Identification of the Causal Agent of Dieback in Blueberry (*Vaccinium corymbosum*) and Its *In Vitro* Control

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ABSTRACT

Objetive:The objective of this study was to isolate, identify, and characterize the causal agent of blueberry (*Vaccinium corymbosum*) dieback and its *in vitro* control.

Design/methodology/approach: Samples were collected from three municipalities in Michoacán with different edaphoclimatic conditions. Using the Agrios protocol, three strains identified as *Pestalotiopsis* sp. were isolated and labeled as CAZr01, CAZh02, and CATg03. The isolates' morphological characteristics were determined. For *in vitro* sensitivity bioassays, the fungicides Robust R®, Tacora 25 Ew®, Cabrio® C, Programic® Mega, and Aliette® Wdg were tested at three doses (low, medium, and high) according to the manufacturer's recommendations, with sterile water serving as the control treatment. The virulence and severity of the isolates were determined through pathogenicity tests on healthy plants. The experimental design was completely randomized, and a Tukey mean comparison test was applied with a 5% probability of error.

Results: The most effective fungicide for controlling mycelial growth was Tacora 25 Ew®, which showed significant inhibition at all tested doses. Of the isolated strains, CAZh02 was the most virulent, causing plant death in the shortest time.

Limitations/implications: This study provides information on the pathogen affecting blueberry and proposes an effective method for its control.

Findings/conclusions: It was found that blueberry dryer is caused by *Pestalotiopsis* sp., which is most efficiently controlled *in vitro* with Tacora 25 Ew®. Among the three isolated strains, CAZh02 was found to be the most virulent.

Keywords: isolates, fungicide effectiveness, Pestalotiopsis.

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INTRODUCTION

Mexico has specialized in the production and export of predominantly blueberries, thanks to the diverse climates and favorable conditions that enhance their growth and yield

[1]. In 2023, blueberry production in Mexico reached 80,133 tons, cultivated across 5,836 hectares, with an average yield of 13.7 tons per hectare and a production value of 5.212 billion pesos [2].

At the national level, Michoacán ranks third with 209 tons of blueberries but generates the highest production value. Within the state, the municipality of Salvador Escalante leads, accounting for 24% of the state's production, followed by Tangancícuaro with 16%, and Los Reyes in third place with 12% of the total production. Ziracuaretiro ranks tenth, contributing 4% of the state's production [2].

In areas where blueberries are cultivated, one of the main production limitations is the prevalence of various diseases, particularly fungal diseases. These significantly impact fruit quality and cause considerable production losses due to the massive death of plants and the decline of orchards. The main fungal pathogens include *Colletotrichum* sp., *Fusarium* sp., and *Alternaria* sp. [3], which cause damage to roots, stems, and leaves.

However, there are other microorganisms of significance that produce similar symptoms and have yet to be identified to establish appropriate control measures. This is the case with blueberries, where symptoms of dieback have been observed, significantly reducing yield and even causing plant death at any age. The objective of this study was to isolate, identify, and characterize the causal agent of blueberry dieback and its *in vitro* control.

MATERIALS AND METHODS

Study Area and Biological Material

Samples were collected from blueberry plants showing symptoms of leaf chlorosis, defoliation, complete branch dieback, and reddish necrosis at the basal part of the stem. These samples were placed in Ziploc® bags labeled with the following information: date, collection order, orchard name, and GPS coordinates from three municipalities in Michoacán, Mexico.

The municipality of Salvador Escalante is located at 19° 24' 23" N and 101° 38' 24" W at an altitude of 2,239 meters; Ziracuaretiro is situated at 19° 21' 31" N and 101° 48' 00" W at an altitude of 2,400 meters; and Tangancícuaro is located at 19° 53' 17" N and 102° 17' 30" W at an altitude of 3,400 meters.

The isolations were performed from stems showing disease symptoms, following the protocol described by [4]. The technique involved washing and disinfecting the stems with 33% sodium hypochlorite, followed by two rinses with sterile distilled water. Five disinfected stem fragments were placed equidistantly in a Petri dish containing Bioxon® PDA culture medium.

For the purification of the isolated strains, hyphal tips were collected using a previously sterilized scalpel. These were then individually transferred to new Petri dishes containing PDA culture medium. Once fungal colonies emerged from the explants and grew on the culture medium, they were transferred again, using hyphal tips, to new dishes with SNA medium for conidia production. After three weeks of incubation, 5 mm diameter discs of SNA medium were taken and placed in test tubes containing 5 mL of sterile distilled water.

The tubes were shaken to obtain a conidial suspension, and 25 μL were taken with a micropipette and deposited at the center of a Petri dish with PDA medium. The suspension

was spread using a previously sterilized inoculating loop and incubated at 25 °C for 24 h. After incubation, 4 mL of sterile 25% glycerol was added, and the suspension was shaken with the micropipette to release the conidia. The solution was then recovered into sterile cryogenic vials and stored at −80 °C until use. From each isolate, 20 μL of conidia suspension in 25% glycerol was inoculated in Petri dishes with PDA, to determine the microscopic and macroscopic characteristics that were obtained with the support of the results obtained by [5] and [6].

In vitro Sensitivity Evaluation to Fungicides

The three isolates were cultured in Petri dishes with PDA medium to obtain young strains, which were then evaluated for *in vitro* sensitivity to the fungicides Robust R® (Benomyl), Tacora 25Ew® (Tebuconazole), Cabrio C® (Boscalid+Pyraclostrobin), Programic Mega® (*Larrea tridentata* extract), and Aliette Wdg® (Fosetil-Al) at three doses: low, medium, and high, along with a control treatment of distilled water. The fungicide doses were determined according to the manufacturer's recommendations. For each strain, a completely randomized experimental design was used with eight repetitions, and two Petri dishes were used for each repetition.

The sensitivity response of the strains to the fungicides was determined based on the final average growth diameter of each strain, measured with a 30 cm long transparent plastic ruler. Measurements were taken every 24 hours, and they were suspended when the mycelium of any treatment reached the filter paper discs.

Pathogenicity Tests

Pathogenicity tests were conducted on healthy blueberry plants of the Biloxi variety with each of the obtained isolates. Three repetitions were performed for each strain, along with a control treatment using distilled water. The substrate used was peat moss mixed with oak soil, sterilized in an autoclave at 121 °C for 60 min. Once the temperature for handling was reached, it was placed in plastic pots that had been previously disinfected with 33% sodium hypochlorite. For inoculation, a mixture was made using the entire Petri dish fully colonized by each of the obtained isolates in 40 mL of sterile distilled water. The mixture was stirred with a mechanical rotor until a homogeneous solution was achieved. Once the inoculum was ready, 5 mL was taken and applied to the root of each plant without causing any damage. For the pathogenicity tests, the variables of severity and virulence were evaluated for each of the isolates. Severity was determined based on the visual symptoms of the foliage, which were scored as follows: 0=healthy plant, 1=plant with yellow leaves, 2=foliar yellowing, 3=apical necrosis, and 4=plant death. Observations were made every 24 hours, starting from the inoculation until the death of the first plant.

RESULTS AND DISCUSSION

From the isolates obtained from material with symptoms of dieback in blueberry plants, three isolates with characteristics matching those reported for the genus *Pestalotiopsis* sp. were obtained. These isolates were assigned the strain codes: CAZr01, CAZh02, and CATg03 (Figure 1).

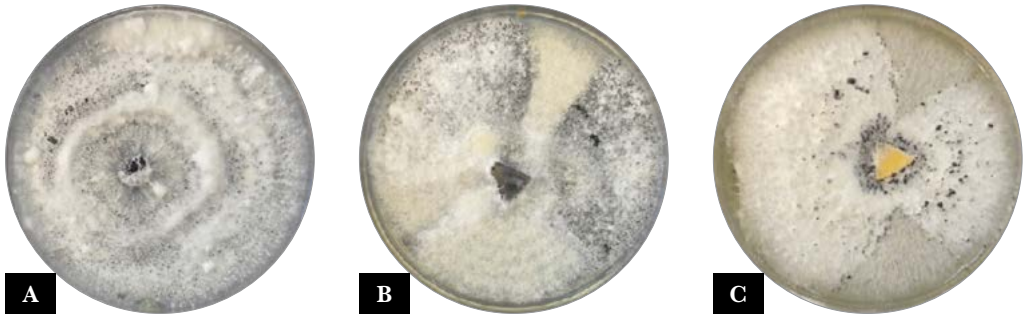

Figure 1. Strains obtained from the samples collected. A) CAZr01 from the municipality of Ziracuaretiro, B) CAZh02 from the municipality of Salvador Escalante, C) CATg03 from the municipality of Tangancícuaro, all from the state of Michoacán.

The strains were identified based on both cultural and microscopic characteristics. Abundant conidia were observed in the different strains from the three municipalities. The conidia had five cells, two hyaline at the ends and three verruculose in the center, as well as the presence of three apical appendages and one basal appendage, except for strain CAZh02, which had two apical appendages and one basal appendage (Figures 2, 3, 4). In terms of macroscopic characteristics, the strains exhibited abundant and cottony mycelial growth that covered the entire Petri dish. The coloration was white-yellowish with the presence of acervuli (black-colored spots). The results of the microscopic and macroscopic characterization of the pathogen align with those reported for the genus *Pestalotiopsis* [7, 8].

Pestalotiopsis species show notable phenotypic diversity and are grouped based on similarities in conidial morphology [5, 8, and 9]. Characteristics such as length, width, pigmentation, and the presence of appendages on the conidia appear to be consistent and useful for the identification of *Pestalotiopsis* [5 and 10]. The conidia of *Pestalotiopsis clavispora*, according to Steyaert [11], are fusiform and straight, with three central versicolor cells, the lower and upper cells being hyaline, and having two to three apical ornamentations, which coincides with the three isolates CAZr01, CAZh02, and CATg03. The conidia showed five cells, three versicolor cells in the center, and two hyaline cells at the ends; with two to three apical ornamentations and one basal ornamentation.

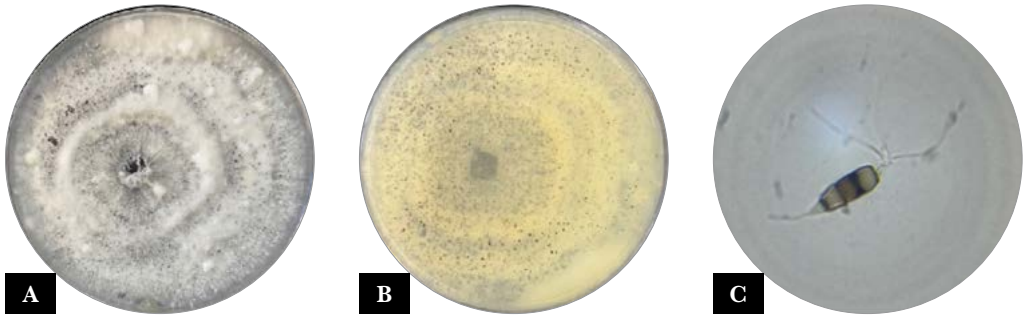

Figure 2. Strain CAZr01 identified as *Pestalotiopsis* sp. grown on PDA nutrient medium: A) Front of the strain, presence of acervuli, B) Back of the strain, C) Conidium of *Pestalotiopsis* sp., 5 cells, 2 hyaline and 3 versicolor, with three apical appendages and one basal appendage.

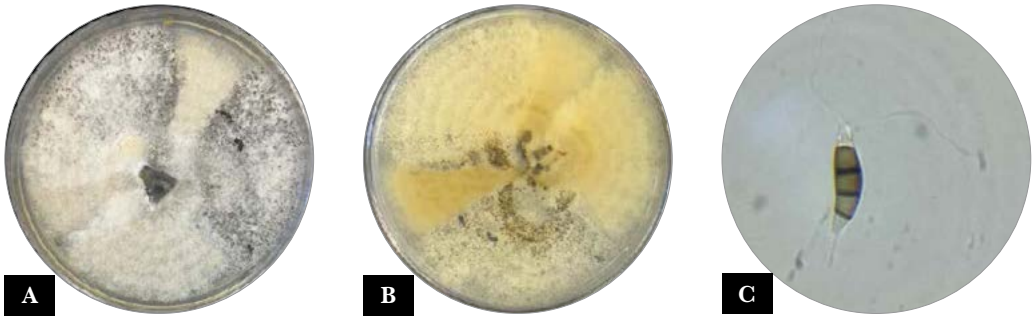

Figure 3. Strain CAZh02 identified as *Pestalotiopsis* sp. grown on PDA nutrient medium: A) Front of the strain, showing the presence of acervuli, B) Back of the strain, C) Conidium of *Pestalotiopsis* sp., with 5 cells: 2 hyaline and 3 versicolor, with two apical appendages and one basal appendage.

Figure 4. Strain CATg03 identified as *Pestalotiopsis* sp. grown on PDA nutrient medium: A) Front of the strain, presence of acervuli, B) Back of the strain, C) Conidium of *Pestalotiopsis* sp., with 5 cells: 2 hyaline and 3 versicolor, with three apical appendages and one basal appendage.

In vitro Sensitivity Bioassay to Fungicides

The *in vitro* sensitivity study of the *Pestalotiopsis* sp. pathogens showed variation. The analysis of variance indicated that there is a significant difference between the treatments of the evaluated fungicides, as indicated below:

In the analysis of variance for the CAZr01 strain, a highly significant difference ($P<0.0001$) was detected for these fungicides. The mean comparison revealed the formation of five groups (Tukey $\alpha=0.05$). The inhibition of mycelial growth of *Pestalotiopsis* sp. with these fungicides ranged from 0.856 to 0.160 cm. The product with the lowest sensitivity to the fungus was Aliette Wdg® at its low dose, as well as Reva Gobex® at its low dose. The highest sensitivity was observed with the fungicide Tacora 25 EW® at its low, medium, and high doses (Figure 5).

For the analysis of variance of the CAZh02 strain, also identified as *Pestalotiopsis* sp., a highly significant difference was detected ($P<0.0001$) among these fungicides. In the mean comparison, six groups were formed (Tukey $\alpha=0.05$), and the inhibition of mycelial growth of *Pestalotiopsis* sp. fluctuated between 1.089 and 0.311 cm. Robust R® presented the least sensitivity at its high, medium, and low doses. In contrast, the fungicide Tacora 25 EW® at its high and low doses was the product that showed the best inhibition of mycelial growth of *Pestalotiopsis* sp. (Figure 6).

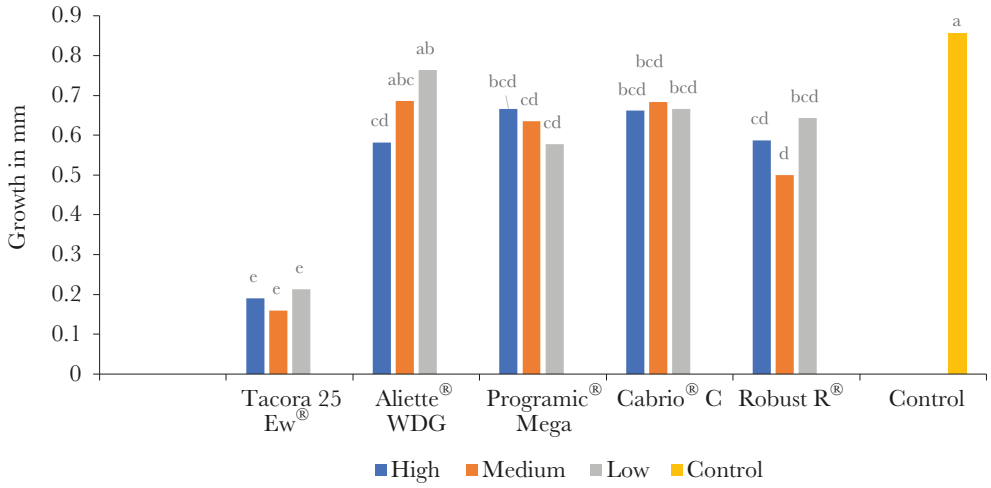

Figure 5. Effect of fungicides on the inhibition of mycelial growth of the CAZr01 strain (*Pestalotiopsis* sp.).

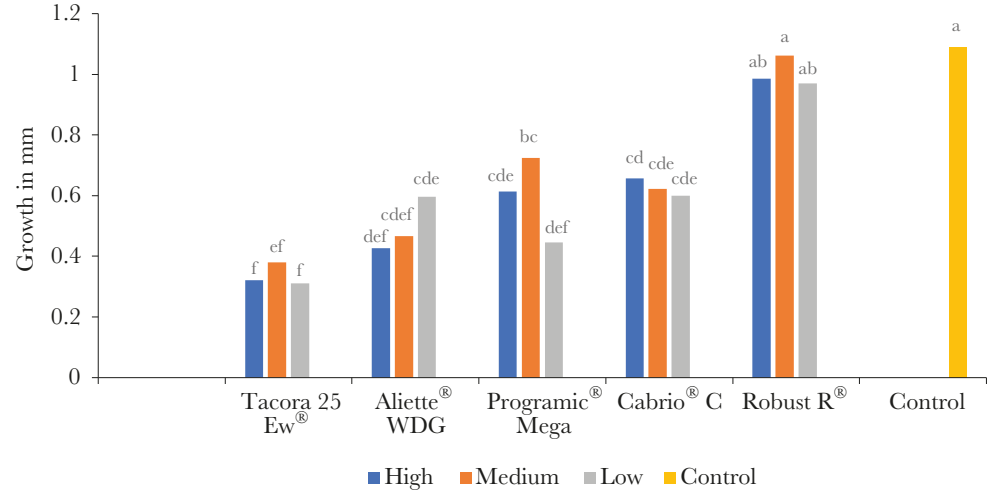

Figura 6. Effect of fungicides on the inhibition of mycelial growth of the CAZh02 strain (*Pestalotiopsis* sp).

The analysis of variance for the CATg03 strain identified as *Pestalotiopsis* sp. showed a highly significant difference ($P<0.0001$) between these fungicides. In the mean comparison, the formation of six groups was observed (Tukey $\alpha=0.05$), with the mycelial growth inhibition of *Pestalotiopsis* sp. fluctuating between 0.234 and 0.846 cm. Robust R® showed the lowest sensitivity at low, medium, and high doses, while the highest inhibition was obtained with the fungicide Tacora 25 EW® at all three doses (Figure 7).

[12] evaluated fungicides for the control of radial growth in vitro of *P. clavispora*, using active ingredients such as Boscalid, Chlorothalonil, Ciprodinil, Fludioxonil, Iprodione, and Pyraclostrobin at doses of 0.025, 0.05, 0.1, 1.0, and 1.5 g mL^{-1}. They determined that increasing fungicide doses significantly reduced the mycelial growth of the phytopathogen. These results are similar to those reported in this study with the product Tacora 25 Ew® at its high and medium doses, which showed the highest inhibition of growth in all three *Pestalotiopsis* sp. strains.

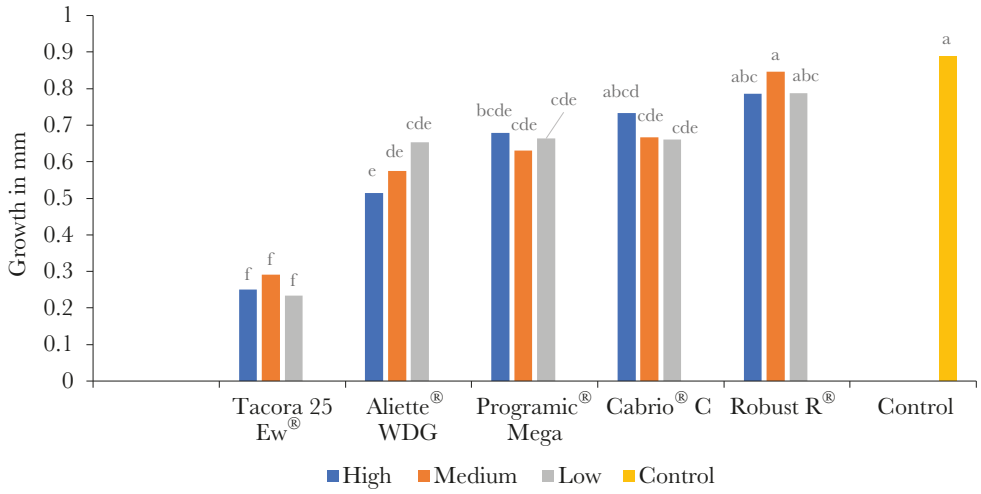

Figura 7. Effect of fungicides on mycelial growth inhibition of the CAZh02 strain (*Pestalotiopsis* sp.).

Pathogenicity Tests in Blueberry Plants

The appearance of symptoms was influenced by temperature and environmental humidity conditions, which directly affected the virulence and severity of the disease caused by the pathogens, allowing the symptoms to manifest in the blueberry plants.

In the block of Biloxi variety blueberry seedlings inoculated with the CAZr01 strain of the *Pestalotiopsis* sp. pathogen, eight days after inoculation, symptoms corresponding to grade II were observed, characterized by apical wilting. After 10 days, the plants reached grade III, showing apical necrosis and defoliation.

The seedlings inoculated with the CAZh02 strain of the *Pestalotiopsis* sp. pathogen, eight days after inoculation, showed symptoms corresponding to grade II, characterized by apical wilting. After 10 days, the plants reached grade III, with brown lesions observed on the branches and apical necrosis. At 26 days post-inoculation, grade IV (plant death) was observed (Figure 8).

Blueberry seedlings inoculated with the strain CATg03 of the *Pestalotiopsis* sp. pathogen, eight days after inoculation, exhibited symptoms corresponding to grade II, characterized

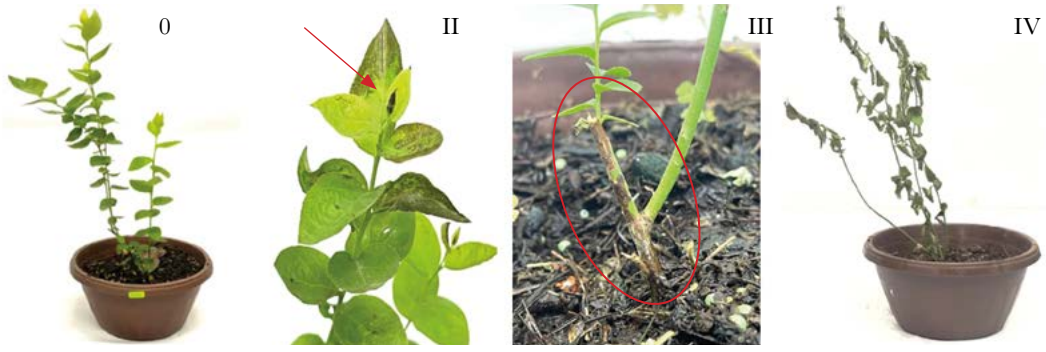

Figure 8. Foliar symptoms of *Pestalotiopsis* sp., strain CAZh02 in blueberry seedlings: Healthy plant (0), apical wilting (II), Brown lesions on the branches and apical necrosis (III), Plant death (IV).

by apical wilting. After 12 days, the plants reached grade III, showing drying of lateral branches, apical necrosis, and defoliation.

The symptoms observed in these pathogenicity tests are very similar to those reported by Espinoza *et al.* (2008), who observed lesions on branches with brown discoloration and necrosis on leaves caused by *P. clavispora*.

CONCLUSIONS

1. In the present study, it was found that the causal agent of the dieback in blueberry was *Pestalotiopsis* sp. 2. In the bioassay performed for the inhibition of mycelial growth of the strains CAZr01, CAZh02, and CATg03, the fungicide Tacora 25 Ew® at all three evaluated doses was the one that achieved the greatest inhibition of fungal growth. 3. In the pathogenicity tests, the first symptoms appeared eight days after inoculation, and at 26 days, the first plant inoculated with strain CAZh02 died, making it the most virulent strain.

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