Mexican Journal of Phytopathology
ISSN: 2007-8080

Crossref
Open access

Scientific Article

Actinomycete extracts against *Pseudomonas syringae* pv. *phaseolicola* in *Phaseolus vulgaris*

Evangelina Esmeralda Quiñones-Aguilar[2], Alfredo Reyes-Tena[1], Gabriel Rincón-Enriquéz[2], Juan Carlos Rico-Aguilar[1], Luis López-Pérez[1]. [1]Instituto de Investigaciones Agropecuarias y Forestales, de la Universidad Michoacana de San Nicolás de Hidalgo. Unidad Posta Veterinaria Km 9.5 Carretera Morelia-Zinapécuaro. Col. El Trébol CP. 58880. Tarímbaro, Michoacán, México. [2]Control Biológico de Enfermedades de Plantas. Laboratorio de Fitopatología, Unidad de Biotecnología Vegetal. Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. (CIATEJ). Camino el Arenero Núm. 1227. Col. El Bajío del Arenal. C.P. 45019, Zapopan Jalisco México.

***Corresponding Author:**
Luis López-Pérez
Correo:
luis.lopez.perez@umich.mx

Section:
Special Number

Received:
November 02, 2024
Accepted:
June 09, 2025
Published:
June 25, 2025

Citation:
Quiñones-Aguilar EE, Reyes-Tena A, Rincón-Enriquéz G, Rico-Aguilar JC and López-Pérez L. 2025. Actinomycete extracts against *Pseudomonas syringae* pv. *phaseolicola* in *Phaseolus vulgaris*. Mexican Journal of Phytopathology 43(4): 73. https://doi.org/10.18781/R.MEX.FIT.2024-02

ABSTRACT

Background/Objective. Common bean (*Phaseolus vulgaris*) is mainly affected by the bacterium *Pseudomonas syringae* pv. *phaseolicola* (PSPH), which can cause severe economic losses. A sustainable alternative for its control is the use of antimicrobial agents such as actinomycetes. This study aimed to evaluate the effect of extracts from three actinomycete strains (ED65, ED66, and ED67) on the control of *Pseudomonas syringae* pv. *phaseolicola* in *Phaseolus vulgaris* under controlled conditions.

Materials and Methods. Two mL of the actinomycete extracts were sprayed onto Azufrado bean plants, inoculated with PSPH. After 16 days, symptoms were assessed by the number of leaves showing spots, defoliation, number of spots per leaf, and disease severity using a damage scale.

Results. A statistically significant reduction ($P < 0.05$), was found in the number of spots and disease lesions in plants sprayed with the extracts. The level of damage decreased significantly in plants treated with the ED65 and ED67 extracts, and the ED66 treatment showed no damage.

Conclusion. According to the results of this study, strain ED66 showed the greatest potential for use as a source of antimicrobial substances for managing halo blight in beans, followed by ED65 and ED67.

Keywords: Antibiosis, Biological control, Bean, Streptomyces, Halo blight

INTRODUCTION

Common bean (*Phaseolus vulgaris*), is a staple food in regions of Africa, Latin America, and countries such as India. In 2020, global production reached 27.546 million tons, with India being the leading producer, contributing 19.8% of the world's total, followed by Myanmar (11.1%), Brazil (11%), the United States (4.9%), China (4.1%), and Mexico (4%) (FAO, 2020). This highlights the global importance of common bean as one of the main crops, making its agronomic management and especially the control of diseases affecting it a matter of vital interest. *Pseudomonas syringae* is a complex species that includes around 60 pathovars with specific virulence across a wide range of hosts (Xin *et al.*, 2018). *Pseudomonas syringae* pv. *phaseolicola* (PSPH), is the causal agent of the disease known as "halo blight" and is considered the main constraint in bean cultivation (O'Leary *et al.*, 2016). This bacterium has been detected in several production regions of the Valley of Mexico and Sinaloa, where its aggressiveness causes yield losses of up to 55% (Mamoucha *et al.*, 2023). Control is generally based on the application of chemical bactericides; however, constant use has led to pathogen resistance and other problems, mainly environmental damage and health risks (Miller *et al.*, 2022; *Zang et al.*, 2023). In light of this issue, it is necessary to find alternative control strategies that prevent the development of pathogen resistance, are environmentally friendly, and above all, support the development of sustainable agriculture.

Currently, antagonistic microorganisms such as actinobacteria and their metabolites are used as a sustainable alternative for the control of diseases in economically important crops (Kour *et al.*, 2019; Trinidad-Cruz *et al.*, 2021). These have gained attention as a sustainable option, reducing the use of pesticides. Actinobacteria make up the largest phylum in the domain Bacteria; they are Gram-positive, have high guanine and cytosine content in their genomes, and possess the ability to form spores (Khushboo *et al.*, 2022). Their morphology ranges from unicellular to filamentous forms (Parte *et al.*, 2020). Actinobacteria are known for their ability to produce a wide variety of secondary metabolites with different effects, among which the promotion of plant growth stands out (Donald *et al.*, 2022; Khushboo *et al.*, 2022). This effect is enhanced by their synthesis of enzymes that solubilize phosphates, fix nitrogen, and produce phytohormones such as indole-3-acetic acid and gibberellic acid, which are important regulators of plant growth (Anwar *et al.*, 2016).

On the other hand, antimicrobial metabolites synthesized by actinobacteria have proven effective in controlling plant diseases both *in vitro* and *in vivo* (Passari *et al.*, 2016; Trinidad-Cruz *et al.*, 2021). Biocontrol mechanisms and/or strategies of actinomycetes include parasitism (El-Tarabily *et al.*, 2006), siderophore production (Shrivastava and Kumar, 2018), production of extracellular enzymes such as cellulases, amylases, and chitinases (Singh and Gaur, 2016), induction of systemic resistance in plants (Sharma and Salwan, 2018), and production of secondary metabolites with antifungal and antibacterial activity (Westhoff *et al.*, 2021; Khushboo *et al.*, 2022). Actinomycetes produce nearly half of all antibiotics used in industry (Bérdy, 2012). *Streptomyces* is the most representative genus in soil (Qin *et al.*, 2015; Olanrewaju and Babalola, 2019) and the main producer of secondary metabolites (Wohlleben *et al.*, 2016). In addition, the efficacy of its volatile and non-volatile metabolites produced in different media (liquid, solid, and semi-solid), as well as their filtrates, has been demonstrated (Yang *et al.*, 2019; Trinidad-Cruz *et al.*, 2021). In this regard, Nguyen *et al.* (2015) and Abbasi *et al.* (2021) showed

inhibition of *Phytophthora capsici* growth using different *Streptomyces* spp. strains *in vitro*. Chen *et al.* (2016) reported that the extract of *S. plicatus* B4-7 inhibited the *in vitro* development of *P. capsici*, *P. cinnamomi*, *P. palmivora*, and *P. parasitica* by 55%, 65%, 43.8%, and 35%, respectively. Yan *et al.* (2019) reported that *S. corchorusii* AUH-1 inhibited the development of *P. capsici* and *P. parasitica* var. *nicotianae* by 67.2% and 78.9%, respectively, following application of an extract obtained by alcoholic fermentation. Zang *et al.* (2023) reported inhibition of *Phytophthora* sp. using extracts of *Streptomyces atratus* in pepper plants. Similarly, Wen *et al.* (2024) reported strong inhibition of *Phytophthora sojae* using extracts of *Streptomyces* sp. Trinidad-Cruz *et al.* (2021), evaluated the effect of actinomycetes ABV38, ABV39, and ABV45 against *P. capsici* in potato dextrose agar and reported inhibition rates of 51%, 28%, and 50%, respectively. Later, when evaluating the culture broth supernatant of strains ABV38 and ABV45, they observed reduced damage in *Capsicum annuum* var. Camino Real plants infected with the pathogen. Arora *et al.* (2018), reported that *Streptomyces* sp. strain C-7 produces nalidixic acid, an antibiotic effective against Gram-negative bacteria, and inhibits the growth of *E. coli* and *P. aeruginosa* under *in vitro* conditions. Al-Dhabi *et al.* (2019) reported that *Streptomyces* sp. strain Al-Dhabi-97 has antimicrobial effects against several bacteria cultured in various solid media, mainly nutrient agar. Based on this, the aim of this study was to evaluate the control capacity of extracts from actinomycetes isolated from agricultural soils against *Pseudomonas syringae* pv. *phaseolicola* (PSPH), in bean plants grown in a greenhouse.

MATERIALS AND METHODS

This research was conducted during the spring–summer period, and the experiment was carried out in a zenith-type greenhouse covered with 800-gauge plastic, allowing 80% of incident light to enter. The greenhouse was located at the facilities of the Institute of Agricultural and Forestry Research of the Universidad Michoacana de San Nicolás de Hidalgo, Mexico. Environmental conditions inside the greenhouse included an average temperature of 32 ± 3 °C and a relative humidity of 46%.

Plant material. For this study, seeds of the *azufrado* bean variety were used. The seeds were first rinsed with running water and then surface-disinfested using 3% sodium hypochlorite for 10 minutes, followed by a rinse with running water. One seed was sown per 2 L plastic container filled with a sterilized mixture (autoclaved at 120 °C, 103 kPa, for 6 hours) of perlite, vermiculite, and agricultural soil in a 2:1:1 v/v ratio. Periodic irrigations with deionized water were carried out to maintain the growth substrate at field capacity.

Biological material. The actinomycetes used in this study were provided by the Phytopathology Laboratory of CIATEJ (Center for Research and Assistance in Technology and Design of the State of Jalisco, Mexico). The actinomycetes were identified as ED65, ED66, and ED67 (Figure 1), and were isolated from agricultural soils cultivated with *Agave cupreata* in the state of Michoacán, Mexico. In previous studies, they showed growth inhibition percentages against PSPH greater than 80% (unpublished data).

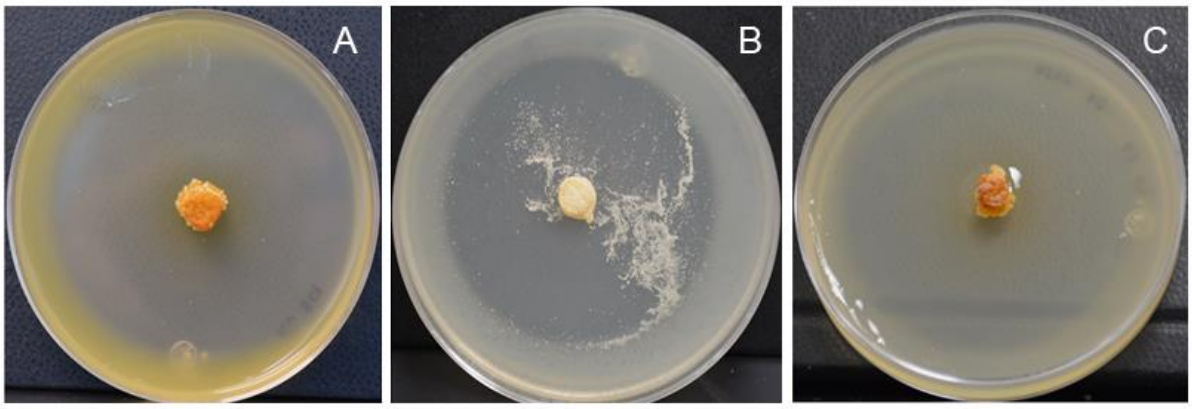

Figure 1. Strains ED65 (A), ED66 (B), and ED67 (C), showing typical morphological characteristics of actinobacteria.

The actinomycetes were cultured on potato dextrose agar with yeast extract (PDA-Y) and incubated at 28 °C for 7 days. Then, two 7 mm agar–mycelium discs were transferred to flasks containing 25 mL of potato dextrose broth with yeast extract (PDB-Y), and incubated in the dark at 28 °C for five days with shaking at 200 rpm. After this period, cultures were centrifuged at 13,000 rpm for 10 minutes, the supernatant was discarded, and the resulting pellet was washed three times with sterile distilled water and resuspended in 30 mL of sterilized deionized water.

Production of filtered actinomycete extract. The actinomycetes were cultured on solid medium following the methodology of Ellaiah *et al.* (2004), with slight modifications as described below: 10 g of semolina moistened with 12 mL of PDB at pH 7 were placed in 250 mL Erlenmeyer flasks, followed by the addition of 2 mL of actinomycete inoculum (1.2–5.6 mg dry cell matter per gram). The mixture was incubated for 12 days at 28 °C. After the incubation period, the extract produced by the actinomycetes was collected by adding 25 mL of phosphate buffer (8.06 g NaCl L^{-1}, 0.22 g KCl L^{-1}, 1.15 g Na_2HPO_4 L^{-1}, 0.2 g KH_2PO_4 L^{-1}) at pH 8.0, as reported by Adinarayana *et al.* (2003), and maintained under agitation at 200 rpm and 4 °C for 16 hours. The contents of each flask were then centrifuged at 13,000 rpm for 20 minutes and filtered through a 0.22 µm membrane (MILLEX® GP). Finally, the filtrate was stored at 4 °C until use.

***Pseudomonas syringae* pv. *phaseolicola* (PSPH).** The PSPH-1448A strain was provided by the Phytopathology Laboratory at CIATEJ. This strain was originally isolated in 1985 in Ethiopia from *P. vulgaris* plants showing symptoms (Teverson, 1991) and has since been used as a model strain in various studies due to its virulence and pathogenicity at both physiological and genetic levels (Joardar *et al.*, 2005; Arnold *et al.*, 2011). To confirm its effectiveness, preliminary pathogenicity and virulence tests were conducted in this study on bean plants, which developed disease symptoms (data not shown). The strain was reactivated and cultured in King B broth under agitation at 200 rpm at 24 °C for 36 hours, then centrifuged for 5 minutes at 10,000 rpm. The pellet was washed with sterile deionized water, resuspended in sterile deionized water, and adjusted to an optical density of OD600 = 2 (2×10^7 CFU mL^{-1}). At the end of the experiment, the pathogen was re-isolated from diseased bean plant tissue and stored at 4 °C in PDA plates, and later preserved in glycerol at −20 °C.

Application of actinomycete extract against *Pseudomonas syringae* pv. *phaseolicola* (PSPH). At 56 days after bean sowing, each plant in the respective treatments (one plant per experimental unit), was sprayed with 2 mL of the PSPH 1448A strain suspension (2×10^7 CFU mL^{-1}). Eighteen hours after inoculation with the phytopathogenic bacterium (PSPH), the plants were sprayed with 2 mL of the extract from actinomycete strains ED65, ED66, and ED67.

Experimental design. The experimental unit consisted of one bean plant in a pot, with six replicates per treatment. All experimental units were arranged in a completely randomized design. The treatments evaluated were as follows: T1) ED65 + Ps1448A, T2) ED66 + Ps1448A, T3) ED67 + Ps1448A, and two controls: a positive control, T4) diseased plant (DP), in which only the phytopathogenic bacterium was sprayed without any actinomycete extract; and a negative control, T5) healthy plant (HP), in which sterilized water was sprayed without any actinomycete extract or phytopathogenic bacterium, ensuring all plants received the same handling.

Response variables. Sixteen days after inoculation with PSPH-1448A, disease symptoms and progression in the plants were recorded based on the number of spots per leaf, the number of necrotic spots on the leaves, and the number of defoliated leaves. Disease severity was determined using a seven-level qualitative scale (0–6), ranging from plants with no foliar symptoms (level 0) to plants dead due to the disease (level 6). The scale was based on the visual description of the disease according to the presence of characteristic chlorotic halos and necrosis on the leaf surface (Candelas-Delgado, 2017).
Statistical analysis. Quantitative data were analyzed using analysis of variance and Tukey's test at a 95% confidence level. Severity scale data, being qualitative, were subjected to a Kruskal–Wallis analysis and median interval at 95%. All analyses were performed using the Statgraphics Centurion XVII statistical package.

RESULTS AND DISCUSSION

The symptoms caused by *Pseudomonas syringae* pv. *phaseolicola* in bean plants, reflected in the number of leaves with necrotic spots (Figure 2), and the number of spots per leaf (Figure 3), were significantly reduced ($P<0.05$) by the application of the ED65, ED66, and ED67 extracts compared to the positive control with the bacterium (diseased plants).

Sixteen days after inoculation with the pathogen, the number of leaves with necrotic spots was reduced by 62% in plants treated with actinomycete extracts compared to the diseased plant treatment. Additionally, plants treated with ED66 showed a reduction in the number of spots per leaf (Figure 3) that was statistically similar to the plants not inoculated with the pathogen (healthy plants).

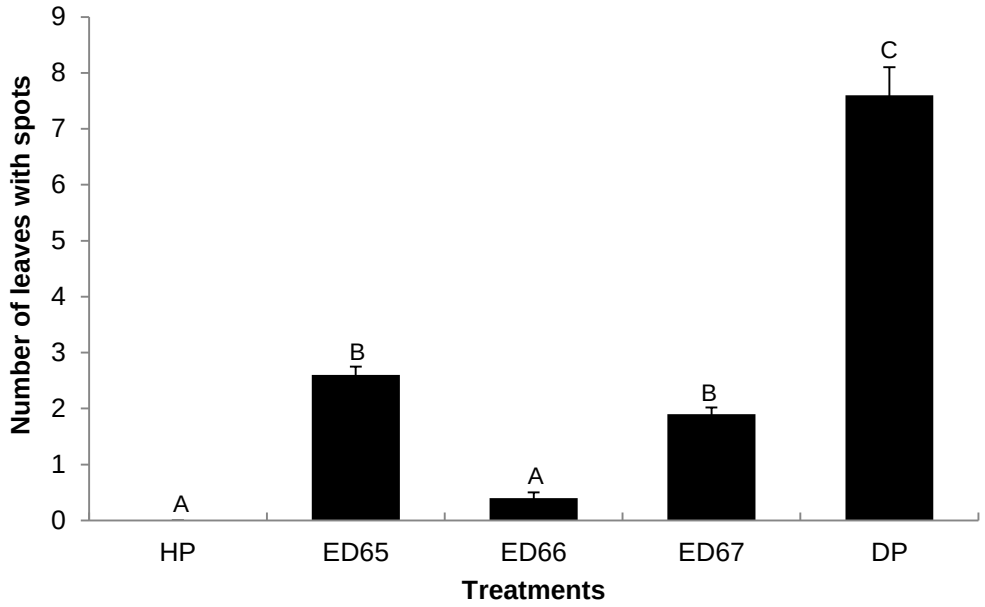

Figure 2. Number of leaves with necrosis at 16 days after inoculation with *Pseudomonas syringae* pv. *phaseolicola* in bean plants. DP = diseased plant; HP = healthy plant. Different letters above the bars indicate statistically significant differences (Tukey, $P<0.05$). The line above the bars (⊥) represents the standard error.

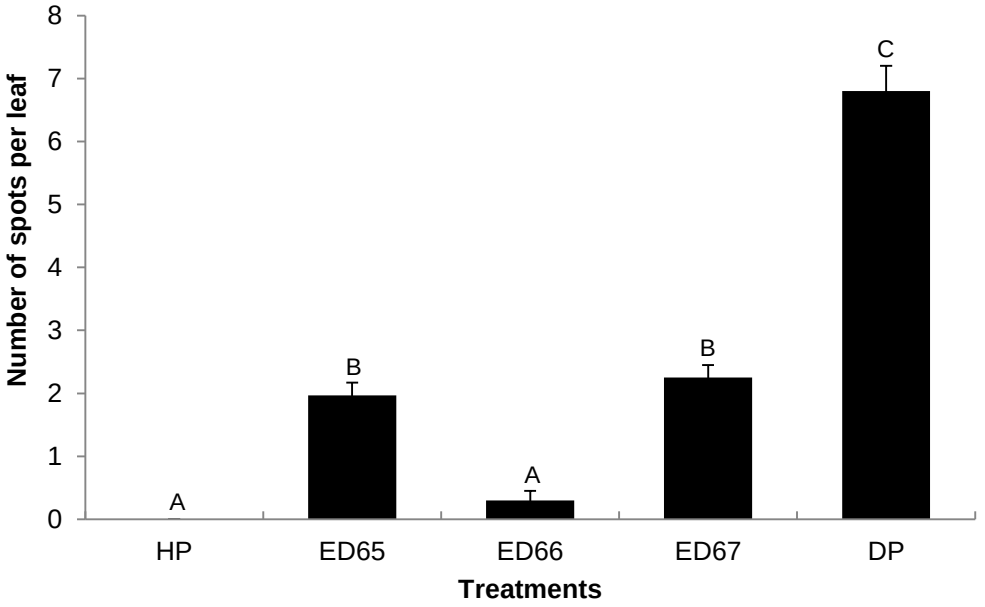

Figure 3. Number of spots per leaf at 16 days after inoculation with *Pseudomonas syringae* pv. *phaseolicola* in bean plants. DP = diseased plant; HP = healthy plant. Different letters above the bars indicate statistically significant differences (Tukey, $P<0.05$). The line above the bars (⊥) represents the standard error.

Spraying with ED67 and ED65, resulted in a similar number of spotted leaves among treated plants, averaging 56% fewer spots than those observed in diseased plants (positive control). Based on this, it is possible that the reduction in necrosis was due to the effect of metabolites present in the extract used (Azman *et al.*, 2017; Zang *et al.*, 2023; Wen *et*

al., 2024). These metabolites may limit the spread, infection capacity, and establishment of the pathogen within the plant, preventing its reproduction in the apoplast and the appearance of symptoms (O'Leary *et al.*, 2016). Another possible mechanism is the induction of the plant's systemic defense system through the synthesis of phaseolin, phytoalexins, and other phenolic compounds triggered by the foliar application of actinomycete extracts, which may contain propagules and exert an indirect biocontrol mechanism against the disease (Sangiogo *et al.*, 2018; Hata *et al.*, 2021).

The number of spots per leaf showed a similar trend across all treatments compared to the number of leaves with spots, with comparable numerical values. Leaves from plants not treated with the extracts had an average of seven spots per leaf at 56 days after inoculation with the pathogenic bacterium. In contrast, application of ED65, ED66, and ED67 reduced the number of spots per leaf to an average of 2.5. This represents a 75% reduction compared to diseased plants and indicates a pathogen control effect from the application of actinomycete extracts (Figure 2). Spraying with ED66 resulted in a reduction in the number of spots per leaf that was statistically similar to that observed in healthy plants without the pathogen. Based on this, the antimicrobial activity of the evaluated strains proved effective for controlling PSPH, possibly due to the presence of bactericidal compounds in the tested extracts. Similar results have been reported for strains of different *Streptomyces* species such as *S. lydicus* (Atta *et al.*, 2015), *S. bingchenggensis* (Jin *et al.*, 2020), and *S. peucetius* (Nguyen *et al.*, 2021).

In the number of defoliated leaves, the analysis showed statistically significant differences among treatments ($P < 0.01$). In plants treated with ED65 and ED67 extracts, the number of defoliated leaves increased, indicating a possible herbicidal effect on the plants (Figure 4).

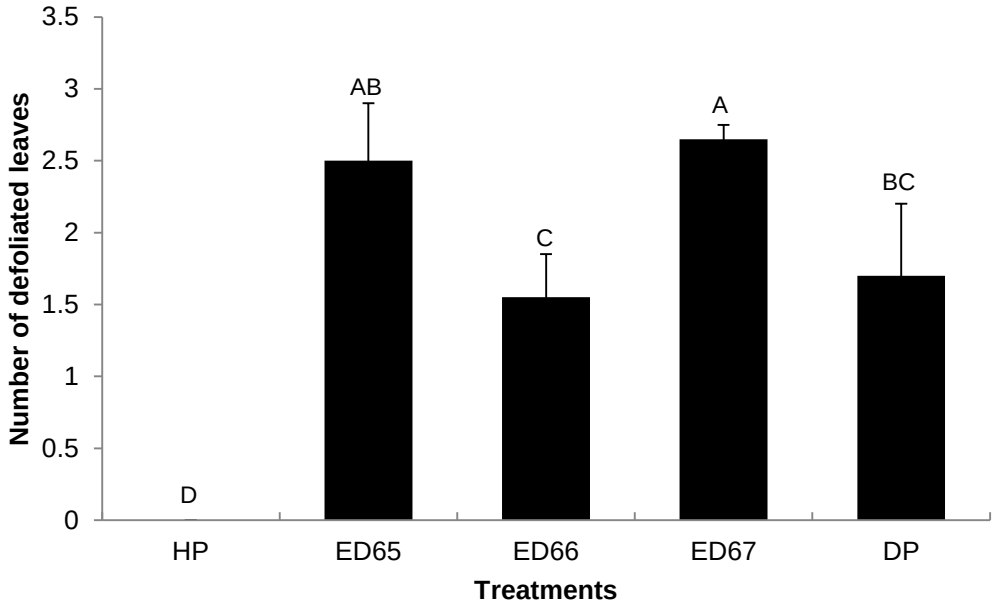

Figure 4. Number of defoliated leaves in bean plants at 16 days after inoculation with *Pseudomonas syringae* pv. *phaseolicola*. DP = diseased plant; HP = healthy plant. Different letters above the bars indicate statistically significant differences (Tukey, $P<0.05$). The line above the bars (I) represents the standard error.

On the other hand, treatment with the ED66 extract showed defoliation levels similar to the plants inoculated with PSPH. This may be due to potential toxicity that caused premature leaf drop and tissue burn (Kim *et al.*, 2020). Some *Streptomyces* species, such as *S. scopuliridis*, have been reported to possess herbicidal properties (Lee *et al.*, 2013). In this study, the results suggest that the extracts could have a negative effect on the plant by reducing the leaf area needed for photosynthetic processes (Bo *et al.*, 2019). Therefore, further evaluations are needed to assess the level of phytotoxicity of the filtered extracts. One alternative for harnessing the antimicrobial metabolites present in these strain extracts as a potential tool for PSPH control is to determine the minimum inhibitory dose of the pathogen, which may avoid causing phytotoxicity (Julian *et al.*, 2020). Another strategy is the characterization and isolation of the bioactive compounds contained in the extracts to identify the compound with the greatest antimicrobial efficacy without causing phytotoxic effects on the plant (Tian *et al.*, 2017).

On the other hand, for the severity level caused by PSPH, the Kruskal–Wallis analysis showed statistically significant differences among treatments (statistic = 22.0906, P = 0.0002) (Figure 5).

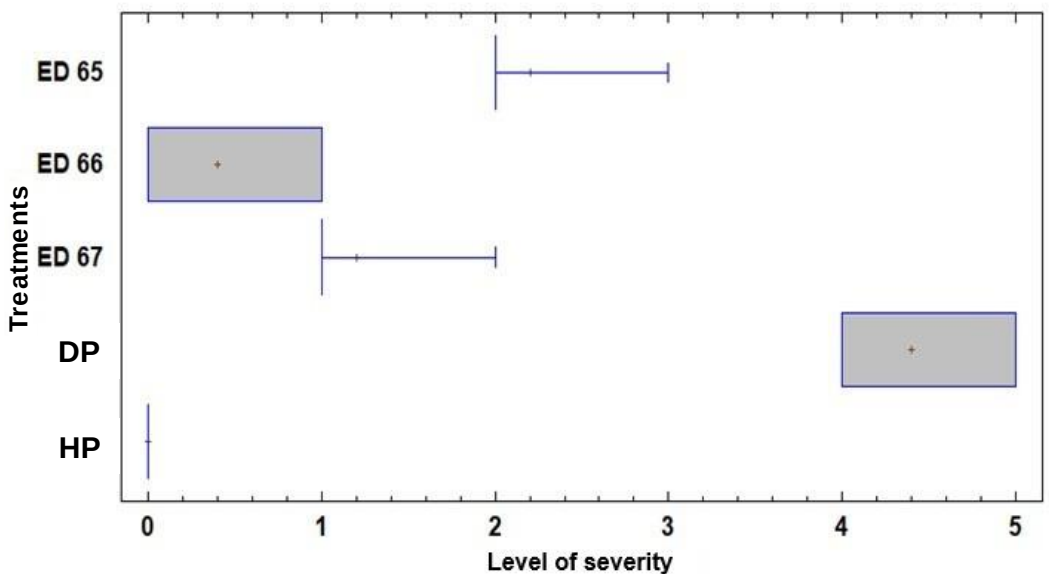

Figure 5. Severity level of halo blight in *Phaseolus vulgaris* at 16 days after inoculation with *Pseudomonas syringae* pv. *phaseolicola*. DP = Diseased plant; HP = Healthy plant.

Plants inoculated only with the phytopathogen (diseased plant), showed the highest severity levels (levels 4 and 5 on the scale). In contrast, treatments with ED65, ED66, and ED67 exhibited lower severity levels than the treatments inoculated solely with the pathogen. Plants treated with the ED66 extract showed few disease symptoms, reflected in the presence of only a few chlorotic and necrotic spots on the leaves. The ED67 extract resulted in a severity level of 1, with minimal chlorosis and necrosis, while the ED65 extract showed a severity level of 2. In plants inoculated with the bacterium, symptoms appeared on the basal leaves despite complete spraying with the extracts (Figure 6). This may be due to the fact that basal leaves translocate nutrients to younger leaves, increasing

their susceptibility (Dordas, 2008). Moreover, appropriate environmental conditions are required for disease development, so that the pathogen can develop and cause symptoms similarly to field conditions, which typically involve relative humidity near 100% and temperatures between 16 and 20 °C (Hirano and Upper, 2000; Arnold *et al.*, 2011; Xin *et al.*, 2016; Sun *et al.*, 2017). Some of these conditions, particularly temperature, did not match the experimental conditions of the present study.

Figure 6. Damage caused by halo blight in bean plants at 16 days after inoculation with *Pseudomonas syringae* pv. *phaseolicola* in plants treated with actinomycetes (ED67, ED66, and ED65). HP = healthy plant (negative control); DP = diseased plant (positive control).

CONCLUSIONS

The actinomycete extracts evaluated in this study showed antimicrobial activity against PSPH, particularly the extract from isolate ED66, which reduced halo blight symptoms in bean plants by more than 90%, as reflected in reduced foliar damage. Although the extracts exhibited phytotoxicity on the leaves, further evaluations are possible to isolate and characterize the bioactive compounds and explore their potential use in the development of control products against PSPH.

Limitations

The results of this research are limited to the working conditions described in the methodology and to the strains used.

Conflict of interest

The authors explicitly declare that there is no conflict of interest related to this research.

Acknowledgments

The authors thank the Scientific Research Program 2022, 2023, and 2024 of the Scientific Research Coordination of the Universidad Michoacana de San Nicolás de Hidalgo; the support of ICTI Michoacán through the program Support for the Strengthening of Scientific, Humanistic, Technological, and Innovation Capacities in Michoacán (Agreement FCCHTI23_ME-4.1.-0011UMSNH/DVSS/77/2023); and the various research projects of the Phytopathology Laboratory of the Plant Biotechnology Unit at CIATEJ for the funding provided for this research.

Author Contributions

Original idea, E.E.Q., G.R.E., L.L.P.; methodology, E.E.Q., G.R.E., L.L.P., J.C.R.A.; sampling and data analysis, J.C.R.A., G.R.E., L.L.P.; initial writing and editing, J.C.R.A., A.R.T., L.L.P.; supervision, G.R.E., E.E.Q.A.; final writing and editing, L.L.P., A.R.T.; funding acquisition, G.R.E., E.E.Q.A., L.L.P. All authors have read and approved the published version of the manuscript.

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