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Bipolaris oryzae associated agent at the leaf spot disease in *Cocos nucifera* hybrid "Brazilian Green Dwarf"

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ABSTRACT

Background/objective. In Tecoman, Colima, Mexico, a leaf spot (LS) disease was detected with an incidence of 92.0% in *Cocos nucifera* hybrid Brazilian Green Dwarf (BGD). The objective was to characterize morphologically, molecularly and biochemically the fungus associated with LS in BGD coconut palm and evaluate its susceptibility to commercial biological fungicides.

Materials and methods. The isolate was characterized morphologically and molecularly. Their growth, sporulation and laccase production were evaluated using different culture media. The *in vitro* mycelial inhibition and mean lethal doses (LD_{50}) of commercial biological fungicides based on antagonistic fungi (*Trichoderma harzianum* and *T. viride*), bacteria (*Bacillus subtilis* and *B. amyloliquefaciens*) and actinobacteria (*Streptomyces lydicus* and *S. jofer*) were determined.

Results. *Bipolaris oryzae* was the associated agent of LS, it produces 25.54 and 22.17 U mg of protein^{-1} of laccase activity in the Sivakumar and wheat bran (WB) media. The WB medium allowed the greatest sporulation. *Trichoderma harzianum* inhibited *B. oryzae* at 100% in the four evaluated doses. *B. subtilis* and *B. amyloliquefaciens* inhibited *B. oryzae* at 100% at the highest tested doses (20 mL L^{-1}).

Conclusion. *Bipolaris oryzae* is the associated agent of LS, it produced the highest laccase activity in Sivakumar and WB culture media. The highest sporulation and daily growth rate were in WB. *T. harzianum* stood out over *T. viride* by inhibiting *B. oryzae* growth by 100%. *B. subtilis, S. lydicus* and *B. amyloliquefaciens* were more effective against *B. oryzae in vitro* compared to *S. jofer*.

Keywords: Antagonism, *Bipolaris*, actinobacteria, bacteria, coconut, Colima.

Introduction

In Mexico, the coconut tree (*Cocos nucifera*) is a culturally and economically important crop for the state of Colima, which came second in terms of nationwide coconut tree production, with 19,587.6 ha harvested and a total production of 20,195.61 t, with a yield of 1.03 t ha^{-1} (SIAP, 2024). Regarding the phytosanitary aspects of the coconut tree, the main pest in the Pacific region of Mexico is the South American palm weevil (*Rhynchophorus palmarum*), the main vector of *Bursaphelenchus cocophilus*, the causal agent of the red ring disease, and *Phytophthora palmivora*, responsible for bud rot (De la Mora *et al*., 2022). These diseases can be found in various coconut tree germplasms, particularly those produced in Colima: "Alto Pacífico" "Ecotipo 4" and "Brazilian Green Dwarf" (BGD). However, not only nematodes and oomycetes cause damage to coconut trees; phytopathogenic fungi can also be found that induce different diseases (Gemaque *et al*., 2009).

The BGD hybrid is the most recently planted coconut tree germplasm in Colima. It was introduced in 2017 and since then, due to its characteristics, has gained popularity amongst producers and companies related to the coconut tree in the Tecoman Valley, due its precocious production after two years, offering a productivity of over 150 drupes per plant and height of 4 to 6 meters, which makes cultural and harvest work easier in comparison with tall varieties and mainly, it is used for the consumption of its water (Benassi *et al*., 2013).

Recently, phytopathology symptoms were found in the BGD hybrid in an open-air plant production nursery in Caleras, Tecoman, Colima. The disease corresponds to a leaf spot (MF), which displays small oval spots with a dark maroon-reddish necrotic at first and with time acquired a light maroon hue, with the presence of a yellowish ring (halo) on the edge of them during both stages, which coincides with a fungal disease, according to the parameters evaluated by Gemaque *et al*. (2009).

The main management of fungal diseases, including the leaf spot in coconut trees in nurseries is via the use of chemical fungicides, mainly with active ingredients such as benomyl (Benlate), chlorothalonil (Daconil) (Palomar and Betonio, 1982), tebuconazole, propiconazole, carbendazim, picoxystrobin, pyraclostrobin, mancozeb, propineb, and others (Ushamalini *et al*., 2019), despite the environmental drawbacks associated with the use of these molecules and the resistance they generate in phytopathogenic fungi.

A green alternative that prevents the resistance of fungal pathogens is biological control with fungicides based on beneficial microorganisms, such as filamentous bacteria and/or fungi, which are often found naturally in the soil and control fungi and bacteria that cause diseases in plants (Ehiobu *et al*., 2022). Biofungicides act through direct competition (exclusion), antibiosis, parasitism or depredation,

resistance induction to the host plant and plant growth promotion (Fenta and Mekonnen, 2024).

Due to the introduction of the BGD coconut tree variety into Colima, the pathogens that can be of economic importance to this germplasm are unknown. Consequently, the agent associated to the MF is unknown. Therefore, the aim of this study was to morphologically, molecularly and biochemically characterize the fungus associated with MF in BGD coconut trees and to evaluate its susceptibility to commercial biological fungicides.

Materials and Methods

Origin of the plant material. In September 2022, BGD variety coconut tree leaves with symptoms of MF were gathered at random in the nursery in a ranch called "La Ceiba," in Caleras, Tecoman, Colima (18°59'31" N and 103°53'19 W). The predominant weather is warm subhumid (Aw) with rains in the summer (600 to 1, 100 mm $year^{-1}$) and 80 masl. The leaves were placed in plastic bags and in an ice chest with cold gels for their immediate transportation to the laboratory to isolate the fungus. At the moment of sampling, the incidence of the disease in trees in the nursery was 92.0%.

Isolation of the associated agent. The infected leaf area was cut into 4×4 cm pieces, and for the disinfection of their surface, they were washed three times for three minutes each time, with NaClO at 2.0% + Tween 20® (500 µL L^{-1}), alcohol at 70.0% and sterile distilled water. Subsequently, the excess humidity was removed with sterile paper towels and they were planted in Petri dishes with agar-water for the proliferation of hyphae and fungal structures. After seven days, hyphae, conidiophores and conidia were observed, which were purified using hypha tips in Potato Dextrose Agar (PDA). Incubation and isolation conditions were 25.0±3.0 °C, 75.0±5.0% of relative humidity (HR) and a photoperiod of 14:10 h light:dark (Conde *et al*., 2008).

Morphological characterization. Under sterile conditions, pure cultures of the isolated fungus were prepared using Riddell's microculture method (1950). Microcultures were observed through an inverted microscope (Axiovert 40 CFL Zeiss, Germany) with the 5, 10 and 40× lenses. Cultural and morphological characteristics were taken into account, such as septated or smooth hyphae, mycelial aspect, shape of the culture's edge, color of conidia in a PDA medium, absence or presence of conidia, and the size and shape of conidia. The description of reproductive structures was carried out following Crous *et al*. (2009), Manamgoda *et al*. (2014), and Valarmathi and Ladhalakshmi (2018).

Molecular characterization. A representative isolate was selected for molecular identification, and genomic DNA was extracted following the protocol by Miller *et al.* (1999). The fungal tissue was ground with liquid nitrogen and a lysis buffer (200 mM Tris HC1 pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was used for phenol-chloroform extraction. The D1/D2 region of the 28S ribosomal subunit was amplified using the oligonucleotides NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Voigt *et al*., 1999). The sequences were inspected with chromatograms using the program SnapGeen Viever™ 7.2.0. The cured sequence was used to compare with the data base in the NCBI GenBank using the program Mega BLAST. The sequence was deposited in the GenBank with accession number PV034038. Additionally, the most similar *Bipolaris*-related sequences were downloaded in FASTA format from GenBank (Table 1). These sequences were aligned using the algorithm CLUSTALW with predetermined parameters (Thompson *et al*., 2003) and were edited manually using the software package MEGA 11 (Tamura *et al*., 2021). A phylogenetic Maximum Likelihood (ML) analysis was carried out with the 2 parameter G+I Kimura model (Kimura, 1980). The gaps were treated as partial deletions with a 95.0% coverage, using an NNI (Nearest Neighbor Interchange) heuristic method to refine the topology of the tree with 1,000 bootstrap replicates. *Fusarium oxysporum* was used as an external clade.

Table 1. Sequences used to generate the phylogenetic tree for *Bipolaris oryzae*, an agent associated to the coconut tree leaf spot.

Specie	Strain number	Host	Country	Gen Bank number	Reference
Bipolaris incurvata	CBS 127221	*C. nucifera*	India	MH875906	A. Kamalakannan *et al*., 2006
Bipolaris oryzae	CBS 127244	No determinado	Holanda	MH875924	Vu *et al*., 2019
B. oryzae	MFLUCC 100715	*Oryza sativa*	Thailandia	JX256384	Manamgoda *et al*., 2012
B. oryzae	MFLUCC100716	*O. sativa*	Thailandia	JX256383	Manamgoda *et al*., 2012
B. oryzae	MFLUCC 100733	*O. sativa*	Thailandia	JX256385	Manamgoda *et al*., 2012
B. oryzae	AR5204	*Panicum virgatum*	USA	KM243277	Manamgoda *et al*., 2014
B. oryzae	B1	No determinado	India	OQ349750	Without publication
B. oryzae	ACCC 36975	*O. sativa*	China	MK051173	Chaijuckam *et al*., 2019
B. oryzae	H2-1	No determinado	Japan	KF590135	Palomares *et al*., 2014
Bipolaris secalis	BRIP 14453	*Secale sereale*	Argentina	KJ415492	Tan *et al*., 2014
B. secalis	UC6	*Vitis vinifera*	Italia	KU554624	Lorenzini *et al*., 2016
B. secalis	K2d	*Sorghum bicolor*	Kenya	PP813561	Without publication
B. secalis	CBS 127712	No determinado	Holanda	MH876123	Vu *et al*., 2019
Curvularia oryzae	CBS 169.53	*C. nucifera*	China	KM243284	Berbee *et al*., 1999

Physiological and biochemical characterization. In an experiment, five semi-solid culture media were evaluated: Czapek-Dox (in g L^{-1}: NaCl 0.75, K_2HPO_4 0.25, $MgSO_4$ 0.125, KCl 0.125, bacteriological agar 18.0 (Sumathi *et al*., 2016),

coconut tree glucose water (CGW) (in g L^{-1}: glucose 10 and blended coconut tree leaves 50), PDA, wheat bran-citrate (WB) buffer (in g L^{-1}: wheat bran 30, NaOH 2.0 and citric acid 9.0 (Sumathi *et al*., 2016) and Sivakumar (in g L^{-1}: glucose 20, yeast extract 2.5, K_2HPO_4 1.0, $MgSO_4$ 0.5, KCl 0.5, $(NH_4)_2SO_4$ 0.5, $CaCl_2$ 0.01, $FeSO_4$ 0.01, $MnSO_4$ 0.001, $ZnSO_4$ 0.001 and $CuSO_4$ 0.002 (Sivakumar *et al*., 2010). The culture media were prepared, sterilized and poured in 100×15 mm Petri dishes. As an inoculum, a mycelium disk developed on agar from a 12-day-old *Bipolaris oryzae* colony on V8-agar juice was placed at the center of the Petri dish. A completely randomized design was used.

The physiological characterization was performed by quantifying the daily growth rate (DGR) and sporulation. The DGR was determined by measuring the diameter of the *B*. *oryzae* colonies for five days. The DGR was calculated using the formula

$$DGR = \chi\Sigma\ (C_{t1} - C_{t1\text{-}1}...n)$$

where, *n* = was the number of evaluations and Ct = the number of days of growth. For sporulation, 10 seven-day-old agar mycelium disks from the evaluated culture media were deposited in 50 mL Falcon® tubes with 10 mL of sterile distilled water with Tween 80® (0.005%). The tubes were shaken in a vortex for 60 s, 10 µL were taken and the number of conidia $disk^{-1}$ mL^{-1} were counted in a Neubauer chamber under a microscope (Axio Vert A1, Zeiss, Germany) with a 40× lens (Manzo-Sánchez *et al*., 2018).

The biochemical characterization of *B*. *oryzae* included the production of laccase (volumetric and specific) and total protein. From agar mycelia disks obtained from the sporulation count, the samples were filtered with sterile filter paper (Whatman Num 1) to avoid conidia and mycelia. Filtration was used to determine laccase activity by the ABTS oxidation method (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (Chan *et al*., 2019) and total protein content with the Bradford method (Kielkopf *et al*., 2020). The laccase activity was reported in U (international unit: the amount of laccase that oxidized 1 µmol of ABTS mL^{-1} min^{-1}) and protein content in mg L^{-1}.

Sensitivity to biological, microorganism-based fungicides. For the bioassays, the following were evaluated: Serenade ASO® (*Bacillus subtilis,* Bayer®), Badixi® (*Bacillus amyloliquefaciens* strain MBI 600, Syngenta®), Magni-Root® (*Trichoderma harzianum,* PlantBio®), Trico® (*Trichoderma viride*, Greenfor®), Blite Free® (*Streptomyces jofer*, enriched with antioxidants, culture medium and chelating agents, AltusBio®) and Domus® (*B*. *subtilis*, *T*. *harzianum* and *Streptomyces lydicus*, CollectTara®). The selection of products was based on their availability

in agricultural supply stores in Tecoman, Colima. The biological products were evaluated at doses (concentration %) of 2.5 (0.25%), 5.0 (0.5%), 10 (1.0%) and 20 mL L^{-1} (2.0%). The doses (mL L^{-1}) were prepared in sterile distilled water (100 mL) + Tween 80® (0.05%). Using a micropipette, 1,000 µL were taken and placed on the surface of PDA solidified medium in 100×15 mm Petri dishes. Five replicates were performed for each dose evaluated in each product. The inoculation of the phytopathogenic fungus was carried out by depositing five µL of a spore solution at a concentration of 9.2×10^4 spores mL^{-1} in the center of the Petri dish. The inoculated Petri dishes were incubated under laboratory conditions at a temperature of 25.0 ± 3.0 °C. 75 ± 5.0% of HR: 10:14 h light:darkness for five days (Chan *et al*., 2014). The percentage of mycelial growth inhibition (%MGI) was determined using the following equation: %; MGI = [(C - T) / C] * 100, where C is the diameter of the mycelium in the control Petri dish (mm), and *T* is the diameter of the mycelium in the treatment dishes with biological fungicide (mm). The ninety lethal dose LL_{90} was determined, which refers to the amount of biological fungicide needed to inhibit the development of the phytopathogen by 90%. It was estimated using the values of the %MGI in each dose evaluated for every product. This estimation was carried out with a Probit analysis (Manzo-Sánchez *et al*., 2018) using the Statistical Analysis System (SAS) software version 8 for Windows (SAS Institute Inc., 2000).

Data analysis. The physiological and biochemical characterization data (DGR, sporulation, laccase activity and production of total protein) and *in vitro* effectiveness (%MGI) were placed under a variance homogeneity ($P < 0.05$) and analysis of variance ($P < 0.05$). Significant differences were found, and a Tukey means comparison was carried out with a significance level of 5%. Additionally, a linear regression analysis (Y=mX+b) was performed between the doses used of the products to evaluate (X) and the percentages of inhibition (Y) to know their relation. The analyses were carried out using the software StatGraphics Plus 4.0 for Windows (Manzo-Sánchez *et al*., 2018).

Results

Morphological and molecular characterization. The symptoms of LS found in BGR correspond to small oval black and maroon oval spots. The posts grow slowly through a slightly yellow halo, causing the weakening and overall yellowing of leaves, leading to their later death (Figure 1). The main cultural and morphological characteristics of the fungus are shown in Figure 2. Macroscopically, the mycelium was white in its first seven days of growth and turning greyish-brown as the mycelium matures, forming the conidiophores and conidia when aged 12 days (Figure 2I and

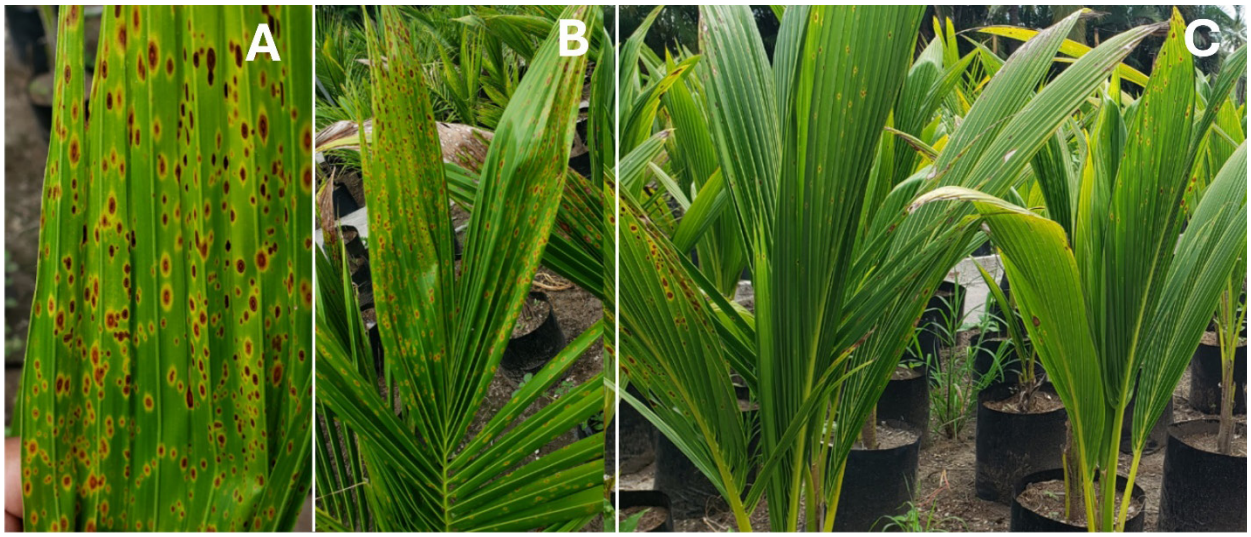

Figure 1. Leaves with symptoms of leaf spot in hybrid coconut tree "Brazilian Green Dwarf". A) Brown oval spots and yellow halo, B) palm leaves with multiple oval spots and C) view of the nursery with plants with leaf spot.

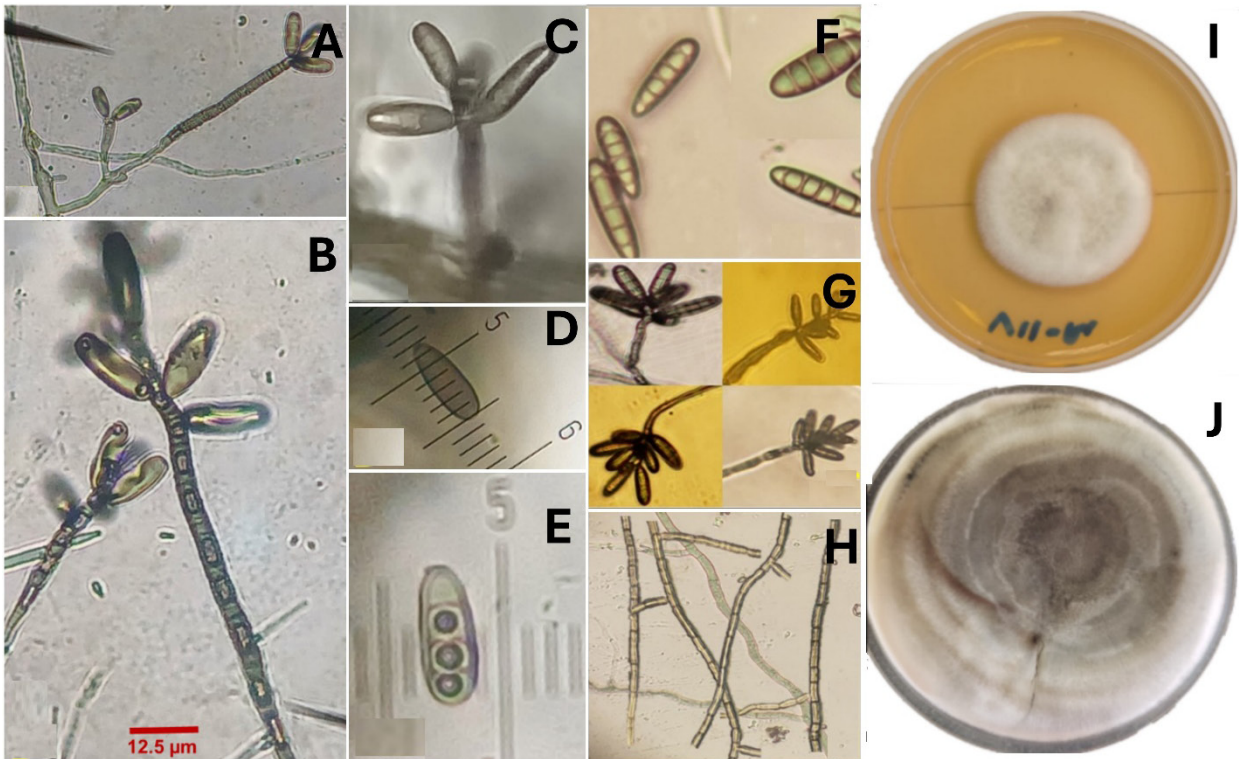

Figure 2. Cultural and morphological characteristics of *Bipolaris oryzae*. A and B) Conidiophores; C to F) conidiospores; G) cluster of conidiophores; H) septated hyphae; I) young mycelium, aged 5 days in medium; J) mature mycelium after 20-day growth in PDA medium.

2J). The hyphae were microscopically septated, bright maroon (Figures 2A, 2B and 2H), straight conidia (Figure 2C, 2D, 2E and 2F), distoseptated (5 – 6 septa), light maroon, ellipsoidal in shape and stretching to its tips in round shapes. The conidiophores are septated and group between 2 and 8 conidia (Figure 2G), with an average of 4.0±1.4 µm (mean ± standard deviation (SD), and the hyphae are also septated (Figure 2H). The length of the conidiophores were 114.8±34.1 µm (mean ±DE *n*=100) and the dimensions of the conidia were 16.1±1.5 µm x 5.2±0.4 µm (mean ±DE *n*=100). The isolations were identified as *Bipolaris oryzae* according to its morphological characteristics (Manamgoda *et al*. 2014; Meghana and Hiremath 2019; Livitha and Christopher 2023).

To confirm the morphological identification of the agent associated to LS, a representative isolation was chosen for the molecular identification and the genomic DNA was extracted using the protocol by Miller *et al.* (1999). The D1/D2 region of the amplicon of the greater 28S subunit of the ribosomal RNA was sequenced (Voigt *et al*., 1999). The sequences of the representative isolation (ACMFCnhEVB_1) were deposited in the GenBank (PV034038). The BLAST analysis of the partial sequences displayed a similarity of 98.27% with the *Bipolaris oryzae* (GenBank OQ349750.1) isolations. To compare the isolation of *B. oryzae* (associate agent MFCnhHVB) reported in this paper with other *Bipolaris* spp. strains, a phylogenetic analysis was performed with sequences from different related strains and species using the software ClustalW and MEGA11 with the Maximum Likelihood method (Figure 3). The analysis was carried out with a total of 14 *Bipolaris* spp. Sequences, nine of which were reported as pathogens (one from *B*. *incurvata* and eight from *B*. *oryzae)*, four from *B*. *secalis* used as a sibling clade and two sibling sequences corresponding to *B*. *incurvata* and *Curvularia oryzae* (Berbee *et al*., 1999; Kamalakannan *et al*., 2006) reported as a pathogen of *C. nucifera* (Table 1). Isolation MFCnhHVB reported in this paper was clustered in the clade of *Bipolaris oryzae* strains B1 (OQ349750.1) and H2-1 (KF590135.1). Based on the sequence data and the phylogenetic analysis, the coconut tree fungus isolated from LS was identified as *B*. *oryzae* (Figure 3).

Biochemical and physiological characterization. *B*. *oryzae* produced the volumetric laccase in a range of 0.66 to 15.34 U L^{-1} in the culture media evaluated (Table 2). The greatest activity (P=0.00001) was found in the Sivakumar media (15.34 U L^{-1}), followed by WB (8.5 U L^{-1}). For the production of total protein by *B*. *oryzae*, no significant differences were found (P=0.6478) between the culture media evaluated. The range of protein found was between 0.40 and 0.78 mg L^{-1} (Table 2). The specific laccase activity was statistically greater (P=0.0005) in the Sivakumar culture media (25.54 U mg of $protein^{-1}$) and WB (22.17 U mg of $protein^{-1}$). DGR fluctuated between 0.87 and 1.7 cm day^{-1}, whereas the media Czapek-Dox (1.75

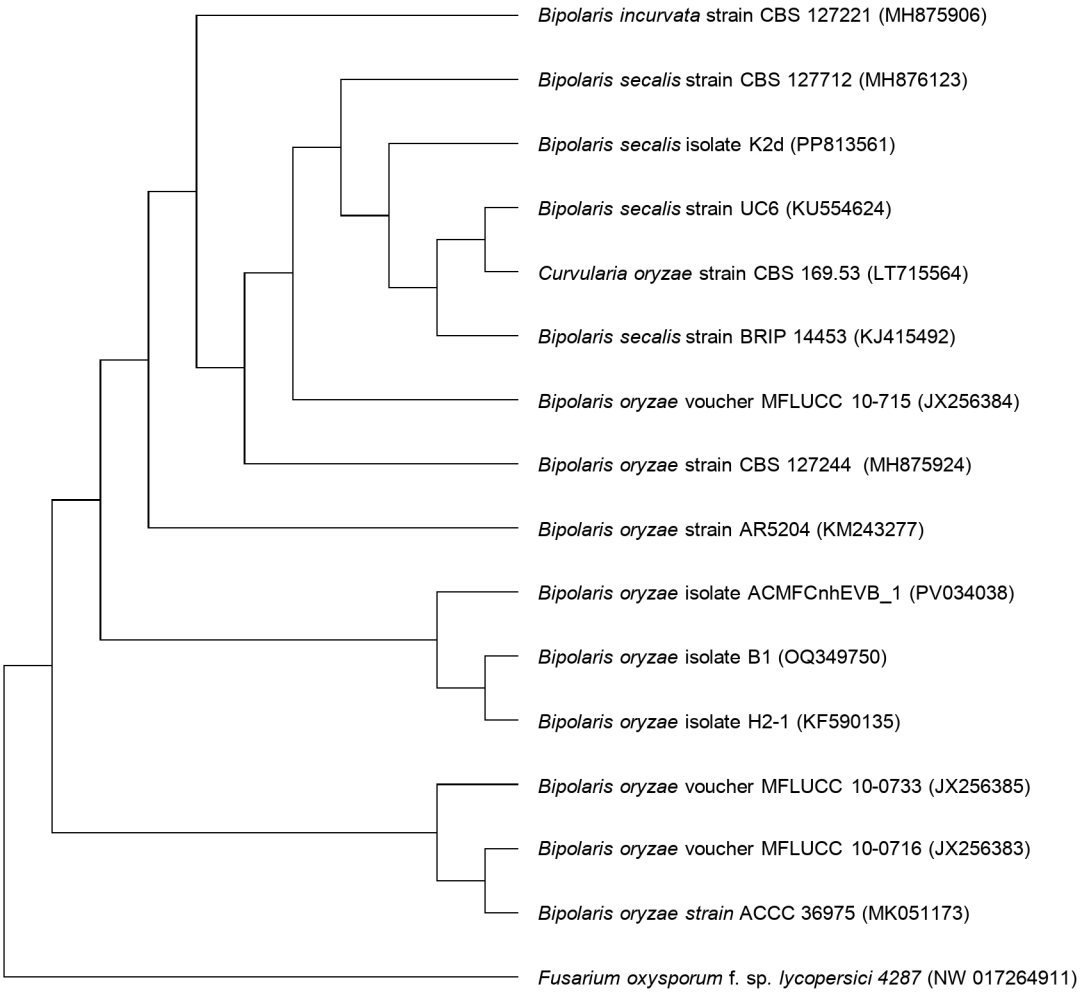

Figure 3. Phylogenetic analysis based on the maximum likelihood method using the 2 parameter Kimura method (G+I). Tree created with the highest probability logarithm (-5759.36) of the sequence of rDNA 28S of the *Bipolaris* strains, similar to those of the agent associated to LS in the BGD coconut tree (*Bipolaris oryzae* ACMFCnhEVB_1) using MEGA11. This tree was rooted using *Fusarium oxysporum* and was used as an external group.

cm day^{-1}) and CGW (1.67 cm día^{-1}) allowed a greater DGR in comparison with the PDA (0.87 cm día^{-1}). The WB medium allowed for a greater sporulation of *B. oryzae* (329 conidia mL^{-1} disk^{-1}) (P=0.00001), in comparison with the Czapek-Dox, CGW and Sivakumar media, which displayed 42.0, 31.0, 37.0 conidia mL^{-1} disk^{-1}, respectively (Table 2).

***In vitro* effectiveness of biological fungicides.** The Magni-Root® biofungicide (*T. harzianum*) inhibited the mycelial growth of *B. oryzae* in the four doses evaluated by 100% (Table 3, Figure 4). Trico® (*T. viride*) caused an inhibition of 100% of *B. oryzae* in the first sampling. However, starting on day 2, the percentage declined up to values of 4.1±2.6 and 0.4±0.2% to a dose of 20 mL L^{-1} and 10 mL L^{-1},

Table 2. Laccase activity, daily growth rate (DGR) and sporulation of *Bipolaris oryzae* in semi-solid media.

Culture media	Volumetric activity (U L^{-1})	Protein content (mg L^{-1})	Specific activity (U mg de $protein^{-1}$)	DGR (cm day^{-1})	Esporulation (conidiosphore mL^{-1} $disk^{-1}$)
Cz-D	2.42±1.51 c	0.72±0.14	3.38±2.37 b	1.75±0.23 a	42.0±7.3 b
CGW	0.66±0.12 c	0.69±0.19	1.44±0.60 b	1.67±0.17 a	31.0±5.7 b
PDA	2.87±0.15 c	0.70±0.23	5.03±0.97 b	0.87±0.18 b	-
WB	8.5±0.64 b	0.40±0.04	22.17±3.15 a	1.32±0.02 ab	329.0±36.5 a
Sivakumar	15.34±2.19 a	0.78±0.24	25.54±7.19 a	1.2±0.13 ab	37.0±5.8 b
F=	23.93	0.63	9.46	4.66	63.37
P=	0.00001	0.6478	0.0005	0.0121	0.00001

Means (± standard error) with different letters are statistically different to each other, according to Tukey's means comparison test (P=0.05). Cz-D=czapek-Dox, CGW= coconut glucose water, PDA=potato dextrose agar, WB=wheat bran.

respectively. At the end of the evaluation (5 days of interaction), the percentages fluctuated between 43.2 and 55.2%, to a dose of 2.5 and 20 mL L^{-1}, respectively. Domus® (*B. subtilis, T. harzianum* and *S. lydicus*) caused inhibitions of 100% in the growth of *B. oryzae* 100% with the dose of 20 mL L^{-1} during the five days of evaluation, whereas the 10 mL L^{-1} dose maintained growth inhibitions of 85.9, 89.7 and 91.9% on days three, four and five days of evaluation, repsectively. Badixi® (*B. amyloliquefaciens*) and Serenade ASO® (*B. subtilis*) inhibited the growth of *B. oryzae* by 100% in the doses of 20, 10 and 2.5 mL L^{-1} five days after evaluation. The 5.0 mL L^{-1} dose obtained the lowest inhibition with the products Badixi®=90.7% and Serenade ASO®=94.2%. BliteFree® (*Streptomyces jofer*) presented an inhibition of 100% against *B. oryzae* in the four doses evaluated, but on day 2, this percentage dropped to values ranging between 18.2 and 1.8%. As the experiment progresses, growth inhibition increased, and by day 4 of the study, the %MGI presented no significant differences between the doses studied, inhibiting the fungus by up to 69% (Table 3, Figure 4).

The LD_{90} in Magni-Root® (*T. harzianum*) could not be calculated because it inhibited the mycelial growth of *B. oryzae* by 100% in the four doses evaluated (Table 4). Both Badixi® (*Bacillus amyloliquefacien*s) and Serenade ASO® (*Bacillus subtilis*) gave negative values, although they displayed %MGI against *B. oryzae* between 83.9 and 100% (Table 3). Domus® (*B. subtilis, T. harzianum* and *S. lydicus*) presented the lowest value with a LD_{90} of 7.52 mL L^{-1}, making it 8.64 and 16.26 times lower than Trico® (*Trichoderma viride*) and Blite Free® (*Streptomyces jofer*) (Table 4).

Table 3. Inhibition of the mycelial growth (%MGI) of *Bipolaris oryzae* by different doses of commercial biological fungicides.

Product	Doses (mL L^{-1})	Evaluation days 1	2	3	4	5
Magni-Root®	20	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	10	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	5	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	2.5	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	F	NC	NC	NC	NC	NC
	P-value	NC	NC	NC	NC	NC
Product	**Doses (mL L^{-1})**	**Evaluation days 1**	**2**	**3**	**4**	**5**
Trico®	20	100.0±0.0 a	4.1±2.6 a	24.8±4.8 ab	44.1±4.1 a	55.2±3.7 a
	10	100.0±0.0 a	0.4±0.2 a	28.8±5.9 a	47.5±4.1 a	58.3±3.0 a
	5	100.0±0.0 a	1.4±1.4 a	6.6±6.6 b	17.4±8.9 b	35.6±7.3 b
	2.5	100.0±0.0 a	0.0±0.0 a	9.5±2.6 ab	29.6±4.8 ab	43.2±4.3 ab
	F	NC	1.59	4.46	5.67	4.71
	P-value	NC	0.2225	0.0148	0.0056	0.0121
Product	**Doses (mL L^{-1})**	**Evaluation days 1**	**2**	**3**	**4**	**5**
Domus®	20	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	10	100.0±0.0 a	75.3±11.6 ab	85.9±6.3 ab	89.7±4.8 ab	91.9±3.8 ab
	5	100.0±0.0 a	50.6±12.0 b	71.8±6.9 b	79.4±5.0 b	83.8±4.0 b
	2.5	100.0±0.0 a	42.3±8.4 b	67.1±4.8 b	75.9±3.5 b	81.1±2.8 b
	F	NC	7.69	7.69	7.69	7.69
	P-value	NC	0.0013	0.0013	0.0013	0.0013
Product	**Doses (mL L^{-1})**	**Evaluation days 1**	**2**	**3**	**4**	**5**
Badixi®	20	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	10	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	5	100.0±0.0 a	83.5±11.0 a	83.9±5.7 b	88.2±4.1 b	90.7±3.3 b
	2.5	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	F	NC	2.23	8.00	8.00	8.00
	P-value	NC	0.1165	0.0011	0.0011	0.0011
Product	**Doses (mL L^{-1})**	**Evaluation days 1**	**2**	**3**	**4**	**5**
BliteFree®	20	100.0±0.0 a	18.2±10.9 a	47.0±8.3 a	61.0±6.1 a	69.1±4.8 a
	10	100.0±0.0 a	1.8±1.3 a	35.6±3.1 a	52.2±2.4 a	61.8±2.0 a
	5	100.0±0.0 a	7.8±4.6 a	42.9±5.4 a	58.3±4.0 a	67.2±3.1 a
	2.5	100.0±0.0 a	9.8±7.2 a	38.2±4.4 a	53.2±3.7 a	61.8±3.7 a
	F	NC	0.95	0.80	0.97	1.12
	P-value	NC	0.4336	0.5062	0.4248	0.3657
Product	**Doses (mL L^{-1})**	**Evaluation days 1**	**2**	**3**	**4**	**5**
Serenade ASO®	20	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	10	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	5	100.0±0.0 a	100.0±0.0 a	89.9±4.5 b	92.6±3.3 b	94.2±2.6 b
	2.5	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	F	NC	NC	5.00	5.00	5.00
	P-value	NC	NC	0.0095	0.0095	0.0095

Means (± standard error) with different letters are significantly different to each other, according to Tukey's means comparison test α=0.05. NC=Not calculated by the software. n= 6 refers to the repetitions per dose.

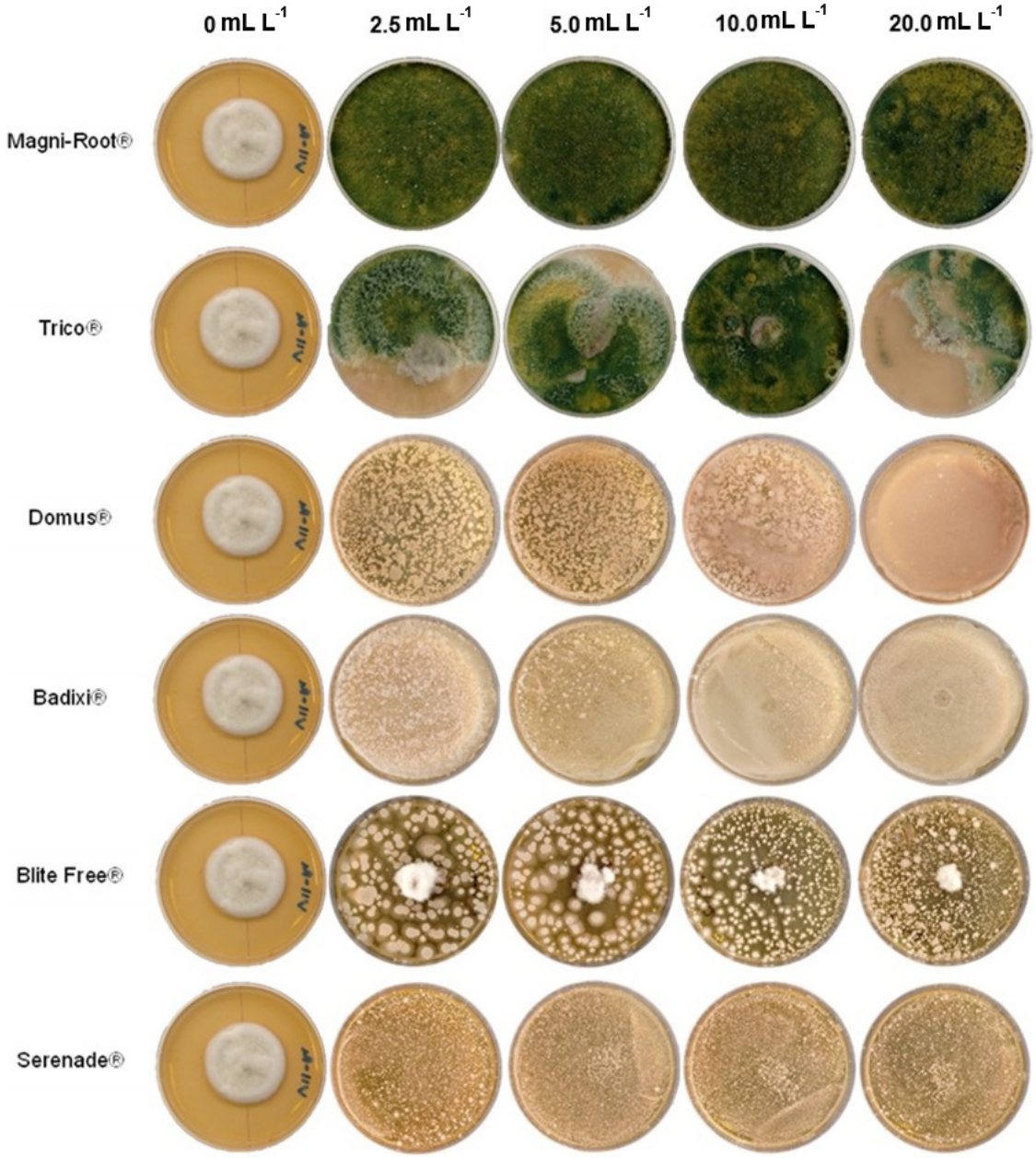

Figure 4. Growth of *Bipolaris oryzae* five days after inoculation in PDA modified with different doses of commercial biological fungicides.

Table 4. Ninety lethal dose (LD_{90}) of six commercial biological fungicides on *Bipolaris oryzae*.

Product	LD_{90} (mL L^{-1})	CI (mL L^{-1})	Slope	Probit ecuation	χ^2	$P>\chi^2$
Magni-Root®	NC	NC	NC	NC	NC	NC
Trico®	65.04	51.5 – 91-0	0.0238	Y= 0.0238X + (-0.2699)	42.5682	0.00005
Domus®	7.52	6.7 – 8.4	0.0956	Y= 0.0956X + 0.5618	9.6654	0.00790
Badixi®	-2.89	-8.90 – -0.14	0.0717	Y= 0.0717X + 1.4891	114.8000	0.00000
BliteFree®	122.31	66.2 – 5242.4	0.0079	Y= 0.0079X + 0.3127	6.6607	0.03570
Serenade ASO®	-6.45	-19.30 – -2.12	0.0667	Y= 0.0667X + 1.7129	71.8874	0.00002

LD_{90}= Ninety lethal dose, CI= Confiance interval, NC= Not calculated by the software.

Figure 5 shows the regression analysis performed on each biological fungicide in relation to the percentage of inhibition and dose used. Magni-Root® (*T. harzianum*) presented the highest ratio between the tested variables (R^2=1.00) in comparison with the remaining products evaluated, which had regression coefficients (R^2) that ranged between 0.05 and 0.5. These results indicate a low ratio between these in the other products.

Discussion

The *Bipolaris* genus has ecologically versatile species, that is, they have saprophytic, endophytic and parasitic habits (Kuan *et al*., 2015; Bengyella *et al*., 2019). The saprobic habit is due to its heterothallic ability to thrive in soil and plant residues (Sun *et al*., 2020). Its endophytic habit is explained by its ability to colonize plant leaves, stems and roots without activating its pathogenicity genes, as well as producing secondary metabolites with important biological functions, such as contributing to the association with the host (Schulz *et al*., 2002). This endophytic ability allows it to produce substances of biotechnological interest, such as organic acids, enzymes and siderophores (Bengyella *et al*., 2019). Its parasitic habit is due to its ability to parasitize plants; most of the 100 described species of *Bipolaris* (Manamgoda *et al*., 2014; Bengyella *et al*., 2019) are plant pathogens associated with leaf spot, root rot, seedling rot and other diseases in grasses, wild plants and even fruit trees. *B. oryzae*, *B. maydis* and *B. sorokiniana* are considered the most devastating species of the genus (Sun *et al*., 2020; Khan *et al*., 2023).

The species of *Bipolari*s have been reported as pathogens of diverse palm genres (*Dypsis*, *Phoenix*, *Cocos*, *Adonia*, *Caryota*, *Elaeis*, and others) causing leaf spot. In *C. nucifera*, only the species *Bipolaris incurvata* (formerly *Helminthosporium incurvatum*) (Elliott *et al*., 2004) has been reported in Hawaii and India

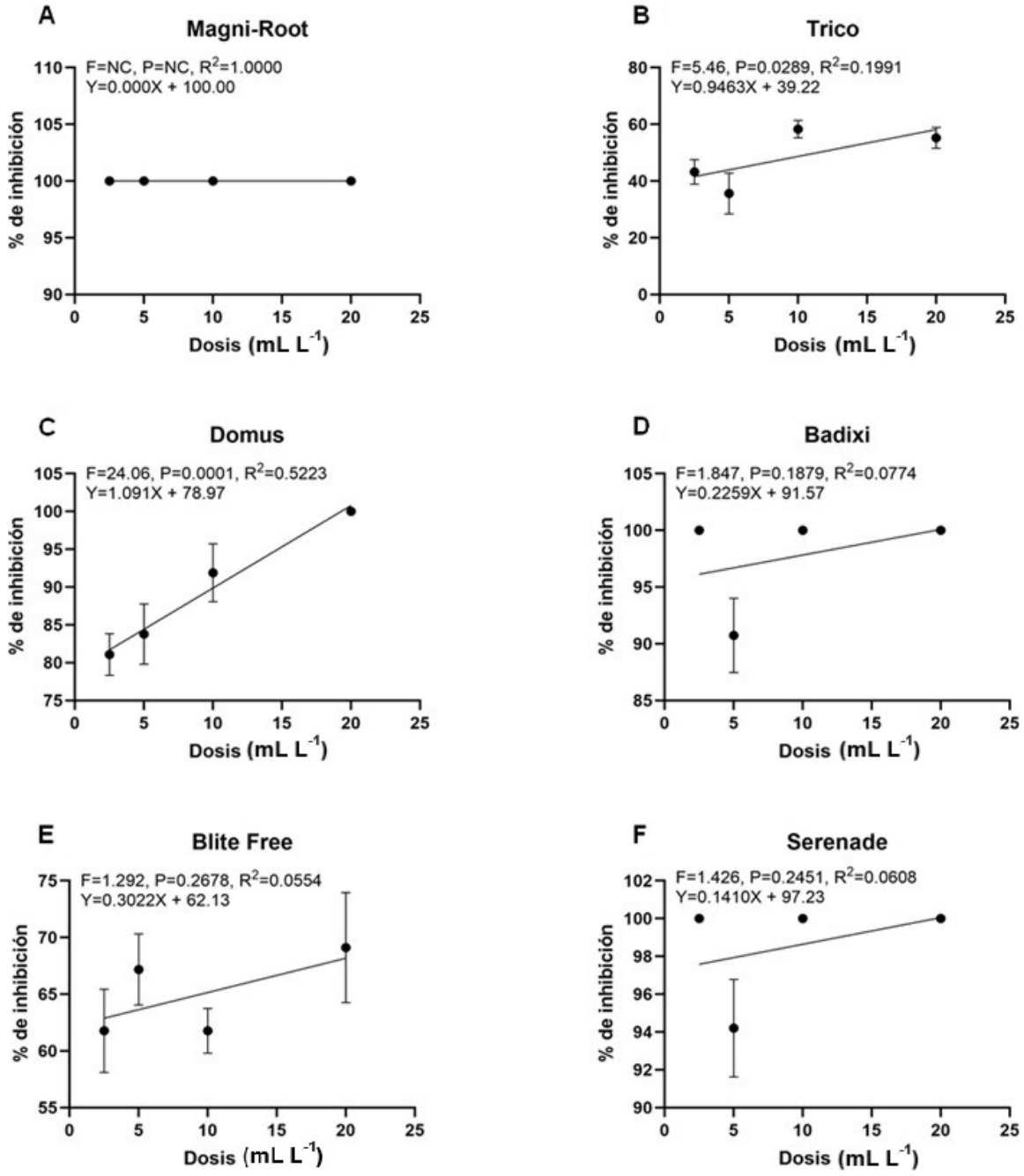

Figure 5. Linear regression between the inhibition (%) in the growth of *Bipolaris oryzae* and the doses of each commercial biological fungicide used.

(Kamalakannan *et al*., 2006); as well as *Bipolaris setariae* (Niu *et al*., 2014) in China and *Bipolaris* sp. in Ghana (Lekete *et al*., 2022). Therefore, this is the first report of *B. oryzae* as an agent associated with leaf spot in *C. nucifera* in Mexico and worldwide.

On the other hand, the laccases (EC. 1.10.3.2) are oxidoreductase enzymes that contain copper and catalyze the oxidation of a single electron in a wide range of organic substrates such as carbohydrates, aromatic compounds, non-aromatic compounds, phenolic compounds and non-phenolic compounds that imply the reduction of oxygen to water. Laccase is an important enzyme in the degradation of lignin in nature and it is mostly synthesized by Basidiomycetos and Ascomycetos fungi (Chan *et al*., 2019). Phytopathogenic fungi can produce several laccase isoenzymes codified by multiple genes. At least 10 functions of laccase genes have been identified in phytopathogenic fungi (Wei *et al*., 2017). *B. oryzae* was able to produce laccases in all the culture media evaluated, including PDA, but its highest activity was found in Sivakumar medium. This could be due to the nutritional content of the medium, particularly $CuSO_4$ (0.002 g L^{-1}), an inducer of multicopper laccase (Chan-Cupul *et al*., 2019).

The ability of *B. maydis* to produce laccase has been the only one reported (Joseph *et al*., 2019). Therefore, this study represents a new contribution to the physiology of *B. oryzae* regarding laccase production, an enzyme related to pathogenesis, sporulation and mycelial melanization in the *Bipolaris* genus (Kandan *et al*., 2013; Lü *et al*., 2017; Wei *et al*., 2017). Fungal laccases, including those produced by *B. oryzae*, are verstatile enzymes with great importance in agriculture and various industries. These oxideases, which contain copper, play a crucial role in the synthesis of lignin and the degradation and maintenance of soil health (Zhai *et al*., 2024). Laccases can oxidize a wide range of phenolic and non-phenolic compounds, making them valuable for applications such as food processing, bioremediation and the textile industry (Upadhyay *et al*., 2016).

On the other hand, in the *Bipolaris* genus, sporulation is an important physiological stage; conidia are more ineffective in plants than the mycelium itself (Hau and Rush, 1980). Therefore, knowing the rates of growth and sporulation of *B. oryzae* in different culture media is a leading activity in the study of the pathogenesis of the fungus in its host. The sporulation of *B. oryzae* was greater in the wheat bran medium, a basic and inexpensive medium. This coincides with a report by Hau and Rush (1980), who used agar with rice powder (15 g L^{-1}), which produced an average of 8.7×10^7 conidia per Petri dish (100×15 mm). Regarding DGR, the glucose coconut tree and Czapek-Dox culture media gave way to the greatest growth speed. This could be due to the coconut tree extract, which emulates the natural substrate of the phytopathogen, whereas Czapek-Dox may be favored by the amount of salts (NaCl, $MgSO_4$, KCl, K_2HPO_4) it contains, since this medium

has previously been used for the growth of *B. oryzae* (Hau and Rush, 1980). Other media reported for the growth of *B. oryzae* are malt extract agar, starch agar, corn flour agar, rice leaf agar and rice powder agar (Arshad *et al*., 2013; Thakur *et al*., 2023).

The *in vitro* bioassays revealed the effectiveness of *T. harzianum* to suppress the *in vitro* growth of *B. oryzae* at 100% in the four concentrations evaluated. By contrast, *T. viride* alone was able to inhibit *B. oryzae* by up to 58.3%. The ability of *T. harzianum* is widely known for the biological control of phytopathogenic fungi, including *B. oryzae*. In this regard, Pérez *et al*. (2013) and Pérez *et al*. (2018), reported the effectiveness of *T. harzianum* on *B. oryzae*, the causal agent of brown spot in rice plants (*Oryza sativa*) by inhibiting *in vitro* growth by 64.4% and reducing the disease by up to 93.1% *in vivo* evaluations. However, not all *Trichoderma* strains are effective; in this regard, Bedoya *et al*. (2021) reported low percentages of mycelial inhibition in *B. oryzae* by *Trichoderma* spp., between 6.1 and 27.5%. The main mechanism for the suppression of *B. oryzae* by *T. harzianum* is mycoparasitism (Abdel *et al*., 2007). In dual interactions, *T. harzianum* has been observed to grow, displace and parasitize *B. oryzae*. However, the possibility of other action mechanisms, such as the production of metabolites and enzymes that affect the cell wall of *B. oryzae* are not ruled out (Khan *et al*., 2020).

Bacteria-based products (Serenade ASO®, Badixi®, Domu® and BliteFree®) turned out to be effective in the mycelial inhibition of *B. oryzae.* In this regard, Zárate *et al*. (2022) suggested that biological control with *B. subtilis* (Serenade ASO®) can be better than chemical control, as they reported mycelial inhibitions in *Bipolaris cynodontis* ranging from 94.7 to 100% under *in vitro* conditions, values similar to those found in this study with *B. oryzae* (94.2-100% of MGI). In another study, Chiangsin *et al*. (2018) reported an *in vitro* mycelial inhibition of 73.5% in *B. oryzae* due to the effect of *B. subtilis*, whereas Karan *et al*. (2022), reported *in vitro* inhibitions between 31.1 and 70.0% with diverse strains of *B. subtilis*. Likewise, *B. amyloliquefaciens* has been identified as a biocontrol agent against *B. oryzae* by reducing its *in vitro* mycelial growth by up to 78.6% and reducing the incidence of the disease in rice plants by 72.6% (Prabhukarthikeyan *et al*., 2019).

Actinobacteria, including *Streptomyces*, are capable of inhibiting the growth of economically important phytopathogens (Sánchez *et al*., 2022). For *Bipolaris oryzae*, inhibitions of less than 50% have been reported, with a minimum inhibitory concentration of 1.25 mg mL^{-1} of *Streptomyces* sp. strain G filtrate, isolated from Iranian soils (Nejad et al., 2014). In another study, Fathi *et al*. (2019) reported the *in vitro* ability of various *Streptomyces* strains to inhibit the mycelial growth of *B. oryzae*, with the highest inhibitions reaching 50.0%. This value was surpassed by the mycelial inhibition percentages observed in this study with *S. lydicus* (Domus®, 81.1–100%) and *S. jofer* (BlireFree®, 69.1%). For *Bipolaris maydis*, mycelial inhibition percentages of 80.0% have been reported due to lactones produced

by *Streptomyces sp.* SN5431 in *in vitro* experiments (Wang *et al*., 2023). This percentage was similar to that found in this study with *S. lydicus* (Domus®, 81.1–100%) and *S. jofer* (BlireFree®, 69.1%).

This study contributes to the phytopathological knowledge of coconut cultivation, specifically of the BGD hybrid, recently introduced to the Pacific region of Mexico. *Bipolaris oryzae* is reported for the first time as the agent associated with coconut leaf spot, and the use of biological control agents based on *Trichoderma harzianum*, *T. viride*, *Bacillus subtilis*, *B. amyloliquefaciens*, *S. lydicus*, and *S. jofer* is proposed. However, it is essential to conduct field (*in situ*) or nursery studies to understand the epidemiology of the disease under applications of the most effective commercial biological fungicides identified in this study.

Conclusions

Based on morphological characterization and molecular identification, *Bipolaris oryzae* was confirmed as the agent associated with leaf spot in the hybrid coconut palm "Brazilian Green Dwarf". *B. oryzae* can produce laccase, an enzyme involved in pathogenesis. The Sivakumar and wheat bran culture media enabled the highest laccase activity of *B. oryzae*. Additionally, the wheat bran medium promoted the highest sporulation (329.0 conidiospores mL^{-1} $disk^{-1}$) and an adequate daily growth rate (1.32 cm day^{-1}). In bioassays with commercial biological fungicides, *Trichoderma harzianum* outperformed *T. viride*, inhibiting *B. oryzae* growth by 100% at all four tested doses. The bacteria *Bacillus subtilis* (100%), *Streptomyces lydicus* (100%) and *B. amyloliquefaciens* (100%) were more effective in the *in vitro* control of *B. oryzae* compared to the actinobacteria *S. jofer* (69.1%).

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