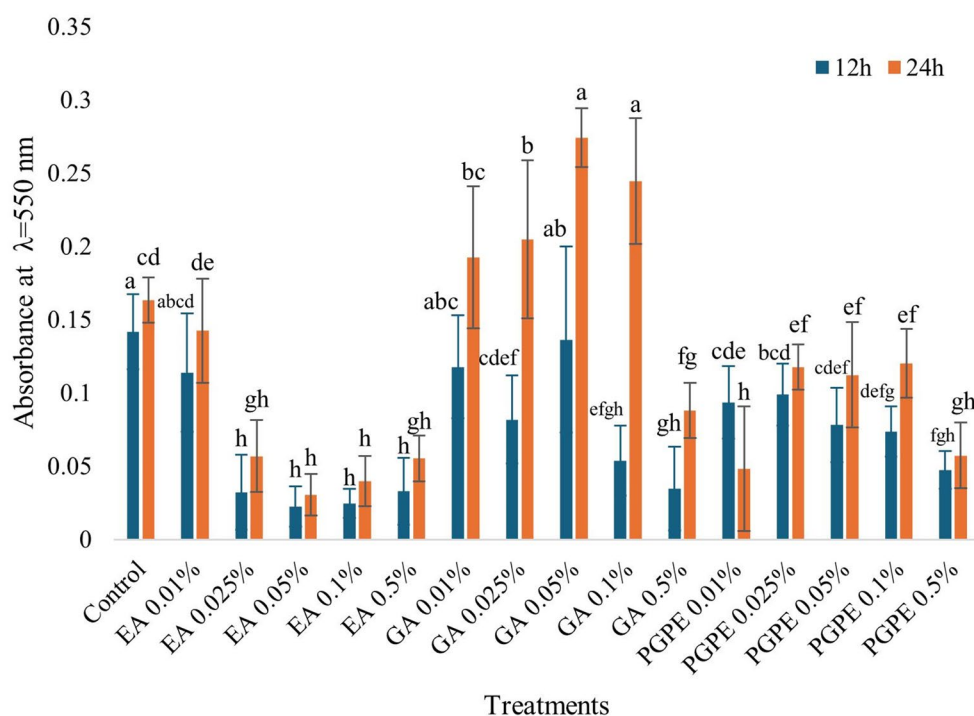


Table 2 Effect of different concentrations of PGPE on SPAD values of *Brassica rapa* leaves against foliar treatments

PGPE concentration in foliar treatments	Mean SPAD value $\pm$ SD	
	DPT 1	DPT 10
0%	62.125 $\pm$ 3.06 ns	58.725 $\pm$ 3.29 ns
0.25%	57.975 $\pm$ 6.25 ns	59.025 $\pm$ 4.09 ns
0.50%	66.075 $\pm$ 8.67 ns	65.225 $\pm$ 8.76 ns
1%	65.475 $\pm$ 1.79 ns	59.55 $\pm$ 7.19 ns
2%	65.15 $\pm$ 4.47 ns	60.1 $\pm$ 7.29 ns
3%	62.95 $\pm$ 2.11 ns	61.35 $\pm$ 3.14 ns

Table 3 Effect of different concentrations of PGPE and xanthan gum (XG) as seed coating treatments on germination percentage

Seed coating treatment	Germination percentage % $\pm$ SD
No Coating	82 $\pm$ 3 ns
0.25% PGPE + 0.25% XG	80 $\pm$ 2 ns
0.50% PGPE + 0.25% XG	78 $\pm$ 4 ns
1% PGPE + 0.25% XG	71 $\pm$ 7 ns
2% PGPE + 0.25% XG	76 $\pm$ 4 ns
3% PGPE + 0.25% XG	73 $\pm$ 4 ns
Only 0.25% XG	85 $\pm$ 2 ns
Hot-water treatment	70 $\pm$ 6 ns

The highest concentrations, i.e., 0.25% and 0.5%, showed a significant decrease in AUDPC values, which reflect that protective treatment of *Brassica rapa* plants with PGPE may help in reducing the development of symptoms of black rot in result of foliar Xcc infection.

Efficacy of PGPE seed coating against seed borne Xcc infection in *Brassica rapa* plants

As black rot is primarily a seed borne disease, seed coating with PGPE at various concentrations could represent a valid protection strategy against the infection of Xcc. The results of seed coating assay shown a significant control of black rot symptoms in mature plantlets with increase in PGPE concentration (Fig. 6). Coating seeds with higher PGPE concentrations i.e., 2% and 3% showed similar results to those of heat-treated seeds. This finding is very promising and reflects the efficacy of this novel approach to combat the black rot in field crops.

Discussion

This study presents the potential of pomegranate peel extract (PGPE) as a biocompatible and sustainable control agent against *Xanthomonas campestris* pv. *campestris*, causative agent of black rot disease in crucifers, through a series of in vitro and in vivo experiments. The chemical analysis of PGPE showed that our extraction method is valid for getting complex organic substance with a variety of phenolic compounds that have previously been studied rigorously for their broad biological, particularly antimicrobial, activities (Siddiqui et al. 2024). Presence of such compounds in our PGPE was found to be concurrently effective in the complex composition through our subsequent experiments. It is well reported that the extraction methods, cultivar and

Fig. 5 Efficacy of PGPE as protective treatment against Xcc foliar infection represented by mean AUDPC values in comparison with copper treated (+) and non-treated (-) controls. Error bars reflect $\pm$ SD and labels assigned representing all pairwise comparison with confidence level of $p < 0.05$

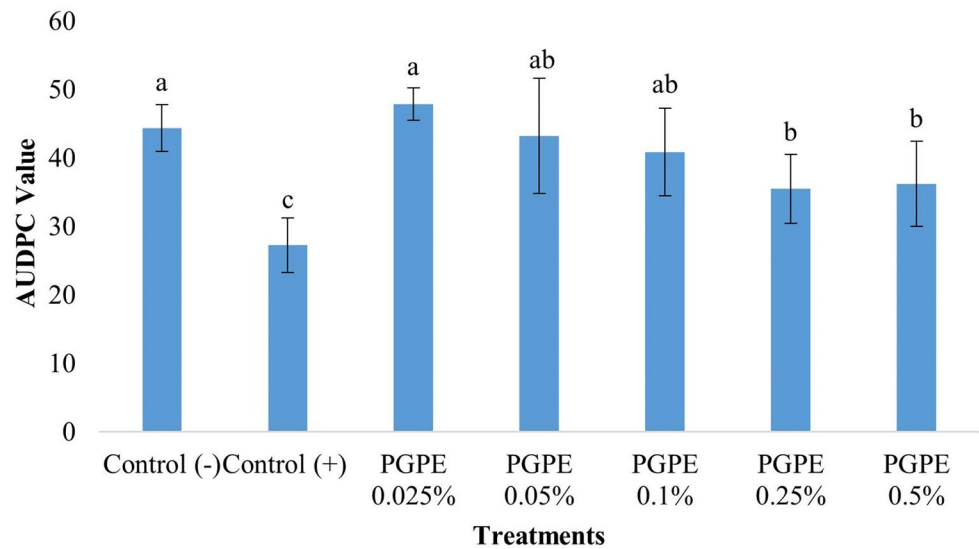
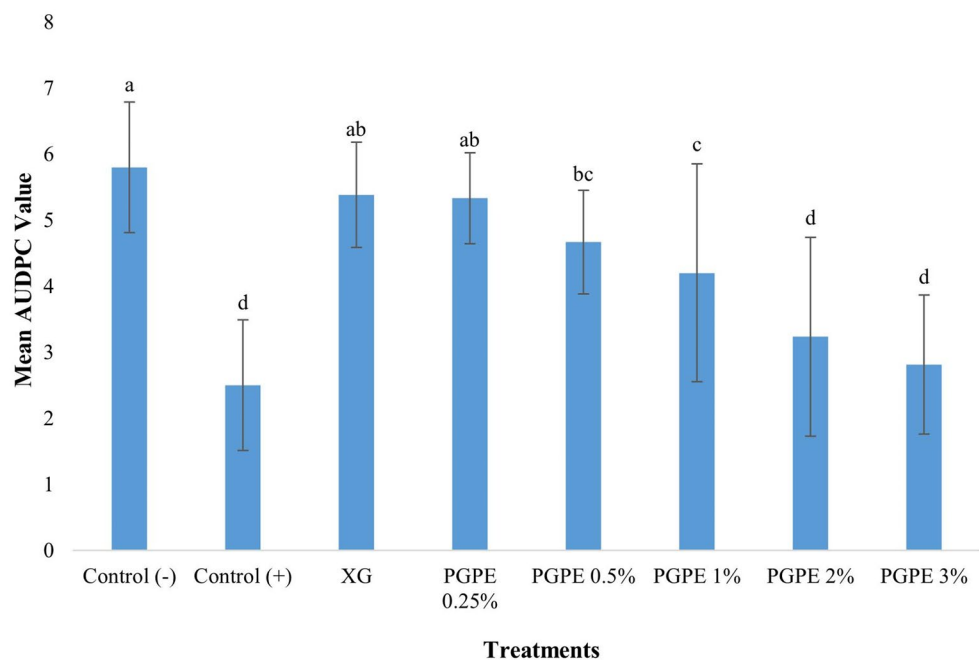


Fig. 6 Effect of different concentrations of PGPE applied as seed coating against Seed-borne Xcc infection in cabbage seedlings in terms of AUDPC values along with hot-water treated (+) and non-treated (-) and one treatment with only coated by Xanthan gum (XG). Error bars reflect $\pm$ SD and labels assigned representing all pairwise comparison with confidence level of $p < 0.05$



other conditions may contribute to variation in levels of certain compounds in the final extract (Singh et al. 2023). But the compounds like Punicalagins, Ellagic acid, Galic acid which are observed to be highly present in our extract are always reported to have some level of antimicrobial properties (Singh et al. 2019). The insights from our in vitro and in vivo experiments, highlight that PGPE is not only effective against Xcc under in vitro conditions but also biocompatible with *Brassica rapa* plants and have shown a protective activity against foliar and seed borne Xcc infection.

The in vitro bacterial growth was consistently reduced with increase in concentration of PGPE and a dose dependent response was observed, contrary to the individual effect of EA and GA. Most significant reduction in bacterial colonies

formation was observed on higher tested concentration of PGPE i.e., 0.5% w/v. These findings are in line with various previous studies where the antibacterial potential of PGPE against various bacteria was observed. For instance, Kharouchi et al. 2018 find that both the water as well as methanol-based extracts of 'Gabsi' pomegranate peel resulted in a dose dependent reduction of *Pseudomonas putidia* under in vitro conditions in terms of log CFU/ml. Likewise, Negi and Jayaprakasha 2003 also reported significant inhibition and reduction of colonies growth of several gram positive and negative pathogenic bacteria. In contrast to PGPE, GA severely inhibited bacterial growth on all tested concentrations while EA couldn't express the strong inhibition under tested concentrations. This could be attributed to severe

disruption of bacterial cells under the effect of GA as compared to EA. Our results are in line with the findings of Quattrucci et al. 2013 who reported a similar antibacterial effect of pomegranate peel extract in comparison with pure ellagic acid and gallic acid against *Pseudomonas syringae* pv. *tomato*. The stronger inhibition under individual effect of GA could be attributed to the ability of GA to suppress the expression of bacterial efflux pump which is an important resistive mechanism with which bacterial cells extrude toxic chemicals (Piddock 2006). Therefore, if the action of these genes could be suppressed by some external agent, the survival of bacteria becomes harder in response to antibacterial agents. A study by (Macêdo et al. 2022), also reflects similar finding that GA has a strong inhibition effect on bacterial efflux pump related genes as compared to EA. Similar findings have been reported in a comparative study for antibacterial potential of GA and EA against human pathogenic bacteria that GA was more effective in inhibiting and causing mortality of bacterial cells (Cota and Patil 2023). The overall synergistic effect of PGPE is interestingly unique finding which could be a result of complex polyphenolic composition and hence PGPE shows promising potential as sustainable control measure against bacterial plant pathogens. The study by Chen et al. 2021; also supports our interpretation where they reported that composite effect of pomegranate peel polyphenols was more evident in terms of restricting the bacterial proliferation of *Ralstonia solanacearum* than that of individual phenolic compounds including EA and GA. However, to understand better about antibacterial mechanism of PGPE against Xcc, the assessment of its effect on swimming and biofilm production by bacterial cells was an important aspect. Our results demonstrate that PGPE as well as GA and EA significantly halt the bacterial motility even at the sub-optimal concentrations for inhibition, and, hence, reduce the swimming capabilities. Xcc cells are motile and have cellular apparatus called flagella which help them in invading host tissues, relocation and movement and hence facilitate develop and spread disease symptoms in host species (Malvino 2024). The findings of our study show that the tested substances and particularly PGPE could have halted the flagellar pathways and reduced the swimming potential of Xcc in a less viscous in vitro growth media. Numerous studies have reported similar findings where pomegranate-based polyphenols or plant extracts caused reduction of swimming motility in various bacterial pathogens, for instance, fermented pomegranate polyphenols against aquatic pathogen gram-negative bacterium *Vibrio alginolyticus* (Yu et al. 2024), pomegranate peel extract against human pathogen gram-negative bacterium *Helicobacter pylori* by inhibiting flagellin gene expression (Hassen et al. 2020), and pomegranate rind extract against uropathogenic *Escherichia coli* by inhibiting flagellin gene expression and

subsequently flagellar propelled motility (Asadishad et al. 2012). Therefore, PGPE is well-known to inhibit the bacterial cells motility and hence could be a promising antibacterial agent against various pathogens. Likewise, we studied the impact of presence of these substances on the production of biofilm by Xcc. Biofilm production is a protective mechanism by which Xcc cells respond against different stressful situations and develop a matrix enclosed cluster of cells to sustain the antibiotics and other stresses (Crossman and Dow 2004). Our results are supported by findings of Chen et al. (2021) as they reported increase of biofilm production over the time in presence of composite pomegranate polyphenols, as well as individual phenolic compounds like gallic acid and ellagic acid which is also evident in our results. However, the decline of biofilm production on increased concentrations of EA, GA and PGPE in our study could be attributed to inhibition of bacterial cells under the higher concentrations of these substances. This finding is in line with results of El-Egami and Sayed 2017; who reported a decline of biofilm production by *Xanthomonas campestris* with increase of pomegranate extracts. This behaviour could also be attributed to the activity of phenolic compounds at higher concentration to be able to disrupt the bacterial quorum sensing and denaturation of extracellular matrix. Various studies have shown that plant-based compounds have ability to disrupt the normal biofilm activity by pathogenic bacteria and inhibit the biofilm formation (Silva et al. 2016). These findings from in vitro experiments highlight the complex interaction of PGPE with Xcc which is important with respect to its antimicrobial potential. Virulence or pathogenicity of bacteria is not limited to any single mechanism and these factors are considered to be most significant in this regard. Approaching a novel natural antimicrobial agent by characterizing and validating action against these mechanisms provides substantial evidence for PGPE to be a promising control agent against Xcc and hence could provide sustainable management solution for black rot disease.

The biocompatibility of PGPE with *Brassica rapa* plants and seeds is a crucial consideration for its practical application in the field. In this study, we observed no significant phytotoxic effects of PGPE for concentrations up to 3% in terms of chlorophyll contents for foliar spray application as well as germination percentage for seed-coating. These findings are in line with Pangallo et al. 2022; who reported no phytotoxic effects on olive plants for pomegranate peels extracts as a sustainable control for Anthracnose. A recent study by Bodor et al. 2024; reported that nutrient-fortified pomegranate peel powders in various combinations did not significantly harmed *Brassica napus* plants and some combinations were found to be beneficial for plants. However, our findings are contrary to those of Campos et al. 2022; who reported phytotoxicity at higher concentrations of

pomegranate extracts in terms of germination percentage and root length for garden-cress seeds. Therefore, the tested levels in our study were found to be suitable for conducting in vivo experiments to assess the potential of PGPE against Xcc in *Brassica rapa* plants.

We utilized two different approaches to study the progress of the disease in *Brassica rapa* plants: one by applying a protective treatment of aqueous suspension of PGPE on the levels which were tested for in vitro studies (i.e., 0.025–0.5%) and other to coat seeds with promising levels from in vitro results (i.e., 0.25% and 0.5%) and higher concentrations which were found non-phytotoxic (i.e., up to 3%). The protective treatment of PGPE showed a decrease in visible symptoms development especially at 0.25% and 0.5% the results were comparable to those of copper treatment, which could be attributed to the fact that contact of bacterial cells with a protective layer of PGPE on leaf surfaces could have halted the penetration into the leave tissues along with inhibition of viable cells and cell swimming, as we observed in in vitro experiments. These findings are supported by results of Elsherbiny et al. 2016 and Almasoudi et al. 2024; who reported similar protective effects of pomegranate peel extract in potato tubers. Likewise, Pangallo et al. 2022 also reported that protective treatment of pomegranate peel extracts significantly controlled the latent infection and symptoms development of olive anthracnose and leaf spot diseases. Additionally, the seed coating with PGPE also showed a decline in AUDPC in a dose-dependent manner with higher concentrations i.e., 2% and 3% showed results comparable to those in plants grown from heat treated seeds. This finding also emphasizes the potential of PGPE for sustainable and feasible control strategy for notorious seed borne diseases like black rot. Our results are supported by the findings of Mandiriza et al. 2018 who proved that seed treatment with plant extracts significantly reduce black rot disease incidence and severity on rape leaves. Moreover, as black rot is mainly seed borne infection, these findings could pave way for an economical and socially responsible control strategy for managing such diseases. The protective effect of PGPE on seeds as well as leaves suggests that it could serve as an alternative to chemical seed and foliar treatments, offering a more sustainable solution to controlling bacterial diseases in crop.

The in vitro and in vivo results demonstrate the multifaceted role of PGPE in inhibiting bacterial growth, motility, and biofilm formation, as well as in protecting *Brassica rapa* plants from foliar and seed-borne infections. PGPE's safe application and significant efficacy make it an attractive alternative to chemical pesticides, paving the way for more sustainable agricultural practices. However, further research is required to optimize its use, valuating efficacy in various combinations and composite formulation with other novel

and effective antibacterial agents, understand the molecular mechanisms involved, and evaluate its long-term field applicability for broader agricultural applications.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest regarding the publication of this article.

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