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Article

Pseudomona viridiflava pathogenicity in en *Agave inaequidens* e and inhibition with essential oils

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Abstract

The bacterium *Pseudomonas viridiflava* (Burkholder, 1930) causes economic damage in beans (*Phaseolus vulgaris* L.); it has been shown that it can affect more than 50 hosts (Saygili *et al*., 2008), among which are alfalfa (*Medicago sativa*), apple tree [*Malus pumila* var. *domestica* (Borkh.) CK Schneid.] (Alimi *et al*., 2011), and broccoli (*Brassica oleracea* L.); however, in *Origanum vulgare*, there is no report of any damage on agave mezcalero (*Agave inaequidens* K.Koch), a species still little studied; this research was based on the evaluation of the effects of the bacterium *Pseudomonas viridiflava* in the process of imbibition in seeds of *Agave inaequidens*; on the other hand, the tolerance of the bacterium to the essential oils of cinnamon (*Cinnamomum verum* Blume), clove (*Syzygium aromaticum* L.), and oregano (*Origanum heracleoticum* Rchb.) was evaluated, as preliminary studies for the ecological, organic, agrochemical-free management strategy in crops affected by *Pseudomonas viridiflava*; it was found that the concentration of 25% of oregano and cloves oil was efficient in the development of the bacterium under *in vitro* conditions; in contrast, in the germination process based on the imbibition of the seed, there was no reduction in the percentage of germination or signs of damage due to the effect of the bacteria.

Keywords:

Agave inaequidens, Agave mezcalero, Pseudomona viridiflava, biological control.

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Introduction

The bacterium *Pseudomonas viridiflava* (Burkholder, 1930) is a gram-negative species with a wide range of hosts and causes symptoms on stems, leaves, and flowers. Although it is considered a phytopathogenic species, it can live as an endophyte, epiphyte, and saprophyte (Lipps and Samac, 2022). In Mexico, it is important in crops of vegetables and fruits such as tomato (*Solanum lycopersicum* L.) (Jones *et al*., 1984), pepper (*Capsicum annum* L.), melon (*Cucumis melo* L.), beans (*Phaseolus vulgaris* L.), lettuce (*Lactuca sativa* L.), grapes (*Vitis vinifera* L.) and citrus (Beiki *et al*., 2016; Al-Karablieh *et al*., 2017).

Pseudomonas viridiflava belongs to the complex of *Pseudomonas syringae* species of economic importance (Bartoli *et al*., 2015); this bacterium forms creamy white colonies, and most species produce fluorescent pigment in King's B medium (Aksoy *et al*., 2017). The isolates are positive for potato soft rot and hypersensitivity reaction in tobacco and negative for the production of levana, oxidase and arginine dihydrolase activity, so it is considered to belong to group II of LOPAT (Levana-Oxidase-Potato Rot-Arginine dihydrolase-Hypersensitivity in Tobacco) (Lelliott *et al*., 1987; Tsuji and Fuji, 2021).

Pseudomonas viridiflava was considered a pathogen with a high potential to infect new hosts (Ramírez *et al*., 2022); therefore, an increase in new hosts affected by *P. viridiflava* has been recorded; for this reason, we considered the evaluation of pathogenicity in seeds and seedlings of *Agave inaequidens*, a species used for the production of artisanal mezcal; in addition, the inflorescence is also consumed, the sap is used for fermented beverages, pulque and its inulin and various parts of the plant are used for medical purposes; nevertheless, it is a species with little research yet.

Agave inaequidens is a native species and is distributed in Jalisco, Durango, Sinaloa, Hidalgo, Colima, Michoacán, State of Mexico, and Morelos in the Mexican Republic, at an altitude of 1 400 and 3 000 m (Torres *et al*., 2019).

This species is threatened by excessive extraction for mezcal production. The most severe threat is located in the state of Michoacán and continues to increase. This species is found in several protected areas, such as Sierra de Manantlán Biosphere Reserve and Monarch Butterfly Biosphere Reserve (Torres *et al*., 2019); however, this did not detract from the protection of the species to prevent its extinction.

On the other hand, chemicals such as streptomycin and oxytetracycline wettable powder plus validamycin A wettable powder (Tae, 2019), which are risky for food safety, continue to be used to control diseases caused by *Pseudomonas viridiflava* (Morris *et al*., 2019). The objective was to characterize and identify a national strain of *Pseudomonas viridiflava*, the possible pathogenicity on *Agave inaequidens* K. Koch in the seed germination stage, and the *in vitro* sensitivity of the bacterium to essential oils of cinnamon (*Cinnamomum verum* J. Presl), clove (*Syzygium aromaticum*), and oregano (*Origanum vulgare* L.) in concentrations of 25, 50, 75, and 100%.

Materials and methods

This research was conducted in the Phytopathogenic Virus Laboratory of the College of Postgraduates, Montecillo *Campus*, Texcoco, State of Mexico.

Isolation of the *Pseudomonas viridiflava* strain

The strain of the bacterium *Pseudomonas viridiflava* is of Mexican origin (national strain); it was activated from a tube preserved in refrigeration in King's B (KB) culture medium and kept in incubation in dark conditions at a temperature of 27 °C ±1 °C.

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Physiological and biochemical characterization

The isolate was reviewed in ultraviolet light (25 W TFL-40 Transilluminator, California, USA), then characterized by the Lopat determinative test (Lelliot and Stead, 1987) and according to the protocols described by Schaad *et al*. (2001).

Bacterial DNA extraction

For DNA extraction, 2% CTAB was used; for identification at the molecular level, 600 µl of 2% CTAB buffer was placed in an Eppendorf® tube, and then a loop of the pure bacterial culture with a growth of 24-48 h in (KB) medium was introduced. It was incubated at 56 °C for 30 min. Then, 400 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added, vortexed for 10 s, and centrifuged at 13 500 rpm for 10 min at 4 °C. From the aqueous phase, 500 µl was recovered in a new tube, and 50 µl of 7.5 M ammonium acetate and 500 µl of isopropanol were added. The mixture was done by immersion. It was kept refrigerated at -20 °C for 1 h.

The centrifuge was set to 13 500 rpm, 10 min at 4 °C, and the supernatant was discarded with caution; it was washed with 800 µl of 80% ethanol and centrifuged at 13 500 rpm for 3 min at 4 °C, the 80% ethanol was discarded again and centrifuged at 13 000 rpm for 3 min at 4 °C, ethanol was discarded for the second time, and the pellet was dried for 30 minutes. Finally, the pellet was resuspended in 50 µl of nuclease-free water and stored at -20 °C. The optimal parameters of nucleic acid concentration and purity were evaluated by spectrophotometry with the help of a NanoDrop 2000 (Thermo Fisher Scientific 2000, USA).

Molecular identification of *Pseudomonas viridiflava* by PCR

Genomic identification was performed by amplifying the 16S ribosomal DNA (rDNA) gene, which generated a fragment of 1 500 bp (Weisburg *et al*., 1991). In a final sample of 10 µl composed of 2 µl Buffer PCR 1X, 0.8 µl of $MgCl_2$ (50 Mm), 0.2µl of dNTPs (10 mM), 0.2 µl of each of the primers FD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGC TCAG-3´)/rP1 (3' CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT-5´), 0.1 µl of GoTaq® DNA Polymerase (Promega), 2 µl of total DNA (50 ng μl^{-1}) and 4.7 µl nuclease-free water. PCR was performed in a MiniAmp™ thermal cycler (Thermo Fisher Scientific), and the amplification conditions were initial denaturation at 95 °C for 2 min, 25 cycles at 95 °C for 2 min, annealing at 42 °C for 30 s, extension at 72 °C for 4 min, and final extension at 72 °C for 5 min. The amplified fragments were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized with UV light in a documentation system.

Biochemical tests for the identification of *Pseudomonas viridiflava*

Gram stain and a 3% KOH test were performed (Arai, 2001). The oxidase test was performed as described by Goszczynska *et al*. (2000). For the determination of oxidative/fermentative metabolism, the methodology indicated by Hugh and Leifson (1953) was followed. The reduction of nitrates to nitrites was carried out as indicated by Hayward *et al*. (1991) and was recorded at 3, 5, and 7 days. The production of arginine dihydrolase was carried out as proposed by Thornley (1960). Starch, phosphatase, and levana hydrolysis were recorded after 48 h. The liquefaction of gelatin was carried out according to what was proposed by Hayward (1994), while the production of indole was carried out by taking a record at 3 and 5 days. All tests were conducted at 27 °C ±1 °C.

Tobacco hypersensitivity reaction test

Bacterial exudate from a pure culture with a growth of 48 h in (KB) medium was used, from which a suspension was made at a cell density of 10^8 CFU/ml, which was compared with the McFarland standard. With the help of a hypodermic syringe plunger (3 ml), the cell suspension was infiltrated into the underside of the tobacco leaf. Through infiltration, it was possible to invade the intercellular spaces of the leaf mesophyll, so the appearance of moisture was observed within the leaf blade.

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Inoculation was conducted under laboratory conditions with natural light, low relative humidity, and average temperatures of 25 °C. Collapsed tissue was evaluated 24 hours later.

Rot test in potato tubers

A moist Sanita was placed at the bottom of a Petri dish, and then two slices of potato tubers were placed; using a sterile dissection needle, bacterial exudate was taken and placed in the center of the potato slice, making an incision; in the other slice, the incision was made but without adding bacterial exudate; it was reviewed 24 h later.

Pathogenicity tests on *Agave inaequidens*

Pathogenicity was evaluated in *Agave inaequidens* seeds at different imbibition times in 1 500 µl of a bacterial suspension at 10^8 CFU mL^{-1} (30 min, 2 h, 4 h, 6 h and 8 h). Twelve seeds were used per treatment, and the same number of seeds were used in the control treatment for each imbibition time; after the imbibition time, the seeds were placed in two-compartment Petri dishes, a moist Sanita with 3 ml of sterile distilled water was placed at the base and they were kept in dark conditions in an incubator at a temperature of 27 °C ±1 °C until the appearance of the radicle; they were subsequently placed in natural light conditions at room temperature.

Cinnamon, clove, and oregano essential oil sensitivity test

Under aseptic conditions, glass Petri dishes measuring 180 x 30 mm were used to which 50 ml of solid nutrient agar medium was added, and they were left to solidify. A bacterial suspension was prepared at 10^8 CFU ml^{-1}, and 300 µl was added to the medium and dispersed with a plastic bacteriological loop; the bacterial solution was left to impregnate the medium.

Subsequently, five circles of filter paper of 0.5 mm diameter were placed, which were impregnated with the essential oils of cinnamon, clove, and oregano in concentrations of 25, 50, 75, and 100%, with the help of the formula Vi Ci= Vf Cf, (C= concentration; V= volume; i= initial; and f= final) (Table 1). The oils were emulsified with 25% ethanol. Each Petri dish worked as a treatment with five replications; the development data of the four cardinal points of each filter paper was recorded.

Table 1. Doses of cinnamon, cloves, and oregano essential oils are used separately to measure the sensitivity of *Pseudomonas viridiflava*.

Treatment	Dose
Control	25% alcohol
Oil (cinnamon, clove, and oregano) 100%	3 ml undiluted
Oil (cinnamon, clove, and oregano) 75%	2.25 ml + 0.75 ml of 25% ethanol
Oil (cinnamon, clove, and oregano) 50%	1.5 ml+ 1.5 ml of 25% ethanol
Oil (cinnamon, clove, and oregano) 25%	0.75 ml + 2.25 ml of 25% ethanol

Data were taken 48 h after the experiment was established and analyzed in the SAS statistical package, version 9.0, in a completely randomized Anova with Tukey's mean separation.

Results and discussion

Characteristics of the colony of *Pseudomonas viridiflava* national strain

The physiological and biochemical characterization of the national strain of *Pseudomonas viridiflava* showed high similarity with the metabolic profile described by Heydari *et al*. (2012); Sarris *et al*. (2012). By means of the Lopat test, the national strain did not produce fluorescent pigments in KB medium; nevertheless, it induced a hypersensitivity reaction in tobacco leaves, presented negative

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results for oxidase and arginine dihydrolase, did not produce levana, and generated maceration in potato tuber slices (Harzallah *et al*., 2004). According to Lelliot and Stead (1987), *Pseudomonas viridiflava* belongs to *Pseudomonas* group II (Table 2).

Table 2. Physiological and biochemical characterization of *Pseudomonas viridiflava*.

Test	Result
Gram stain	-
Fluorescence	-
Levana	-
Oxidase	-
Potato rot	+
Hypersensitivity in tobacco	+
Catalase	+
Gelatin hydrolysis	+
Nitrate reduction	-
Starch hydrolysis	-
Oxidative/fermentative	o
Glucose	+
Lactose	+
Maltose	+
Trehalose	+
Inositol	+
Sorbitol	+

Hypersensitivity reaction in tobacco

The tobacco leaf blade that was infiltrated with the bacterium suspension presented lesions 48 h after inoculation.

Rot in potato tubers

The potato tubers evaluated present rot 24 h after inoculation; the degradation of the plant tissue was observed; when touched with the dissection needle, the tissue was soft, and there was necrosis, as well as an advance in the natural oxidation process of the tissue.

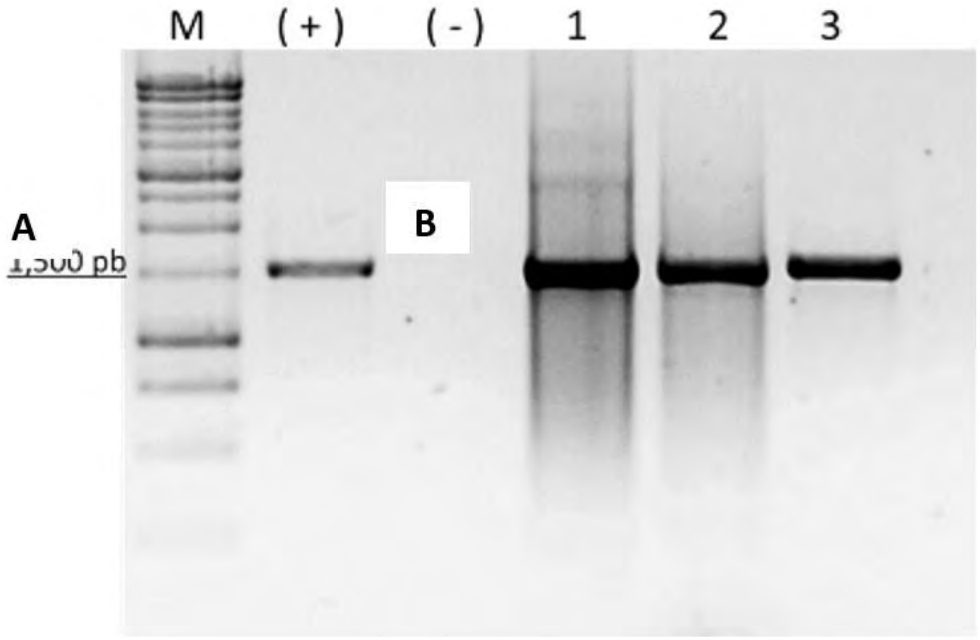
Molecular identification

By extracting DNA from the bacterium under study, it was possible to obtain the amplification of the 16 S gene with 1 500 bp. Various studies have considered the risk of establishing phylogenies based on sequences of a single gene; however, the 16 S gene is the most widely used for bacterial identification (Lieckfeldt *et al*., 2000) (Figure 1).

Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v15i8.3841

Figure 1. 1.5% agarose gel electrophoresis of PCR products obtained with universal oligonucleotides FD1/rP1 (~1 500 bp) from phytopathogenic bacteria. Amplification of the 16 S ribosomal gene, lanes: (M) molecularmarker 1 Kb, (+) positive control, (-) negative control (H_2O). 1) control *Pseudomonas marginalis*; 2) *Pseudomonas viridiflava* and 3) control *Clavibacter michiganensis* sub. *michiganensis*.



Effect of *Pseudomonas viridiflava* on the germination process of *Agave inaequidens*

The seeds of *Agave inaequidens* that were imbibed with the bacterial solution at different times did not reduce their germination percentage due to the effect of the development of the bacterium *Pseudomonas viridiflava*. On the third day of the imbibition of the seeds with the bacterial suspension, the first seeds began to appear with the emergence of the radicle; nonetheless, most were in the region of the controls. By day 12, 96% of the *Agave inaequidens* seedlings had developed without the presence of damage caused by the bacterium *Pseudomonas viridiflava* (Figure 2). Therefore, it was considered that the bacterium does not affect the germinative development of *Agave inaequidens*.

Revista Mexicana de Ciencias Agrícolas

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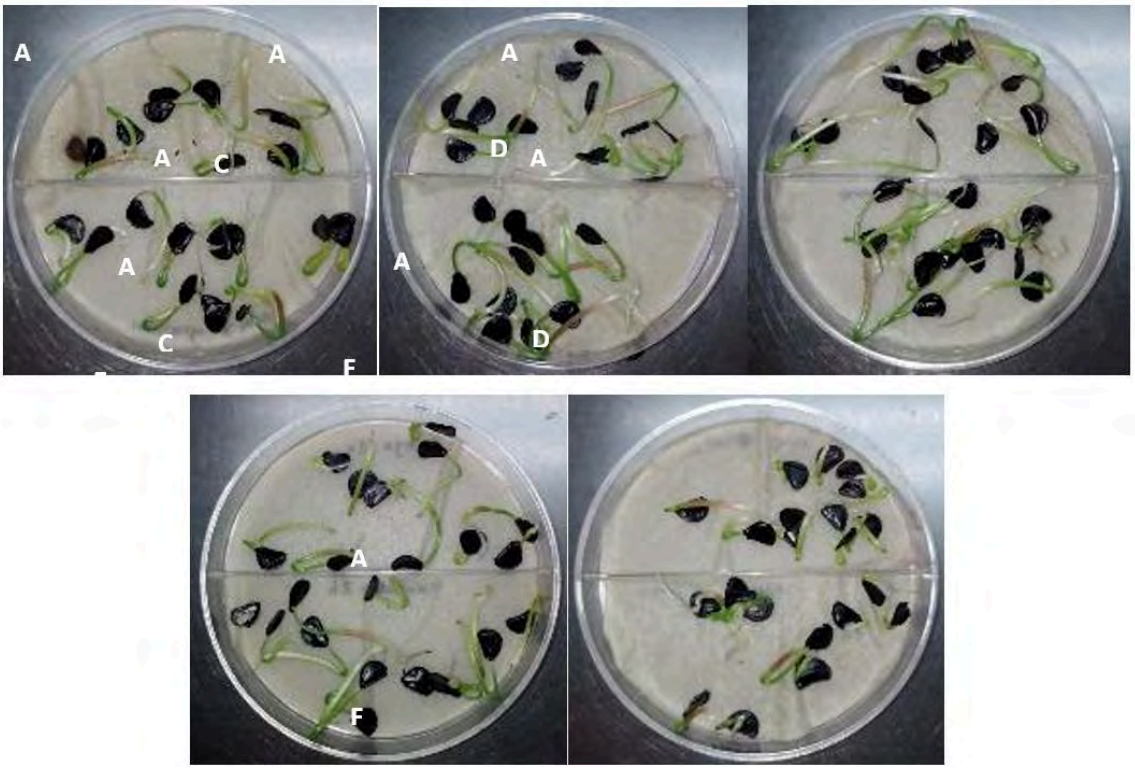
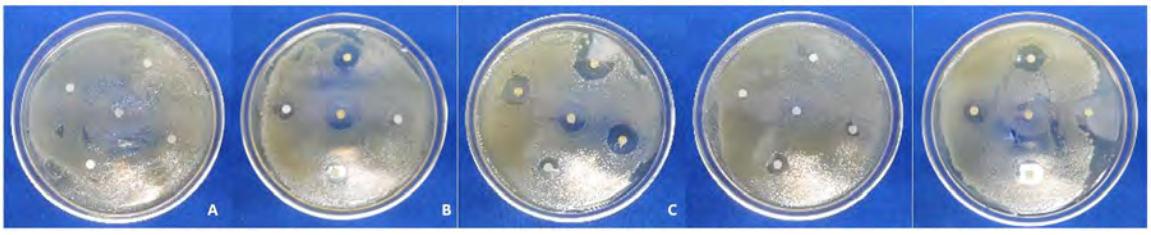
Figure 2. *Agave inaequidens* seeds inoculated on day 12 with a suspension of the bacterium *Pseudomonas viridiflava* at different imbibition times: A) controls at each time; B) 30 min; C) 2 h; D) 4 h; E) 6 h and F) 8 h.



Sensitivity of *Pseudomonas viridiflava* to essential oils

The cinnamon oil treatments in their four different concentrations presented statistical differences between the highest dose (100%) and the lowest (25%); however, an important fact is that the dose of 25% concentration was the most effective despite what one would think due to the degree of concentration of the compounds present in the oil, which are cinnamaldehyde and eugenol; nevertheless, the importance of oil being emulsified so that it is activated at a less dense concentration to obtain a better control response is highlighted (Figure 3).

Figure 3. Evaluation of cinnamon (*Cinnamomum verum* J. Presl) oil on the development of *Pseudomonas viridiflava* in a solid medium (nutritive agar), with the treatments: A) control; B) 100%; C) 75%; D) 50% and E) 25%.



Plant-based essential oils have been shown to have antimicrobial, antioxidant, antiparasitic, anti-inflammatory, antidiarrheal, and antifungal properties in humans (Van-Zyl *et al*., 2004). These results may be associated with the presence of eugenol and cinnamaldehyde in essential oils, which directly affect the bacterial membrane by inhibiting the growth of fungal and bacterial

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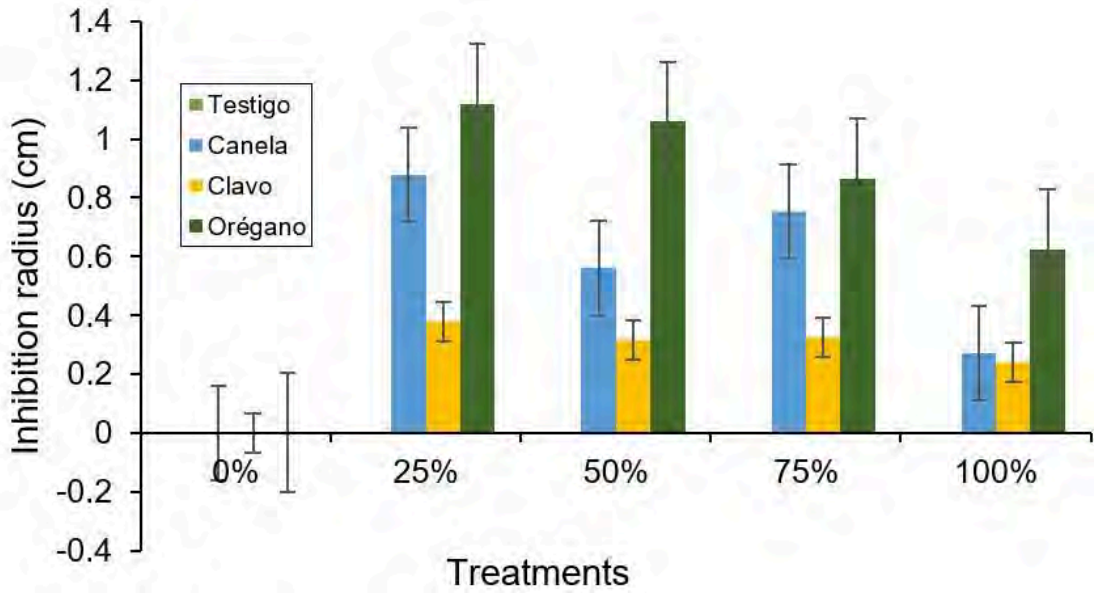
microorganisms (Cava *et al*., 2012). For their part Tong *et al*. (2005) determined that essential oils can inhibit the tricarboxylic acid (TCA) cycle of bacterial respiration metabolism, affecting the oxygen consumption of bacteria and causing their death.

The clove treatments at the analyzed doses behaved statistically similarly; the main chemical component in their oil was eugenol, a compound mentioned above in cinnamon.

Oregano oil in the four different concentrations achieved the highest inhibition radius of bacterial development; it is worth noting the inhibition capacity at the lowest concentration, which is an important contribution by reducing application costs in the case that it is effective under *in situ* conditions (Figure 4).

Figure 4. Growth inhibition radius of *Pseudomonas viridiflava* due to the effect of the essential oils of cinnamon, cloves and oregano in concentrations of 25, 50, 75, and 100%.



Using essential oils from plant species provides a positive alternative to antibiotics that can generate resistance and harm health (Esquivel *et al*., 2010). Caballero *et al*. (2016) showed that clove (*Syzygium aromaticum*) and cinnamon (*Cinnamomum zeylanicum*) essential oils at concentrations of 0.05, 0.1 and 0.2% inhibit the growth of fungal microorganisms such as *Aspergillus flavus* (Erosa *et al*., 2021), which is why the present research suggests experimentation with other doses of the oils to reach the ideal concentrations.

Conclusions

Pseudomonas viridiflava did not generate pathogenicity in the seeds of *Agave inaequidens*; for their part, the oils that caused a greater inhibition of the development of the bacterium were oregano at 25%, followed by cinnamon at 25%, so they are considered candidates to be evaluated in greenhouse conditions and open-field plantations in the main hosts of the bacterium.

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