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Epidemiological etiology of *Erysiphe* sp. and putative viral and phytoplasma-like symptoms in Ayocote bean (*Phaseolus coccineus*)

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ABSTRACT

Introduction/Objective. Ayocote bean (*Phaseolus coccineus*) has potential as a source of resistance in breeding programs because it exhibits greater tolerance to plant pathogens than *P. vulgaris*. However, its sanitary characterization is insipient; therefore, the purpose of this work was to carry out an etiological-epidemiological diagnosis, with emphasis on presumptive symptoms of viral and phytoplasmic organisms, and a typical fungal signs of powdery mildew.

Materials and Methods. A plot (50 x 62 m) of flowering Ayocote bean was selected. It was divided into 80 (8 x 10) quadrats (6 x 6 m) and 720 subquadrats (2 x 2 m). From 25 plants with powdery-mildew-type leaf symptoms, mycelium was collected with adhesive tape for light microscopy observation and taxonomic identification. Length-width measurements were made on 60 conidia. Pure mycelium collected *in situ* and *ex situ* from 1-5 leaflets/plant was used for genomic analysis by PCR with universal primers ITS1 and ITS4. Samples were sequenced in Macrogen Inc. Korea. A total of 63 plants and 121 trifoliate leaves with viral and phytoplasmic symptoms were collected by direct sampling. In 88/121 samples, genomic analysis was performed by PCR with universal primers for *Potyvirus* (1), *Begomovirus* (2), and Phytoplasmas (1). Sequence editing and analysis were performed in SeqAssem and BLASTn/GenBank. Phylogenetic constructions were developed in Mega 11 with MUSCLE, Maximum Likelihood (ML), and HKY substitution model (1000-Bootstrap). Putative powdery mildew severity (%), flower damage (%), *Macrodactylus* sp. adult density, and plant vigor (%) were evaluated in 80 quadrats

(3subquadrats/quadrat) with App-Monitor®v1.1 configured with a 5-class scale. In GoldenSurfer® v10, Kriging geostatistical analysis was performed to determine the spatial interrelationship between these variables.

Results. *Erysiphe vignae* was identified as associated with powdery mildew of *P. coccineus*. The fungus, with hyaline, ovoid to ellipsoid conidia measuring 31.74 ± 0.3419 µm x 15.11 ± 0.1579 µm, without the presence of fibrosin bodies, had 100% genomic homology. This is the first report in Mexico. With average July-August temperature and relative humidity of 16.3 °C (±5.8) and 92.8 % (±10.7), respectively, powdery mildew leaf incidence and severity were 65.3 and 22.7 % (±16.9, range: 0 - 66.5 %), respectively. The most inductive focus (60-80 % severity) had an aggregate e 4-quadrat pattern (96 m^2, *lag* = 4 and σ^2-s = 450). Inoculum dispersal was significantly associated with dominant North-South winds and plant vigor (*lag* = 4 and σ^2-s = 470). Flower damage was inconclusive in its spatial association with powdery mildew and *Macrodactylus* sp. suggesting uncorrelated events. No *Potyvirus*, *Begomovirus*, or Phytoplasmas were detected associated with yellowing, leaf distortion, mosaic, internode shortening, and other symptoms observed *in situ*. This confirms the relative tolerance/resistance reported for *P. coccineus*.

Conclusion. *E. vignae* (Erysiphales: Erysiphaceae) associated with *P. coccineus* is reported for the first time in Mexico with moderate to intense epidemic level, which indicates its susceptible condition to this fungus. However, negative results for *Potyvirus*, *Begomovirus*, and Phytoplasmas, validate the apparent tolerance/resistance of *P. coccineus* to these organisms.

Key words: *Erysiphe vignae*, *Macrodactylus* sp. powdery mildew, *Potyvirus*, *Begomovirus*.

Introduction

Phaseolus genus (Fabaceae: Papilionoideae) is widely distributed in the world, although the origin centers are Mesoamerica and South America (Graham y Ranalli, 1997). Among the 56 landrace species to Mesoamerica in this genus (Mora-Aguilera *et al*., 2023), 'Ayocote' bean (*Phaseolus coccineus*) is the third most economically important after the 'ancho' bean (*P. lunatus*), and common bean (*P. vulgaris*) (De Ron and Santalla, 2013; Santalla *et al.,* 2004). Other landrace materials or cultivars of *P. coccineus* are cultivated in Central America, South America, Africa, Asia, and Europe, in addition to Mexico (Giurca, 2009; Watanabe and Tojo, 2006).

The Ayocote bean is a vigorous and semiperennial plant that provides 18.4 % of protein, an amount lower than average of common beans (Pérez *et al*., 2002), but high tolerance to cold and plant pathogens such as *Colletotrichum* sp*., Uromyces* sp., *Sclerotinia sclerotiorum* y *Xanthomonas campestris* (Vargas *et al*., 2014; Giurca, 2009; Schwartz *et al*., 2006). Notoriously, since Japanese genotypes were introduced, this bean has been susceptibility to *Pythium myriotylum* showing stem and root necrosis in commercial plantations, reaching 93-100 % incidence under controlled inoculations (Watanabe and Tojo, 2006). However, this fungus has not been reported in Mexico for this crop. Regarding viral diseases, *Cowpea mild mottle virus* (Betaflexiviridae: Carlavirus) has been reported in symptomatic conditions (chlorotic lesions, moderate and severe mottling, moderate mosaic, and tissue deformation), and asymptomatic from Jalisco y Nayarit (Chiquito-Almanza *et al*., 2018); however, the viral agent prevalence in Ayocote bean is lower 20 %, whereas in common bean it is over 97 % in western Mexico (Chiquito-Almanza *et al*., 2021).

These studies support that *P. coccineus* exhibits greater tolerance to pathogens than *P. vulgaris*. This tolerance condition can be due to restricted genetic manipulation of the specie, allowing natural adaptation to parasitic stress factors. On the other hand, *P. coccineus* exhibits greater allogamy compared to the common bean, leading to greater genetic diversity expressed as phenotypic plasticity, productive cycle duration, color, and weight of bean-grain (Vargas *et al*., 2014). The parasitic tolerance of Ayocote bean has justified the incorporation into breeding programs in Mexico, although emphasizing to improve productive features. The first cultivar developed by Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) was 'Blanco Tlaxcala' with this approach, keeping natural tolerance to rust and anthracnose (Vargas *et al*., 2014; Pérez *et al*., 2002). Nonetheless, *P. coccineus* has been of concern as a resistance source for *Sclerotinia sclerotiorum* y *Bean golden yellow mosaic virus* management (Osorno *et al*., 2007; Schwartz *et al*., 2006).

In the experimental field from Colegio de Postgraduados, Campus Montecillo, Estado de México, in a plot of *P. coccineus* with 3100 m^2 planted for seed production and genetic improvement purposes, high incidence of symptoms presumptive to powdery mildew was detected, in addition to presence of distortion and leaf yellowing putatively associated to virus or phytoplasmic infection. Due to the recognized tolerance of Ayocote bean to plant pathogens, in order to characterize the parasitic behavior for future breeding programs, this work aimed to determine the etiology of described symptomps; to analize the spatial contagious behavior of presumptive powdery mildew, the most prevalent disease, for determining the epidemiological fitness considering that study region falls within the origin and diversification center of *P. coccineus*.

MATERIALS AND METHODS

Experimental plot. On July 2022, summer-autumn crop cycle, a plot (50 x 62 m) of Ayocote bean on flowering stage from Colegio de Postgraduados, Campus Montecillo Texcoco, Estado de México (19°46´80´´N, 98°89´76´´W) was selected. The plants, second-year resprouts, exhibited severe symptoms of presumptive powdery mildew and viral/phytoplasmic type (Figure 1). The characterization plot showed irregular density crop, discontinue canopy, moderate agronomic management, weeds control, and flood irrigation. A 13mpx image was captured through a 50 m vertical flight using a DJI® Phantom-3 drone for spatial sampling design. The plot was divided into 80-quadrants and 720-subcuadrants with 6x6 m and 2x2 m, respectively, to assess the powdery mildew severity type and other prevalent symptoms (Figure 1A). Field quadrant marking was carried out using peripheral wooden-stakes and slat-net made (Figure 1B). A HOBO u23 Pro v2 weatherproof data logger to measure temperature (°C) and relative humidity (HR) setting at 30 min intervals between 16-July and 11-August 2022 was placed at plot centroid.

Etiological and genomic identification of the fungus

Morphological identification of the fungus. A targeted sampling was performed in 25 plants with different severity levels of the powdery mildew symptom, the only fungal symptom detected. On symptomatic leaves, using the adhesive tape-aqueous solution technique, microscope slides *in situ* and laboratory were prepared for structure review under a compound microscope (40x) and fungal identification at genus level using taxonomic keys (Barnet and Hunter, 1998). Sixty conidia were measured with ImageJ® software and the average ± standard deviation of conidia length and width were calculated using a sample determined by Muestra-N Cuanti v.1.0 (CP-LANREF, 2020. Unpublished). Microscopic observations were compared with specialized taxonomic descriptions (Takamatsu, 2014; Cook and Braun, 2009; Glawe, 2008).

DNA extraction and genomic identification of the fungus. A targeted sampling was performed in 10 plants spatially distributed on the plot. Per plant, 3-5 leaves with signs of the fungus were collected and placed independently in 'polipapel' bags for transporting to laboratory. Leaves immediately were photographed. Only one leaflet was selected by quantity and quality of symptom-sign criteria. In order to avoid fungal contaminants, one leaf per plant was scraped from surface mycelium in early infections showing active signs and severe growth. Collected mycelium was placed in an Eppendorf tube with 600 µL of AP buffer and two 1/8"

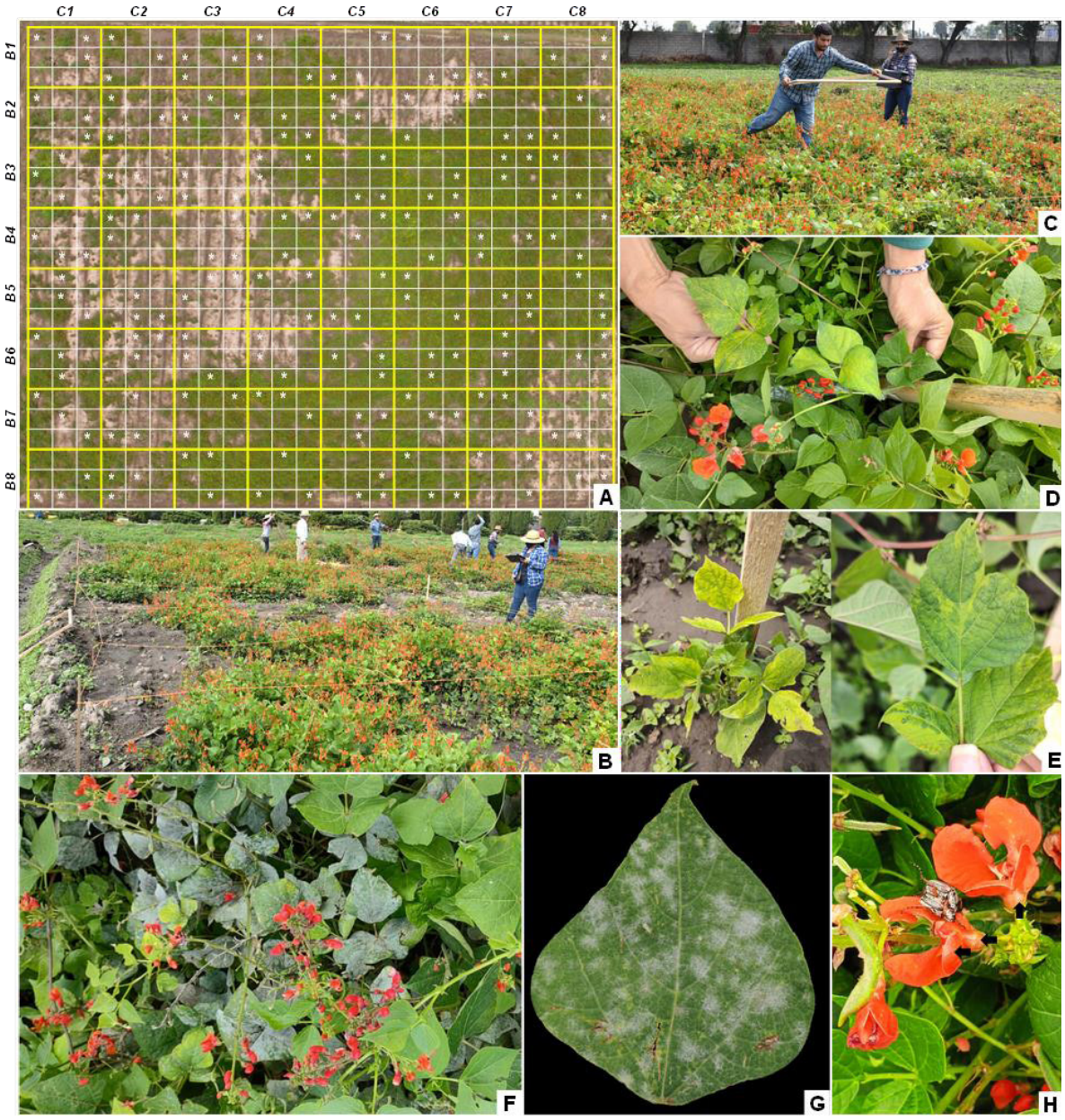

Figure 1. Sampling methodology to identify and assess severity of fungal, presumtive viral/phytoplasmal symptoms, and entomological signs on *Phaseolus coccineus*. **A**. 13mpx image at 50m using DJI® Phantom-3 drone, showing quadrantization of experimental plot. Yellow-lines correspond to 6x6 m quadrants, and white-lines to 2x2 m subquadrants. Asterisks indicate randomly selected subquadrants/quadrants; **B**. Field quadrant marking with wooden-stakes and a slat-net; **C.** Selection of subquadrant by placing wooden frame 1x1 m for assessment guidance; **D**. Dotted mosaic symptoms (left) and vein clearing (right), putative to virosis. Plants marked with stakes for traceability samples; **E**. Generalized yellowing with growth reduction (left), mosaic with leaf deformation (right) presumptive viral; **F**. Leaf symptom with white fungal mycelial growth putative to powdery mildew; **G**. Front leaflet showing white mycelial growth. **H.** *Macrodactylus* sp. adults, and flowering color-morphology of *P. coccineus*. Note some petals showing small white-spots (see arrows).

pellets. Each tube was considered as a sample/plant. The mycelium in tube was macerated in a disruptor at 30 frequencies for 15 min. The pellets were removed. One µL of β-mercaptoethanol was added, vortexed for 10 s and incubated at 56 °C for 45 min. After that, 400 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added, homogenized by vortex, and centrifuged at 13,500 rpm for 10 min at laboratory temperature. Five hundred µL of aqueous phase was recovered, 500 µL of isopropanol and 50 µL of ammonium acetate were added, mixed by immersion, and incubated at -20 °C overnight. Subsequently, it was centrifuged at 13,500 rpm for 10 min. The supernatant was discarded and washed with 500 µL of ethanol at 80%. It was centrifuged at 13,500 rpm for 3 min. The supernatant was discarded, and the pellet was dried for 1 h and finally resuspended in 50 µL of nuclease-free water and stored at -20 °C. Per sample, the concentration and purity of total nucleic acids were quantified with NanoDrop 2000 (Thermo Fisher Scientific 2000, EUA). For PCR, ITS1 and ITS4 primers amplifying the ITS region of the DNA were used (Table 1). The reaction mixture consisted of 10 µL of each primer, 0.2 µL of Taq *DNA* Polymerase (Invitrogen), 2 µL of DNA (40 ng μL^{-1}) and 16.5 µL of nuclease-free water. PCR conditions consisted initial denaturation at 95 °C for 5 min, 31 cycles with denaturation at 94 °C for 1 min, alignment at 55 °C for 1 min, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized in a photodocumenter (UVP, BioImaging Systems, Epi Chemi II Darkroom). Five of 10 samples were amplified and sent for sequencing to

Table 1. Primers, sequences, and amplicon size for genomic identification of *Potyvirus*, *Begomovirus*, Phytoplasmas and eukaryotic microorganisms in *P. coccineus* plants exhibiting signs of powdery mildew and putative virus and phytoplasma symptoms.

Organism	Primer[x]	Sequence	Size (bp)	Reference
Fungus	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	~500	White *et al*., 1990
Potyvirus	NIb2F NIb3R	GTITGYGTIGAYGAYTTYAAYAA TCIACIACIGTIGAIGGYTGNCC	350	Zheng *et al*., 2008
Begomovirus	PBL1v2040 PCR1c	CARTGRTCKATCTTCATACA CATATTTACRARWATGCCA	500~650	Rojas *et al*., 1993
	AV494 AC1048	GCCYATRTAYAGRAAGCCMAG GGRTTDGARGCATGHGTACATG	~550	Wyatt y Brown, 1996
Phytoplasmas	P1 P7	AAGAGTTTGATCCTGCAGGAT CGTCCTTCATCGGCTCTT	1,800	Deng y Hiruki, 1991; Samart *et al*., 1996
	FU5 RU3N	CGGCAATGGAGGAAACT TTCAGCTACTCTTTGTAACA	890	Lorenz *et al*., 1995

[x] Universal primers were used.

Macrogen®, South Korea. Four sequences of five amplicons had quality required for identification studies (FAC6, FAC7, FAC8 and FAC9). All sequences were edited with SeqAssem software (https://acortar.link/r0FdRx) and compared in BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with GenBank sequences to determine pathogen identity based on percent identity and *e*-value.

Phylogenetic analysis. Once *Erysiphe* sp. was identified, in order to determine the phylogenetic relationship among other species of this genus, as well as to confirm identity, the sequences obtained were aligned against sequences downloaded from GenBank using the MUSCLE algorithm in Mega 11 (Table 2). The phylogenetic analysis was performed with the same software, using the Maximum Likelihood (ML) statistical method, and Hasegawa-Kishino-Yano substitution model with 1000 Bootstrap replications. *Oidium* sp. (accession number: EU377475) was included as an outgroup. The analysis included 12 sequences of approximately 561 bp.

Table 2. Sequences obtained from the NCBI Genebank used to construct the phylogenetic tree for comparison with amplicon sequences obtained from four samples of powdery mildew fungus present in *P. coccineus* plants.

Powdery mildew causing species	Genbank accesion	Plant Host	Location and Date of report
Erysiphe diffusa	FJ378880	*Glycine max*	Australia, 2006
E. fallax	LC228618	*Carica papaya*	USA, 2017
E. manihoticola	MT106660	*Manihot esculenta*	Argentina
E. pisi	KR912079	*Pisum sativum*	China, 2016
E. vignae	MW293895	*Vigna radiata*	Australia, 2019
E. vignae	MT628286	*V. radiata*	Australia, 2019
Erysiphe sp.	JQ730709	*Senna septemtrionalis*	Mexico, 2012
Oidium sp.	EU377475	*Knautia arvensi*	USA, 2009

Genomic identification of viruses and phytoplasma

Plant sample collection. Plants exhibiting presumptive symptoms of *Potyvirus*, *Begomovirus* and Phytoplasma were selected *in situ*. Symptoms included faint mosaics, moderate leaf deformation, generalized yellowing, leaf chlorosis, vein clearing, leaf reduction, and violaceous coloration on leaves, peduncles and stems. Plants selected were marked with wood-stakes and labeled for later sampling. The sampling was conducted at two different periods; in the first, two trifoliate leaves were collected in 32 plants, each leaf as a replicate (64 samples), and in the second, 31 plants were collected.

Nucleic acids extraction (RNA and DNA). Leaf tissue of the 64 samples from first sampling was macerated in liquid nitrogen. A total of 0.1 g was weighed in 32 samples selected by symptoms and total RNA was extracted using the modified Green and Sambrook method (2012). For the second sampling, total RNA and DNA extraction was performed using the 2 % CTAB method (Yu, 2012; modified by CP-LANREF, 2021). Per sample, concentration and purity of nucleic acids were quantified with NanoDrop 2000 (Thermo Fisher Scientific 2000, USA).

***Potyvirus* genomic identification**. Nucleic acid of 28 and 18 samples from the first and second sampling, respectively, were analyzed. NIb2F and NIb3R universal primers (Table 1) which amplify part of the coding region of the *Potyvirus* genome (350 bp) for the nuclear inclusion protein B (NIb) were used (Zheng *et al*., 2008). Firstly, cDNA synthesis of total RNA was performed and subsequently RT-PCR according to Gonzalez-Cruces *et al.* (2022) protocol. A pre-RT-PCR reaction mixture was performed with 9.75 µL of nuclease-free H_2O, 500 nM per primer and 2.5 µL of total RNA, incubated at 85 °C for 3 min. For RT-PCR, a mixture with 2 mM mix dNTP's, 1X buffer-RT and 100 U of reverse transcriptase enzyme (M-MLV-RT), all from Promega Corp. USA was added to the pre-RT-PCR reaction obtaining a total reaction volume of 25 µL. RT-PCR conditions were 44 °C for 60 min for incubation, followed of 92 °C for 10 min. The final reaction volume was 25 µL consisted of: 12.5 µL of Gotaq® G2 Hot star mix, 500 nM of each primer and 2.5 µL of cDNA. A program with initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 45 s, alignment at 45 °C for 45 s, extension at 72 °C for 45 s, and final extension at 72 °C for 5 min was performed.

***Begomovirus* genomic identification**. Leaf tissue from 17 samples (second sampling) was analyzed. Two universal primer pairs for the *Begomovirus* genus were used: PBL1v2040 / PCR1c (Rojas *et al*., 1993) and Av494 / Ac1048 (Table 1) (Wyatt and Brown, 1996). PCR mix with PBL1v2040 / PCR1c primers were performed with a final volume of 25 µL: 1X GoTaq® buffer, 0.2 mM dNTP mix, 300 nM of PBL1v2040 - PCR1c primers, 0.5 U of GoTaq® G2 and 2 µL of DNA. The final volume was measured with nuclease-free water to 25 µL. The program included initial denaturation at 95 °C for 3 min, 30 cycles with denaturation at 94 °C for 30 s, alignment at 50 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. The PCR was performed on the Arktik Thermal Cycler (Thermo Fisher Scientific). For AV494 / AC1048 primers, the PCR mix consisted of a final volume of 25 µL: 15 µL of H_2O, 5 µL of 5X Buffer Green, 2 µL of $MgCl_2$ (7. 5 mM), 0.5 µL of dNTPs (10 mM), 0.5 µL of each primer (10 mM), 0.25 µL of GoTaq® DNA Polymerase (U μL^{-1}) (Promega) and 2 µL of DNA. PCR conditions included initial denaturation at 95 °C for 4 min, 30 cycles with

denaturation at 95 °C for 1 min, alignment at 55 °C for 2 min, extension at 72 °C for 60 s, and a final extension at 72 °C for 10 min.

Phytoplasma genomic identification. Leaf tissue from 42 samples (second sampling) was analyzed. The primers P1 / P7 were used for the first nested PCR step (Samart *et al*., 1996; Deng and Hiruki, 1991). The PCR mix was prepared for a final volume of 25 µL consisting of: 12 µL of nuclease-free H_2O, 2.5 µL of 1X Buffer, 0.6 µL of $MgCl_2$ (50 mM), 0.5 µL of dNTPs (10 mM), 2 µL of each primer (10 mM), 0.4 µL of Taq DNA Polymerase (U μL^{-1}) (Invitrogen), and 5 µL of DNA. An initial denaturation program was performed at 95 ºC for 5 min and 35 cycles at 95 ºC for 30 s, alignment at 52 ºC for 75 s, extension at 72 ºC for 90 s, and final extension at 72 ºC for 10 min. The PCR was performed in the T-100 thermal cycler (BioRad).

Primers FU5 and RU3N targeting the amplified 16S region were used in the second nested PCR step (Lorenz *et al*., 1995). PCR-direct products obtained were diluted (1:30) in sterile distilled water and used as template DNA with the same concentrations described above. An initial denaturation program was performed at 94 ºC for 2 min and 40 cycles at 94 ºC for 30 s, alignment at 56 ºC for 40 s, extension at 72 ºC for 90 s, and final extension at 72 ºC for 5 min. PCR was performed on the T-100 thermal cycler (BioRad). PCR product was analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized with UV light in photodocumenter (UVP, Biolmaging Systems, Epi Chemi II Darkroom).

Epidemiological Analysis

Sampling and assessment. Per quadrat, 3/9 subquadrats were selected as sampling units to assess variables of disease intensity and *Macrodactylus* sp. adults incidence (Coleoptera: Scarabaeidae) as a potential agent spread of presumptive powdery mildew on flower structures. Subquadrant selection was randomized using MS Excel Aleatoriza v1.0 (CP-LANREF, 2022. Unpublised) (Figure 1A). A 1x1 m portable wooden-frame was placed at centroid by subquadrant to delimit the area of assessment variables (Figure 1C-D). For sampling units having less than 25% leaf canopy, sampling was addressed to a different section inside the respective subquadrant.

Assessment variables. App-Monitor® v1.1 Android®, available in PlayStore® (CP-LANREF, 2022) was used to characterize the experimental plot and assesement variables. Geographic coordinates, state, municipality, locality, crop name, crop area, phenology, technological level, irrigation, and planting density were entered. A severity sampling of powdery mildew, flower damage and plant canopy was set up

through a 5-classes arithmetic scale: ***1***=0%, ***2***=0.01-25%, ***3***=25.01-50%, ***4***=50.01-75% and ***5***=75.01-100% (Figure 1F-G). Furthermore, *Macrodactylus* sp. adults were counted (Figure 1H). Regardless of plant number available, assessments were performed considering of canopy of visible plant tissue within sampling portable wooden-frame.

Geospatial analysis. The records in App-Monitor® v1.1 were exported in MS Excel®. Data matrix was structured in spatial array: X = rows (quadrant-i), Y = columns (subquadrant-j), and Z = variables-k for geostatistical analysis in *Golden Surfer*® v10. The maximum value obtained with the 5-class arithmetic scale for powdery mildew severity, flower damage and plant canopy were chosen to calculate a value for each quadrant based on three subquadrants assessed. For *Macrodactylus* sp. the cumulative insects per quadrant were determined. Geostatistical analysis by variable was performed using the Kriging method and displayed as contour maps. Spatial dependence and autocorrelation were calculated using omnidirectional variograms (360°) fitted to Spherical model. Variographic indicators *lag-distance* and *sill* or plateau (σ^2-s) to determine spatial dependence at plot level were obtained (Gonzalez-Cruces *et al*., 2021).

Results and Discussion

Fungal morphological and genetic identification. Symptoms observed *in situ* were irregular white powdery spots on the leaf surface, predominantly on middle and lower leaves of the plant. Yellowish spots were identified in advanced symptoms. Slight distortion and reduction of leaf area was observed on young leaves due to a greater fungus abundance. Flowers showed slight leaf distortion and whitish spots but no powdery appearance. An abundant presence of *Macrodactylus* sp. was also observed, mainly in flowers. This pest affects several crops such as maize and bean (Aragón *et al*., 2021), and therefore could be a potential spreader of fungi due to feeding and mobility habits.

The powdery appearance was confirmed by compound microscopy corresponding to signs of fungus characterized by conidia, conidiophores, and hyphae (Figure 2A-C). Fungal structures were not obtained in flowers. The conidiophores were cylindrical and erect, with straight or slightly curved basal cell followed by two shorter apical cells. Conidiogenesis *Pseudoidium*-type was exhibited (asynchronous conidial maturation, one at a time) (Figure 2B-C). Hyaline, ovoid to ellipsoid conidia of 31.74 ± 0.3419 µm x 15.11 ± 0.1579 µm, and absence fibrosine bodies (Figure 2A-E). Germ tubes with 1 to 2-fold the length of conidia, terminal or subterminal development, and lobed apices (Figure 2D-E).

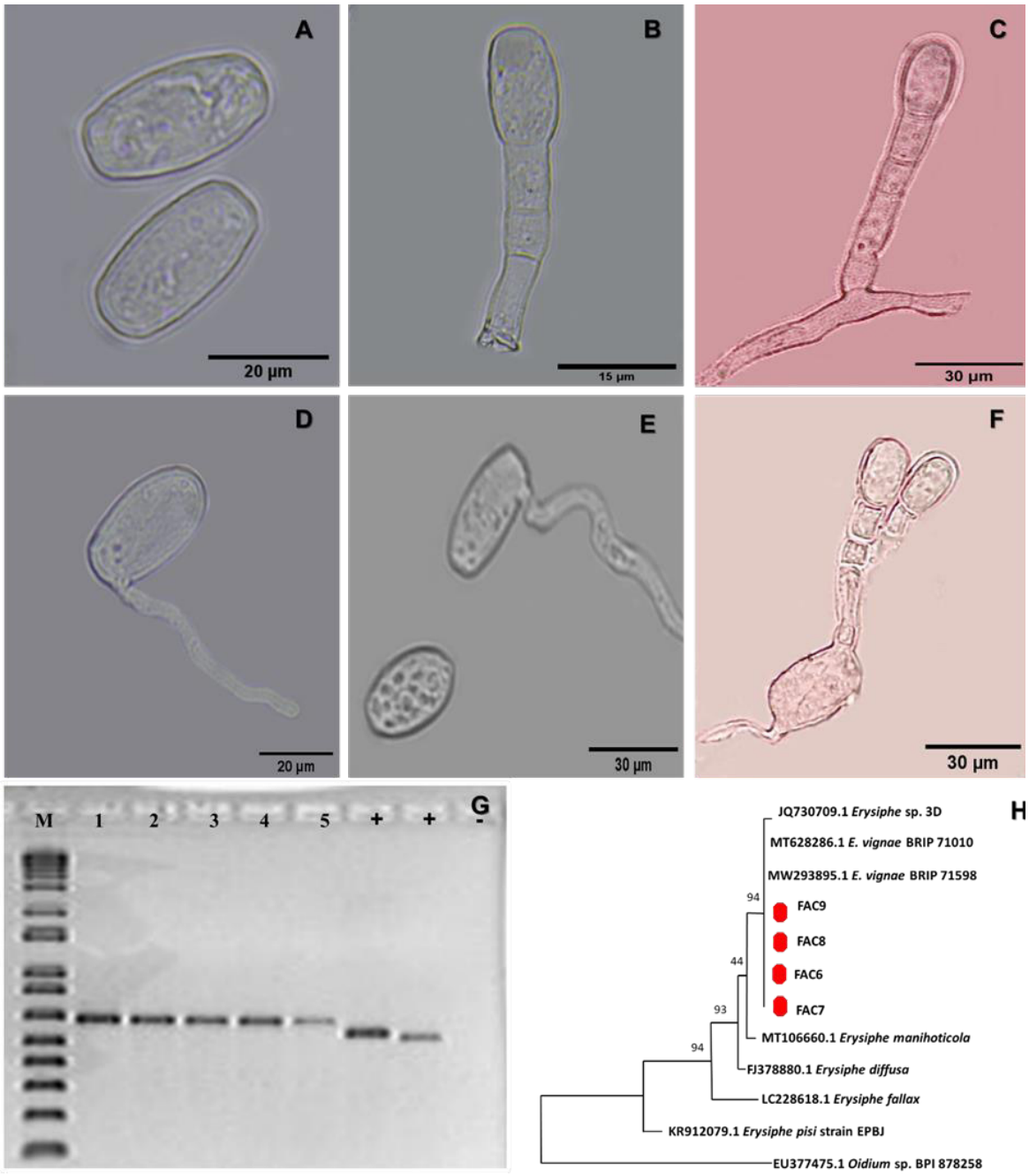

Figure 2. Morphological and genomic identification of powdery mildew in Ayocote bean (*Phaseolus coccineus*). **A**. hyaline, ovoid to ellipsoid conidia; **B-C**. cylindrical and erect conidiophores; **D-F**. germinating conidia; **G**. Amplification of the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (~500 bp) of five samples of cenicilla DNA (1-5), two positive PCR controls (+) belonging to the ITS region of *Alternaria* and *Fusarium* genera, 1 kb molecular weight marker (M) plus Invitrogen and negative PCR control (-); **H**. phylogenetic tree performed by Maximum Likelihood (ML) and the Hasegawa-Kishino-Yano substitution model with 1000 Bootstrap replications, based on ITS region of fungal sequences belonging to *Erysiphe* genus (Table 2). The study sequences are: FAC6, FAC7, FAC8 and FAC9 (red dot). *Oidium* sp. (accession number: EU377475) was included as outgroup.

Morphological characteristics determined that the fungus belongs to the *Erysiphe* genus (Bradshaw *et al*., 2020, Tovar-Pedraza *et al*., 2018; Takamatsu, 2014; Cook y Braun, 2009; Glawe, 2008). Sexual structures were not observed. Agarose gel electrophoresis confirmed amplification of internal transcribed spacer region (ITS) of nuclear ribosomal DNA (~500 bp) in five fungal DNA samples, including DNA from positive PCR controls belonging to *Alternaria* and *Fusarium* genera (Figure 2G). Four sequences were suitable for BLASTn comparative analysis with GenBank collections. The genus was confirmed by the BLASTn, hence *Erysiphe vignae* was determined as causal agent of powdery mildew in Ayocote bean with 100 % homology (*e*-value = 0). Sequences were registered in GenBank with accession numbers OQ448664 (FAC6), OQ448665 (FAC7), Q448666 (FAC8), and OQ448667 (FAC9). Furthermore, at 94 % reliability, the four sequences clustered closely to two sequences belonging to *Erysiphe vignae* (MW293895, MT628286) associated to *Vigna radiata* (Fabaceae) from Australia (Figure 2H). Morphological and genomic results in this study were consistent with reported features by Kelly *et al.* (2021) y Deng *et al.* (2022) for this specie.

In addition to reports of *E. vignae* infecting *Vigna radiata* (Kelly *et al*., 2021), in other fabaceae such as black bean (*V. mungo*) in Australia, espelon (*V. unguiculata*) in Brazil, and common bean (*Phaseolus vulgaris*) in South America and China have also been reported (Deng *et al*., 2022; Kelly *et al*., 2021). In Sinaloa, Mexico, *E. diffusa* has been reported as causal agent of common bean powdery mildew disease reaching incidences up to 10% in the planted area (Félix-Gastélum *et al*., 2017; Félix-Gastélum *et al.*, 2011). However, *E. vignae* is not reported affecting fabaceae in Mexico (Schoch *et al*., 2020). Therefore, this study provides the first report of *E. vignae* associated to Ayocote bean (*Phaseolus coccineus*) in conditions of the central Mexican Altiplano. This genus, as well as Erysiphales in overall, constitute an obligate parasite having relative host-specificity (Deng *et al.*, 2022; Félix-Gastélum *et al*., 2017), thus, fungal-plant association can be assumed as causality indicator. However, in the future, etiological studies conducting to determine the pathogenesis and to evaluate the behavior of *P. coccineous* stocks to fungal infection, analogously to common bean studies (Deng *et al.*, 2022).

Overall, Ayocote bean has been recognized for the resistance and tolerance to pests, diseases, and cold (Chiquito-Almanza *et al*., 2021; 2018; Schwember *et al*., 2017; Vargas *et al*., 2014; Giurca, 2009; Osorno *et al.,* 2007; Schwartz *et al*., 2006). For instance, Ruíz-Salazar *et al*. (2019) highlight resistance to common blight (*Xanthomonas axonopodis* pv. *phaseoli*) and anthracnose (*Colletotrichum lindemutianum*) in Ayocote bean. However, *E. vignae* is not reported by some research. Powdery mildew, caused by other *Erysiphe* species, represents a yield restricting factor for some common bean growing regions worldwide, reaching losses up to 69 % (Deng *et al*., 2022; Félix-Gastélum *et al*., 2017).

Genomic identification of viruses and phytoplasmas. *Potyvirus* was not detected with the universal primers used (Zheng *et al*., 2008) in a total of 46 leaves showing symptoms of mosaic, yellowing, leaf deformation and vein clearing (Figure 1D, F). Analogous, the genus Begomovirus was not detected using two pairs of universal primers (Wyatt and Brown, 1996; Rojas *et al*., 1993) in 17 samples showing symptoms of yellowing and slight deformation leaf (Figure 1E). In common bean, both genera were reported in Mexico, and therefore were included for this study, as well as by the coincidence with some origin centers and cultivation between common bean and Ayocote bean (Mora-Aguilera *et al*., 2023). Particularly, the sampling for Potyvirus was increased due to the higher prevalence of *Bean common mosaic virus*, probably associated to seed transmission efficiency and several aphid species present (Hemitera: Aphididade) (Gonzalez-Cruces *et al*., 2022; Chiquito-Almanza *et al*., 2021). Begomovirus occurrence, i.e. *Bean golden yellow mosaic virus*, has decreased since the 90's when it had an epidemic impact on bean crop (Chiquito-Almanza *et al*., 2021; Rojas *et al*., 1993). Worldwide, 23 viral species belonging to nine genera have been reported in common bean (*Potyvirus, Comovirus Nepovirus, Sobemovirus, Alfamovirus, Cucumovirus, Tobamovirus, Begomovirus* y *Curtovirus*), which 11 predominant species belonging to Potyvirus genus are the main causing viral diseases (Meziadi *et al*., 2017).

The Ayocote bean viral etiological researchs in Mexico are scarce (Chiquito-Almanza *et al*., 2021; 2018). Osorno *et al*. (2007) suggests that *P. coccineus* exhibits resistance to *Bean golden yellow mosaic virus*, due two genes. A recessive gene conferring resistance to leaf chlorosis and a dominant gene avoiding deformation of virus-infected seed pods. However, *Cowpea mild mottle virus* (Betaflexiviridae: Carlavirus), transmitted by *Bemisia tabaci* (Hemitera: Aleyrodidae), has been reported in Jalisco and Nayarit under symptomatic (chlorotic lesions, severe and moderate mottling, moderate mosaic, and leaf deformation) and asymptomatic conditions (Chiquito-Almanza *et al*., 2018). This virus was not analyzed in this study and therefore must be considered in future studies. However, findings in this and other studies suggest tolerance and/or resistance of *P. coccineus* to viral pathogens. For instance, a regional study in western Mexico revealed less than 20 % incidence of plants exhibiting viral symptoms in this species, reporting only *Cowpea mild mottle virus*, whereas more than 97 % in common bean were associated to at least 1/9 species of five identified virus genera (Chiquito-Almanza *et al*., 2021).

Diagnosis was also negative for phytoplasma in 42 samples analyzed using universal primers and nested PCR (Samart *et al*., 1996; Lorenz *et al*., 1995; Deng y Hiruki, 1991). The analysis was repeated several times, with PCR protocol setting and resampling *in situ*, due to the symptoms observed, overall associated with these organisms. These symptoms included leaf deformation, brown or violet coloration, evident purplish coloration on the veins of back younger leaves,

stunting and internodes shortening, slight leaves yellowing in middle stratum, and abortion and/or delay of flower emissions (Figure 3A-B). However, agarose gel electrophoresis of the PCR products obtained only showed bands of the positive control (Figure 3C). Currently, *'Candidatus* Phytoplasma asteris' has only been reported on fabaceae *Macroptilium lathyroides* (Alves *et al*., 2018) and *Crotalaria juncea* (Bianco *et al*., 2014), exhibiting leaf reduction, yellowing, and stunting symptoms. The leafhoppers *Empoasca kraemeri*, *E. fabae*, and *E. papayae* (Hemiptera: Cicadellidae) have also been reported associated with a viral and phytoplasmic symptom complex in common bean (Lozano-Gutiérrez *et al*., 2017; Sánchez-Castro *et al.*, 2016). The presence of leafhopper adults was reported in this study, although identification was not performed. Therefore, future researches are needed to discard phytoplasma(s) presence in Ayocote bean using a greater number of production units and plant samples, in order to determine the association of these mollicutes as potential insect-vectors. Rather than productive, the main relevance of *P. coccineus* might be the potential use as resistance source in breeding studies of common bean, for which a comprehensive phytosanitary characterization of this specie is required.

Geospatial analysis. Geostatistical analysis by Kriging method and omnidirectional variograms of powdery mildew epidemic inductivity, flower damage, *Macrodactylus* sp. density and plant canopy, clearly evidenced the high infective capacity of powdery mildew (*E. vignae*) in Ayocote beans, even though the presumptive viral and phytoplasmic symptoms were not etiologically confirmed (Figure 4A-C). Powdery mildew incidence was 65.3% (subquadrant unit) and average severity 22.7% (±16.9, range: 0 - 66.5%). These severity levels are moderate to high, therefore *E. vignae* must be considered a high-risk pathogen for *P. coccineus*. Spatial contagion of powdery mildew exhibited an aggregate pattern conform by four quadrant as maximum spatial dependence in the main outbreak (96 m^2, *lag* = 4, and σ^2-*s* = 450), and 1-2 quadrants in secondary outbreaks (48 m^2, *lag* = 2 and σ^2-*s*= 350, Figure 4A). The main outbreak, exhibited by the red color continous in the scale, had a severity range of 60 - 80% and a noticeable border effect associated with highest plant canopy (range 50-75 %) (Figure 4D). This statement is evidenced by overall coincidence of the highest value colorimetric contours on the scale between the quadrants (*c*) 6-10 and blocks (*b*) 2-6 (Figure 4A and 4D). The infectious foliar of these fungi is consistent with their biological nature, which ensuring re-infection at higher biomass with microclimatic conditions suitable for inductive pathogenic, such as reduced exposure to direct sunlight, which inhibits spore germination (Craig and Weyne, 2012; Hückelhoven and Panstruga, 2011). In this work, powdery mildew occurred at average temperatures and relative humidity (July-August) of 16.3 °C (±5.8) and 92.8 % (±10.7), respectively. However, the directional spatial

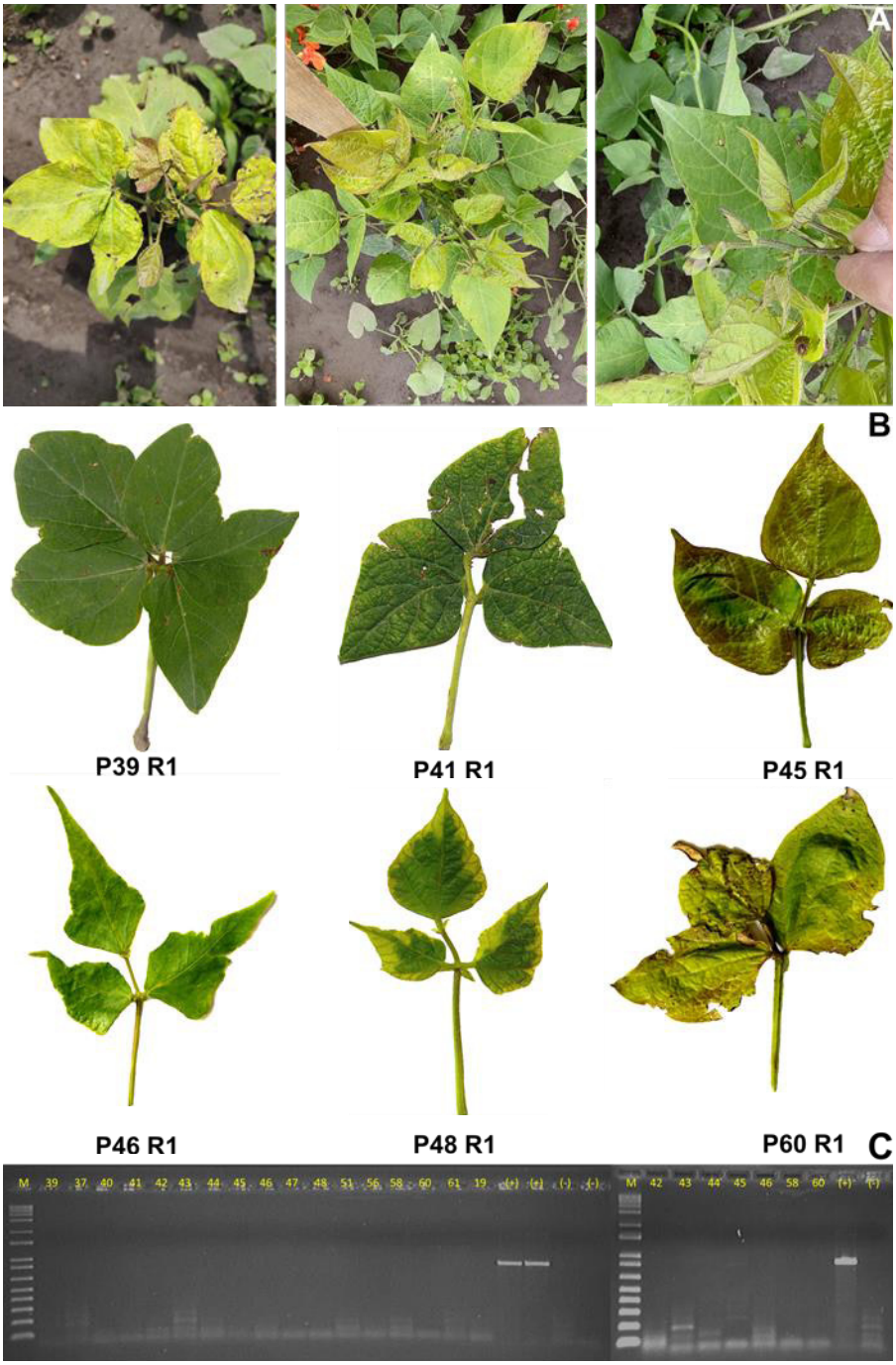

Figure 3. Presumptive field symptoms of phytoplasma infection in Ayocote bean (*Phaseolus coccineus*). **A**. Putative symptoms to phytoplasmas. Including leaf deformation, brown or violet coloration, obvious purplish coloration on veins of back young leaves, stunting, slight yellowing of leaves; **B**. Examples of six trifoliate leaves showing presumptive symptoms of phytoplasmas, included in PCR diagnosis; **C**. Agarose gel electrophoresis of 24 samples processed by nested PCR. PCR-amplified positive controls only.

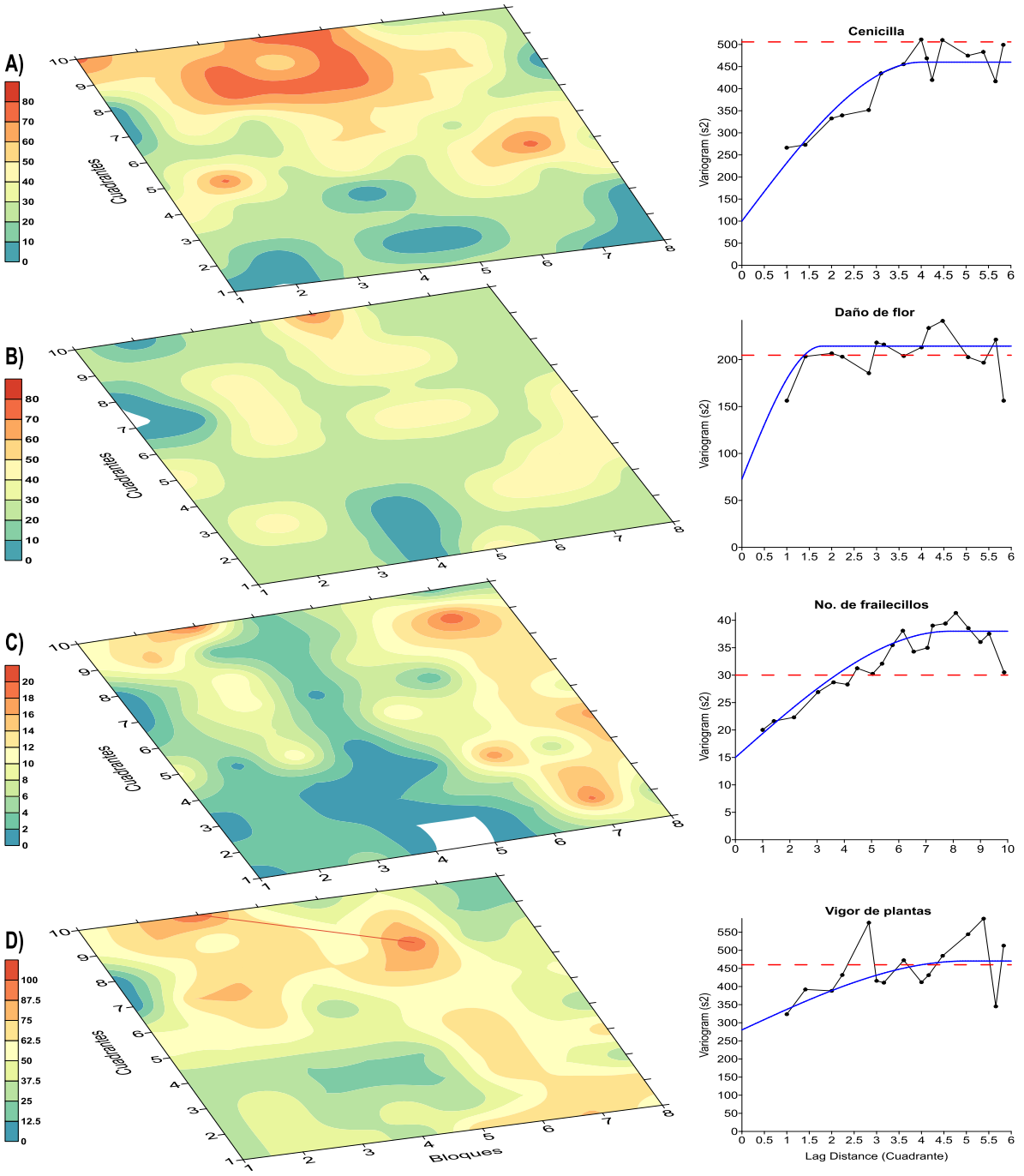

Figura 4. Geostatistical Kriging contour maps and variograms of phytosanitary variables and vigor in Ayocote bean. **A**. Powdery mildew severity, **B**. Flower damage, **C**. Density of *Macrodactylus* sp. adults and **D**. Plant canopy. For adult analysis, cumulative of three subquadrants per quadrant was calculated. For the analysis of powdery mildew severity, flower damage, and plant canopy, the maximum damage obtained per quadrant was calculated. Omnidirectional variograms were obtained by the Spherical method. X-axis = distance-*lag* in quadrants and Y-axis = variance (σ^2).

dependence, and aggregate structure; also validated that dominant North-South winds effect in the gradient conformation. Moreover, the high epidemiological fitness of Erysiphales is well known by the abundant and intermittent conidial production with easily released and spread by air (Deng *et al*., 2022; Glawe, 2008). Plant canopy heterogeneity, probably due to the second growing year or phenotypic plasticity (Vargas *et al*., 2014), explained the conformation of differentiated plant canopy aggregates and identification of spatial dependence (*lag* = 4 and σ^2-*s* = 470) (Figure 4D). In epidemiological terms, flower damage was not associated conclusively with powdery mildew incidence. This agreed with signs absent at the microscopic level. The spatial distribution of this damage had randomly distributed, moderate outbreaks with dependence upon 1-2 quadrants and 30 - 40 % severity (*lag* = 1.5 y σ^2-*s* = 250) (Figure 4B). No colorimetric correspondence between flowers damage and powdery mildew incidence maps (Figure 4A) suggested events without infectious correlation.

Similarly, the spatial density of *Macrodactylus* sp. adults, although heterogeneous, two main large aggregates were located between 1-10 *c* and 6-8 *b*, and between 5-10 *c* and 1-3 *b*, containing one and four foci (> 18 insects/centroid), respectively (Figure 4C). A contour map of flower damage dispersion or powdery mildew on leaf, as originally hypothesized, was not associated with *Macrodactylus* sp. spatial distribution. No feeding damage was detected in flowers, where adults were more prevalent (Figura 1H). The *Macrodactylus* sp. role as potential pollinator, important in *P. coccineus*, was not evaluated. The adults have been reported feeding on flowers, fruits, pollen grains, and sweet sap secretions of several crops such as maize and common bean (Aragón *et al*., 2021). *Macrodactylus* sp. involvement in *E. vignae* dissemination is not conclusive. For this purpose, comprehensive spatio-temporal studies need to be conducted.

Conclusions

The pathogen associated with powdery mildew on *Phaseolus coccineus* was *Erysiphe vignae* (Erysiphales: Erysiphacea). This is the first report on Ayocote bean in Mexico. Powdery mildew exhibited an incidence of 65.3 % and average severity of 22.7 % (±16.9, range: 0 - 66.5 %). These severity levels were from moderate to high, and therefore *E. vignae* must be considered a pathogen of high-risk for *P. coccineus*. Aggregate pattern of *E. vignae* infection was associated with plant canopy and directionality of dominant winds. The pattern was not associated with other damage such as whitish spots on flowers and occurrence of *Macrodactylus* sp. (Coleoptera: Scarabaeidae). *E. vignae* can also be a risk for common bean (*P. vulgaris*) in Mexico because it has not been reported. Potyvirus and Begomovirus

species and phytoplasma-type mollicutes associated with yellowing symptoms, leaf distortion, moderate mosaic, internode shortening, and purple coloration of leaves and stems were not detected, validating the tolerance/resistance reported for *P. coccineus*. Potential nutritional importance of the Ayocote bean, as well as the implementation in breeding resistance programs, requires the encouragement of etiological-epidemiological research in *P. coccineus*.

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