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Pathogenicity, aggressivity, and inoculum load induced by two ITS-haplotypes of *Neopestalotiopsis rosae*, isolated from strawberry (*Fragaria x ananassa*) and blackberry (*Rubus* sp.)

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ABSTRACT

Background/Objective. In strawberry (*Fragaria* x *ananassa*), the main commercial rosaceous fruit in Mexico, the occurrence of *Neopestalotiopsis rosae* (Ascomycota: Sporocadaceae) was recently reported in Michoacán. The objective was to analyze, using an etiological-epidemiological approach, the implication of two *N. rosae* ITS-haplotypes, associated to five isolates from strawberry and blackberry (*Rubus* sp.) selected for regional prevalence, in the pathogenesis, aggressivity, and inoculum load in strawberry cv. San Andreas.

Materials and Methods. Three isolates of Haplotype 1 / Strawberry (H1): F142P40S, F6P35R, F17P34R, and two of Haplotype 2 (H2) / Blackberry: F142D93R and Z13P27R were cultured in V8 / 25° / 14 d until conidiomata formation, and in PDA / 25° / 7 d to obtain liquid and solid inoculum, respectively. Eight three-month-old strawberry plants cv. San Andreas were evaluated per isolate; two trifoliate leaves / 4 plants were inoculated with 4 mL spray 1 x 10^6 conidia mL^{-1}, and 4 plants with 5 x 5 mm mycelium PDA-blocks per crown wound. Sterile water was sprayed on 8 control plants. Plants were kept in a semi-covered greenhouse. Starting at 72 hai, every 3 d / 3 months, the following variables were measured: Number of lesions (LN), Lesion diameter (LD), Number of sporulating lesions (EL), Plant height, Crown diameter, and Chlorophyll units SPAD 502 (CU). The *incubation period* ***(Pi)***, *latency period* ***(Pl)***, and *generation period* ***(Pg)*** were estimated for each haplotype. Temperature and RH were recorded every 30 min with HOBO U23. Pathogenicity was validated using Koch's postulates. Isolates H1 and H2 were confirmed culturally by ITS1 / ITS4. ANOVA, in split plots design, and Tukey (p = 0.05) were conducted in RStudio.

Results. The five isolates of *N. rosae* were more infectious on **leaves** (p = 0.0001) but exhibited variable and significant aggressivity (p = 0.0001) on LN, EL, and LD, with expression of circular leaf lesions, light brown color, dark edge, and reddish halo on the upper and lower surface. H1 isolates were more pathogenic than H2 (e.g., 3504 vs. 270 LN / plant), with H1 - F142P40S being more aggressive (p = 0.05), with an average LN = 187.5 and EL = 8.45 at 2 and 26 days after inoculation (dai), respectively. In contrast, H2 - F142D93R and H2 – Z13P27R had LN = 20 and 25 at 87 and 95 dai, respectively, with a maximum average range of 0.19 - 0.37 EL > 73 dai. Similarly, in **crown**, all isolates were pathogenic, but with less aggressivity and no statistical difference. A reddish-dark discoloration and internal necrotic rot was observed in crown and low root density. H1 - F17P34R was conspicuous with a maximum NL = 20 and LE < 0.1 at 85 dai. In **leaf**, the speed of symptoms expression and sporulation rate, although variable, was higher in isolates H1 vs. H2, determined, respectively, by 2 vs. 4 dai ***Pi***, 14 - 16 (3 d) vs. 33 - 40 (7 d) dai ***Pl***, and 14 - 73 (59 d) vs. 33 - 56 (23 d) dai ***Pg***. The **crown** infection was slower with 28 d ***Pi*** in both haplotypes. Contrasting aggressivity among isolates had no significant effect on plant height and crown diameter, but did have an effect on CU reduction, with a maximum of 14.5 in H1 - F142P40S compared to control. ITS of 33 isolates confirmed the H1 and H2 pathogenicity.

Conclusion. Through significantly contrasting LN, EL, CU, ***Pi***, ***Pl***, and ***Pg***, it was demonstrated the pathogenic variability of two *N. rosae* ITS-haplotypes, prevalent in Michoacán, obtained from strawberries and blackberries commercial cultivars. A novel mechanistic understanding of pathogenesis was addressed. It is reported for the first time that *N. rosae* isolates, associated with blackberries, were pathogenic to strawberries but with less aggressivity. Sustainable regional management must consider the variable capacity of the fungus and its interaction with other pathogens with vascular capacity.

Keywords: Berries, Chlorophyll Units, Incubation Period, Latent Period, Physiology

INTRODUCTION

The strawberry and berry agroindustry in Mexico represents a commercial value of $3.789 billion and generates one of the highest rates of direct and indirect employment in the country (DGSIAP, 2025; González *et al*., 2019). Strawberry production, followed by blackberry production, has maintained steady growth, with 582,992.46 tons produced on 13,742.86 hectares in 2024 (DGSIAP, 2025). The berry production system is characterized by high dynamism with constant changes in varieties, the incorporation of technological innovations for yield purposes, and an intensive flow of plants for plantation renewal, particularly in strawberries. This has contributed to the gradual increase of various phytosanitary problems in addition to the endemic 'dry rot' caused by a fungal complex with regional prevalence of *Fusarium* spp. and whose losses can reach up to 50% (Garay-Serrano *et al.,* 2021; Ceja-Torres *et al*., 2014; 2001).

In 2017, with a low-intensity epidemic, symptoms not previously reported in Mexico were observed in strawberries, consisting of crown rot and leaf blight in young plants and the onset of fruiting in Albion, Festival, and other commercial varieties in Jacona and Zamora, Michoacán. The first diagnosis, including Koch's postulates, was made by Raúl Soto, demonstrating the involvement of *Neopestalotiopsis* sp., possibly *N. rosae*, due to reports of this species in cv Festival in Egypt (Essa *et al*., 2018) (R. Soto. 2018. Personal Communication, Agroquímica Jacona). On this basis, several research groups initiated confirmatory studies with an emphasis on strawberries. Thus, Rebollar-Alviter and colleagues confirmed *Neopestalotiopsis rosae* (Rebollar-Alviter *et al.,* 2020). However, in 2019, *Pestalotiopsis* sp. had already been reported in strawberry crops in Puebla, Mexico (Morales-Mora *et al*., 2019 and 2020), and previously in 2005 in *Rosa* sp., another Rosaceae species (https://www.ncbi.nlm.nih.gov/nuccore/AY904051.1/). In one of the first reports in berries, this genus was reported in blueberries in China associated with leaf blight (Luan *et al*., 2008).

In the most recent taxonomic review of *Pestalotiopsis* (Maharachchikumbura *et al*., 2014), *Neopestalotiopsis*, together with *Pseudopestalotiopsis*, emerges as a new genus based on morphological characteristics and genomic analysis with LSU (Large Subunit of Ribosomal DNA) and multilocus with *Internal Transcribed Spacer* (ITS), *β-tubulin* (TUB), *and Translation Elongation Factor* 1-α (TEF). Because these three genera share four-septate, elongated, straight or slightly curved conidia with 3-5 appendages from the hyaline cell, morphological differentiation is based on the conidiogenous cell and the coloration of the three central cells combined with the ITS, TUB, and/or TEF gene regions, depending on the level of taxonomic study required (Maharachchikumbura et al., 2014; 2012). TUB, and/or TEF gene regions, depending on the level of taxonomic study required (Maharachchikumbura *et al*., 2014; 2012), it is possible that *Neopestaliotiopsis* was present prior to the first epidemic reports in strawberries in the Zamora region of Mexico. Based on the proposal by Maharachchikumbura *et al*. (2014),

In 2018, CP-LANREF, based on the work of R. Soto, integrated the status of *N. rosae* into its etiological-epidemiological research, with an emphasis on *Fusarium* and *Trichoderma*. To this end, it carried out regional sampling in strawberry and blackberry crops under the premise that *Neopestalotiopsis* could not be restricted to strawberries, due to the predominance of these crops in the Michoacán berry-growing region and that it could possibly be associated with *Fusarium* due to the eventual collapse of the plant similar to *'dryer*'. This etiological assumption is based on the fact that *Pestalotia*, the genus (conidium

5-septate) from which *Pestalotiopsis*, *Neopestalotiopsis*, and *Pseudopestalotiopsis* (Maharachchikumbura *et al*., 2014) includes saprophytic, endophytic, and parasitic fungi generally considered secondary associated with leaf or fruit spots on tropical and subtropical perennial plants (Maharachchikumbura *et al*., 2014 and 2012), with no history at the vascular level. It was not until recently, mainly in strawberries, that vascular symptoms were recorded, induced by root and crown rot with eventual leaf blight and wilting caused by *N. rosae* (Olivares-Rodríguez *et al*., 2024; Lawrence *et al.,* 2023; Sun *et al*., 2021; Wu *et al*., 2020; Rebollar-Alviter *et al*., 2020; Essa *et al*., 2018).

In 2019, the presence of *N. rosae* in strawberries and blackberries was confirmed based on morphological and ITS studies (G. Mora. CP LANREF, Unpublished; Montes-Hernández *et al*., 2021). In this study, the objective was to analyze, using an etiological-epidemiological approach, the involvement of two ITS haplotypes of *Neopestalotiopsis rosae*, associated with five isolates from strawberries (*Fragaria x ananasa*) and blackberries (*Rubus* sp) selected for prevalence in the CP-LANREF regional survey, in the process of pathogenesis, aggressiveness, and inoculum load in strawberry cv. San Andreas. Preliminary reports of this work were published in SMF/RMF Supplement (Montes-Hernández *et al*., 2024a and 2024b).

MATERIALS AND METHODS

Origin of isolates and identification of *Neopestalotiopsis rosae*. In 2018, five representative isolates were obtained from an extensive epidemiological diagnosis in the Los Reyes - Zamora region, Michoacán, with 44 tissue and rhizosphere samples. The samples were obtained from 18 strawberry (*Fragaria x ananassa*) and blackberry (*Rubus* sp.) plantations and were selected based on contrasting agricultural management and different cultivated varieties (CP-LANREF, 2019. Unpublished). The isolates were identified morphologically and genomically with the ITS gene using the molecular processes and sequencing described in the following sections (Montes-Hernández *et al*., 2021). The experiment was conducted in a greenhouse under controlled conditions located in Tequila, Jalisco, km 15 on the Guadalajara-Tepic highway.

Preparation of inoculum. To induce the growth of the five isolates (H1: F142P40S, F6P35R, and F17P34R, H2: F142D93R, and Z13P27R), V8 medium was prepared from 150 g of Campbell V8 juice, 40 g of agar, and 1,000 mL of demineralized water + 0.25 g of chloramphenicol after sterilization. Five replicates were seeded for each isolate and incubated at 25 °C for 14 days to obtain conidiophores. Colonies were scraped with sterile water to remove the conidia. One mL of the solution was taken, poured into a Falcon tube with 15 mL of sterile water, homogenized, and a 100 µL sample was taken to count the conidia until a concentration of $1x10^6$ conidia per mL was obtained. A total of 16 mL per isolate was obtained. For the solid inoculum, 5 x 5 mm mycelium squares were used. The five isolates were seeded on PDA medium (40 g Potato Dextrose Agar, 20 g agar, 1,000 mL demineralized water + chloramphenicol after sterilization) with three replicates.

***In vivo* pathogenicity test on leaves and crown.** The pathogenicity test was performed on 48 three-month-old strawberry plants (cv. San Andreas) transplanted into 1 L pots with sterile, moist peat moss. A circular acetate plate was placed over the exposed crown to prevent contamination of the upper plant tissue by mycelium and spores from the . Eight strawberry plants were used per isolate (40 plants); four were inoculated by spraying the

leaf tissue and four were inoculated by wounding the crown. For leaf spray inoculation, two trifoliate leaves were selected per plant. The conidia suspension was sprayed onto the selected leaflets, completely covering the upper and lower surfaces. Four milliliters of suspension were applied per plant, immediately after which a 1-kg polypropylene bag was placed over the plant for three days, creating an optimal microclimate to ensure the infection process. Visual symptoms on the upper and lower surfaces, number of lesions, and sporulating lesions on each of the leaflets were evaluated. A total of 144 leaflets were evaluated.

For inoculation by wounding the crown, a wound was made with a sterile scalpel, and then a square of medium with the colony was placed on it using a sterile wooden stick. The internalization of the fungus was evaluated in each plant by making a longitudinal cut in the crown. Eight plants were used as controls. The evaluation of the experiment began 72 hours after inoculation (hdi), and six variables were evaluated every three days: chlorophyll units with SPAD 502 plus (KONICA MINOLTA), plant height, crown diameter with a digital vernier caliper (Truper), lesion diameter (mm), number of lesions, and number of sporulating lesions. The *incubation period (**Pi**), latency period (**Pl**)*, and *generation period (**Pg**) sensu* Vanderplank were estimated. In addition, temperature (°C) and relative humidity (RH) were recorded with a HOBO U23 Pro V2 data logger set to take readings every 30 min.

Reisolation of *Neopestalotiopsis rosae* and monosporic culture. To complete Koch's postulates, one leaflet, stem, crown, and roots with putative symptoms were selected per inoculated plant to make 5 x 5 mm cuts. Each cut was disinfected with 70% alcohol and 2% sodium hypochlorite for 2 min for each substance, and rinsed three times with sterile demineralized water. The completely dry tissue was seeded in PDA boxes (90 x 15 mm), placing five tissue fragments per box. It was incubated in a bioclimatic chamber for 5 days. Upon observing mycelium growth, it was transferred to new boxes (90 x 15 mm) with PDA medium using dissection needles and incubated until conidiophores appeared. To perform the monosporic culture, part of the conidioma was taken with a loop and seeded in a single streak on Agar-Water medium (20 g Agar, 20 mL lactic acid, 1 L water, 0.250 g Chloramphenicol). Isolates were purified using the hyphal tip method and transferred to PDA and V8 media for cultural characterization. The cultures were incubated at 25 °C for 16 days to promote mycelial growth and conidioma development. The results were compared with preliminary studies characterizing *N. rosae* in five culture media (Montes-Hernández *et al*., 2021). A photographic collection of 96 colonies of 16 dds was generated using a Canon EOS Rebel T6 camera. Finally, the monosporic isolates were preserved using three methods: slanted PDA tubes refrigerated at 4 °C, silica gel, and 10% glycerol in a freezer at -20 °C (Mendoza-Ramos *et al.,* 2021).

DNA extraction, PCR, and sequencing. A total of 33 monosporic isolates of *Neopestalotiopsis rosae* from the re-isolations were selected based on colony morphology, source of isolation, and colony prevalence: nine root isolates, 10 neck isolates, eight stem isolates, and six leaflet isolates. DNA extraction was performed using the 2% CTAB protocol (Maharachchikumbura *et al.,* 2011) with slight modifications. DNA fragments were amplified using the ITS1 (TCCGTAGGTGAACCTGCGG) / ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al*., 1990) used in multigene analyses of this genus (Maharachchikumbura *et al*., 2011). For endpoint PCR, the final volume of the reaction mix was 25 µL [1X PCR buffer (10X) INVITROGEN, 1 mM $MgCl_2$

(INVITROGEN 25mM), 0.50 mM PROMEGA deoxynucleotide triphosphates (dNTPs), 200 nM of each primer, 0.2 units of Platinum® Taq DNA polymerase (Invitrogen), and 2 µL of DNA (40 ng μL^{-1})]. The PCR conditions in the BioRad T-100 thermocycler consisted of an initial denaturation cycle at 95 °C for 5 min, followed by 31 amplification cycles at 94 °C for 1 min, 55 °C/1 min and 72 °C/1 min 30 s, and a final extension at 72 °C/5 min. The amplified fragments were analyzed by electrophoresis on a 1.5% agarose gel and 1X TBE. Once the amplification of the fragment of interest at 550 bp was confirmed, the respective PCR products were sequenced using the commercial service of Macrogen Inc., Korea (http://www.magrogen.com).

Statistical analysis. In MS Excel 2020, a matrix with 12 total variables was integrated. Temporal analyses were performed by variable classified by inoculation method, haplotype, and isolate. In RStudio, an analysis of variance was performed in a split-plot design and comparison of means with Tukey ($p \leq 0.05$) to determine the effects of inoculation method and isolate on the number of lesions and sporulating lesions of *Neopestalotiopsis rosae* on *Fragaria x ananassa*.

RESULTS AND DISCUSSION

The morphological and genomic identification of five isolates of *N. rosae*, four from strawberries and one from blackberries with 100% homology, were associated with *N. rosae* in *Fragaria x ananassa* cv. San Andreas plants. The isolates formed two haplotypes (H), with F142P40S, F6P35R, and F17P34R belonging to H1; and Z13P27R (blackberry isolate) and F142D93R to H2. The five isolates were differentially pathogenic to *Neopestalotiopsis rosae* in *Fragaria x ananassa* cv. San Andreas plants (Figure 1).

***In vivo* pathogenicity in leaves and crown.** The pathogenicity experiment on 40 inoculated plants and 33 isolates obtained from the pathogenicity test confirmed 100% homology with *Neopestalotiopsis rosae*. By inoculation method, spraying $1x10^6$ conidia mL^{-1} of *N. rosae* on strawberry plant leaflets exhibited symptoms associated with light brown circular lesions with dark edges and a reddish halo on the upper and lower surfaces of the leaflet (Figures 1A and 1D). These symptoms are consistent with Olivares-Rodríguez *et al.* (2024) in the same strawberry variety. In mature lesions, acervuli with dense conidial sporulation were observed (Figures 1B, 1D, and 1F). The absolute controls showed no signs or symptoms (Figure 1E).

Inoculation by wounding with mycelium placed at the crown level did not show symptoms of external necrosis. However, longitudinal sections revealed internal lesions with reddish coloration and downward directionality toward the plant's root system (Figure 2). The highest pathogenicity for both inoculation methods was shown with isolate F142P40S from H1, while F142D93R from H2 induced greater damage through spray inoculation. In the roots, a dark color change and a clear reduction in root density were observed (Figure 2). In general, no qualitative differences (symptom characteristics) were observed between isolates; however, the intensity of damage (development of lesions and sporulating lesions, loss of tissue in a shorter time) did show differences between inoculation method and isolate.

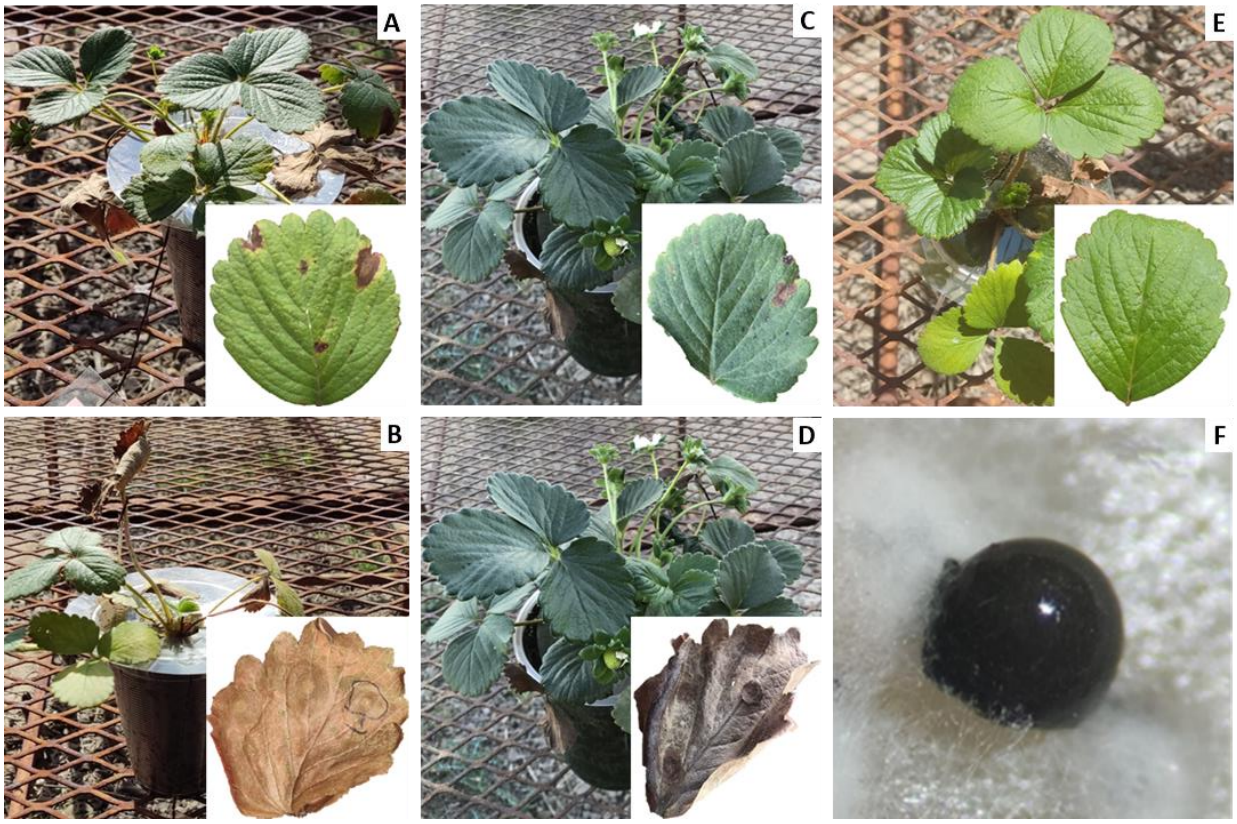

Figure 1. Expression of lesions and sporulating lesions on strawberry leaves cv. San Andreas inoculated by spraying with isolates of *N. rosae*. **A** and **B.** NL and LE 21 days after inoculation (ddi) with F142P40S. **C** and **D.** Number of lesions (NL) and sporulating lesions (LE) at 77 ddi with isolate Z13P27R **E.** Absolute control, **F.** Conidioma of *N. rosae* on PDA.

Pathogenesis of isolates. The pathogenic progress of *N. rosae* isolates obtained from strawberry, in both haplotypes, showed greater pathogenicity and damage to *Fragaria x ananassa* cv. San Andreas plants, even in haplotype 2 but with attenuated aggressiveness (Figure 3). Compared to the control, H1 represented by isolate F6P35R was the most pathogenic and severe, with maximum damage and loss of leaflets after 19 dpi, followed by F142P40S at 40 dpi; and F17P34R, an isolate of moderate-high aggressiveness with maximum leaf damage after 63 dpi (Figure 3). In the case of haplotype 2 isolates, both showed low aggressiveness. The strawberry isolate F142D93R was moderate, reaching maximum damage at 63 ddi. The blackberry isolate Z13P27R showed lower aggressiveness, possibly because it is a strain isolated from *Rubus* sp., suggesting incompatibility in the pathogenesis process in a different host such as *Fragaria x ananassa* cv. San Andreas (Figure 3).

Statistical comparison of the aggressiveness of the isolates based on the number of lesions (NL) and sporulating lesions (LE) showed effects of inoculation method ($p < 0.0001$), isolate ($p < 0.0001$), and their interaction ($p < 0.0001$). Spray inoculation induced significantly more lesions in H1 isolates than in the others. H2 isolates in both inoculation methods and all isolates inoculated by wounding showed no significant differences from the control (Figure 4A). Interestingly, at the sporulation level, the F142P40S isolate was statistically more pathogenic and aggressive than the other isolates for H1 and H2. The F142D93R isolate was the least aggressive, with no significant differences compared to the isolates inoculated by wounding (Figure 4B).

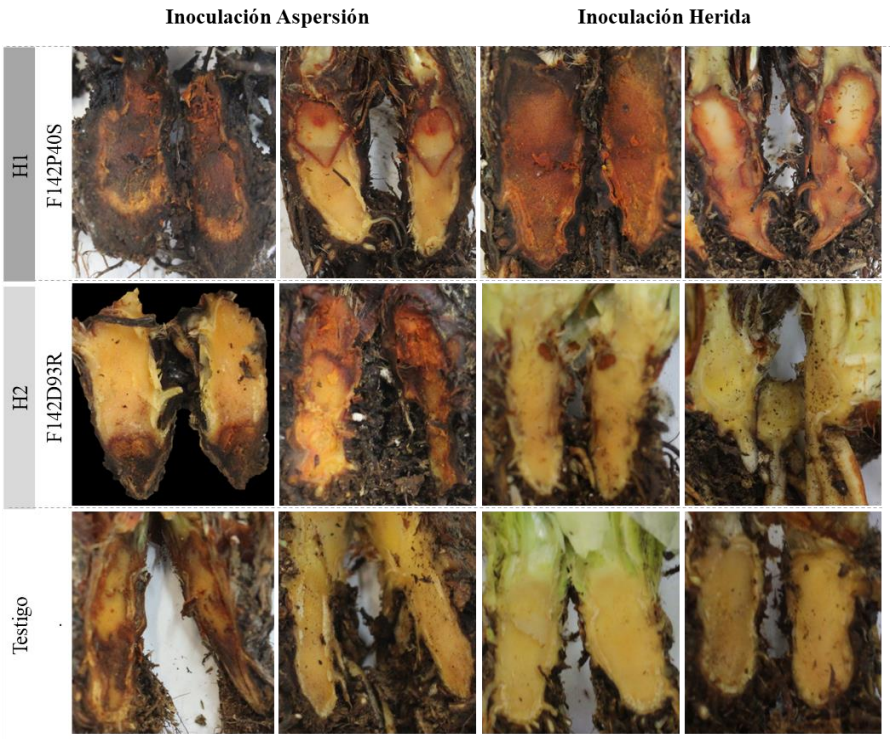

Figure 2. Longitudinal section of crown of strawberry plants cv. San Andreas at 115 ddi inoculated with *N. rosae*, F142P40S (H1) and F142D93R (H2). In wound inoculation (B), H1 caused total necrosis. The crown shows predominantly light brown to dark reddish-brown color changes. No color change was detected in control plants.

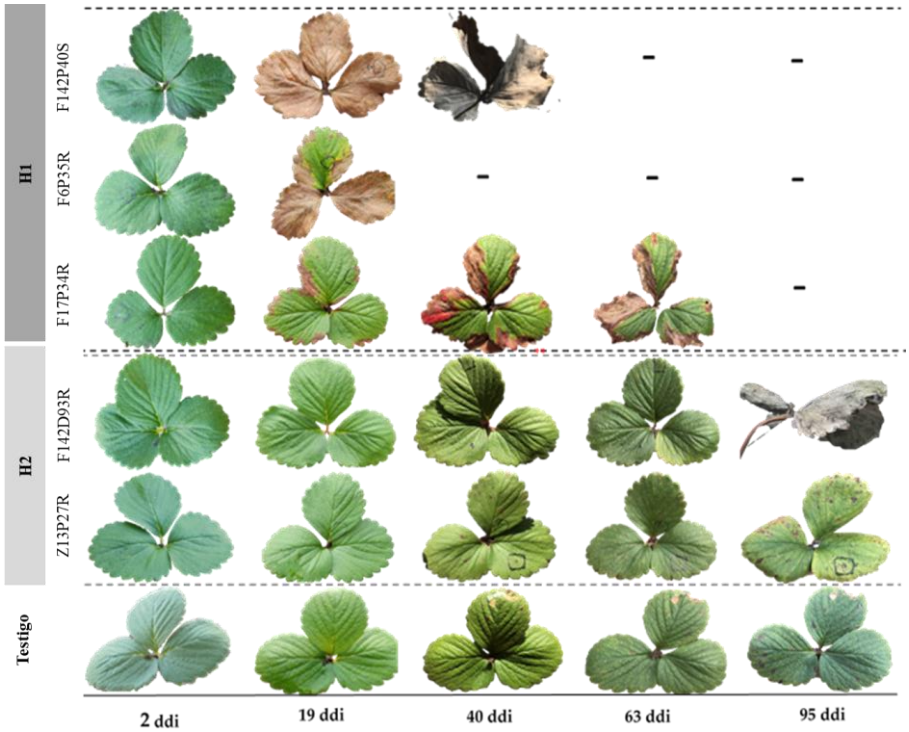

Figure 3. Progress of lesions on leaf blades of strawberry plants cv. San Andreas inoculated by spraying with five isolates of *N. rosae* corresponding to haplotypes H1 and H2 at 2, 19, 40, 63, and 95 ddi. The dashes represent leaf loss due to total tissue deterioration caused by the aggressiveness associated with each isolate. For example, in F6P35R, leaf loss is reached at 40 dpi.

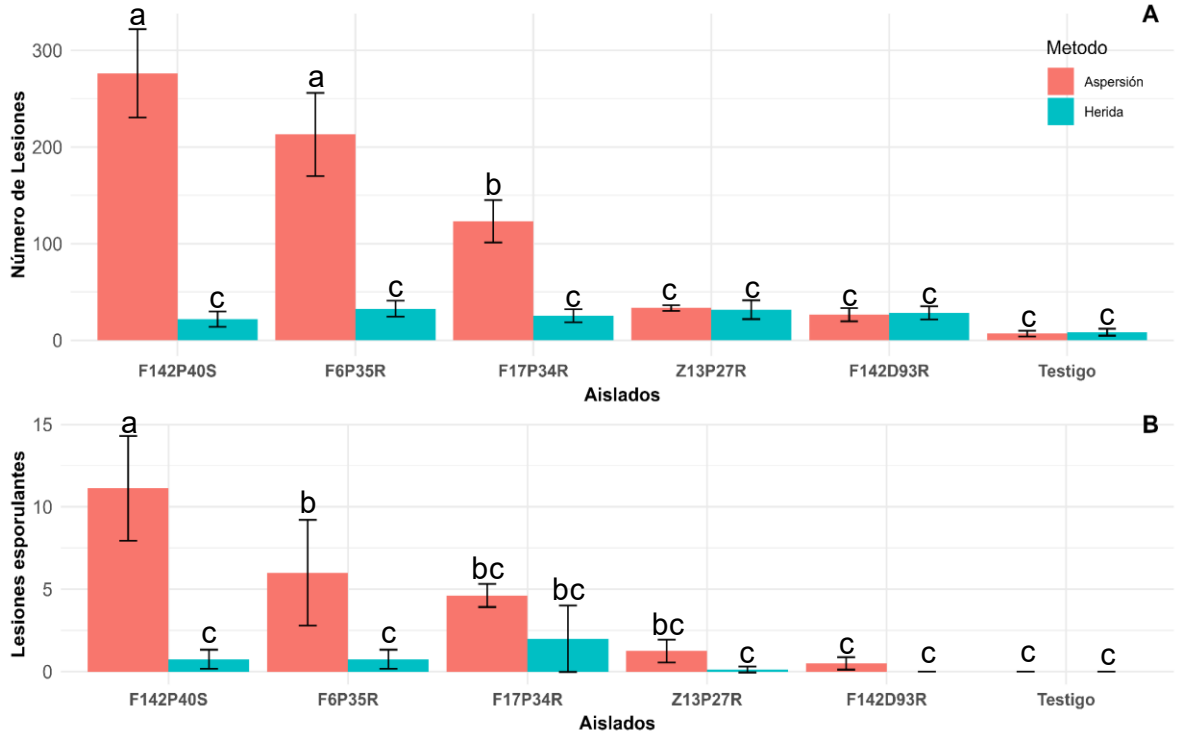

Figure 4. Analysis of variance (Tukey, $p < 0.05$) in split plots, where the large plot is the inoculation method and the small plot is the isolate for the variables number of lesions (**A**) and sporulating lesions (**B**). Different letters indicate significant differences at $\alpha = 0.05$.

Temporal dynamics of *Neopestalotiopsis rosae*. The average temporal dynamics of five isolates associated with two haplotypes (H1 and H2) of *N. rosae* was variable and contrasting in lesions and sporulation. Lesions induced by H1 isolates began at 2 ddi with a range of 50–196 lesions/plant. Isolates F17P34R and F6P35R had maximum increases up to 7 ddi, subsequently showing a decreasing trend up to 23–75 ddi. An average of 3,504 lesions/plant was recorded for H1 (Figures 5A and 6A). Comparatively, in H2 isolates, the number of lesions was highly variable, ranging from 2 to 33 ddi with a significantly lower average of 270 lesions/plant (Figures 5B and 6B).

H1 isolates sporulated more rapidly between 14 and 16 ddi. However, the duration of spore production differed; for example, isolate F17P34R sporulated for at least 30 days longer, up to 75 ddi (Figures 5A and 6A). H2 isolates showed longer spore production between 33 and 47 ddi, and specifically isolate Z13P27R had sporulating lesions until the end of the experiment (Figure 5B and 6B). The control plants showed no symptoms. Overall, the temporal dynamics confirmed the pathogenicity-aggressiveness of H1 associated with specialization to strawberry tissue, and the moderate-low pathogenicity-aggressiveness of H2, particularly of the isolate obtained from blackberry (Z13P27R).

Periods associated with the pathogenesis of *N. rosae*. The evaluation of the number of lesions (NL) and sporulating lesions (LE) allowed the pathogenesis process of *N. rosae* haplotypes to be determined by quantifying the incubation periods (Pi) from the deposition of the initial inoculum to symptomatic expression; latency (Pl) from inoculum deposition to the onset of sporulation; and generation (Pg) from the expression of the first sign to the end of sporulation, were dependent on °C and RH (Figures 5 and 6). In spray inoculation, NL and LE showed variability between isolates. Haplotype 1 (H1) was more pathogenic

and aggressive in both variables, with maximum levels in isolate F142P40S with NL = 187.5 at 2 ddi and LE = 8.45 at 26 ddi (Figure 5A). Comparatively, H2 showed maximum levels NL = 20 at 87 ddi with isolate F142D93R, and LE = 0.37 at 89 ddi in isolate Z13P27R (Figure 5B).

Notably, thresholds of °C = 21.8–26.1 and RH = 65.2–92.01 were more inductive conditions for H1 in the period from 2 to 58 ddi; while °C = 18.2–21.9 and RH < 64.9 were optimal conditions for H2 from 61 to 100 ddi (Figure 5C). The association of pathogenesis process periods was **Pi** = 2 ddi, Pl = 14–16 ddi, and Pg = 12–59 ddi in H1, notably more aggressive compared to **Pi** = 4 ddi, Pl = 37–47 ddi, and Pg = 44–46 ddi in H2 (Figure 5D).

In crown wound inoculation, NL and LE showed low variability among isolates. The onset of symptoms was delayed compared to spray inoculation. Isolate F142P40S (H1) was the most pathogenic and aggressive with the highest level NL = 15.66 at 87 ddi and LE = 0.33 at 68 ddi (Figure 6A). Relatively, the blackberry isolate Z13P27R (H2) showed maximum levels NL = 24.22 at 79 ddi and LE = 0.046 at 68 ddi (Figure 6B). The thresholds of °C = 18.4–24.8 and RH = 52.8–82.7 were the most inductive conditions for H1 in the period from 26 to 93 ddi; similar temperature conditions were inductive for the expression of lesions of H2 isolates. However, they were not favorable for the development of sporulating lesions (Figure 6C).

The association of periods of the pathogenesis process was **Pi** = 28 ddi, **Pl** = 30–42 ddi, and **Pg** = 40–69 ddi in H1, notably more aggressive and stable compared to **Pi** = 28 ddi, **Pl** = 68 ddi, and **Pg** = 11 in H2; It has the longest latency period (Figure 6D). Studies of *N. rosae* suggest that laboratory conditions of 25 °C are favorable for mycelium growth and conidioma expression, as observed in the case of haplotype 1 isolates (Maharachchikumbura *et al.,* 2014). In the field, symptom expression in Albion strawberry plants was reported between 23-27 °C with high relative humidity (> 80%) under macro tunnel conditions (Rebollar-Alviter *et al.,* 2020).

Globally, there are no studies that comprehensively determine the pathogenesis cycle of *N. rosae*. Some studies infer results from Pi, for example, Rotondo *et al*. (2023) on four-week-old strawberry cv. Cabrillo strawberries four weeks old and inoculated at a concentration of $1x10^4$ conidia mL^{-1} recorded lesions at 5 ddi; while Wu *et al*. (2020), in the Xiang-shui strawberry variety at the three- to four-leaf stage and a concentration of $1x10^6$ conidia mL^{-1} reported the appearance of the first visual symptom at 10-14 ddi. This suggests that both pathogenicity and aggressiveness are attributes dependent on the cultivar, inoculum load (conidia concentration mL^{-1}), and climatic inductivity. This is the first study to estimate the three periods of pathogenicity associated with *Neopestalotiopsis rosae* in strawberry cv. San Andreas, a variety widely cultivated in Mexico for attributes such as fruit quality, flavor, and disease tolerance (Eurosemillas, 2022).

Effect of *N. rosae* inoculation on physiological characteristics. The physiological variables evaluated did not show significant changes during the *N. rosae* infection process from the incubation and latency period (2–14 ddi). Height was reduced by 0–2.1 cm, attributable to the loss of evaluable leaves, even in the control. The crown diameter showed constant increases of 0–0.2 cm with no significant difference by inoculation method or isolate (Table 1).

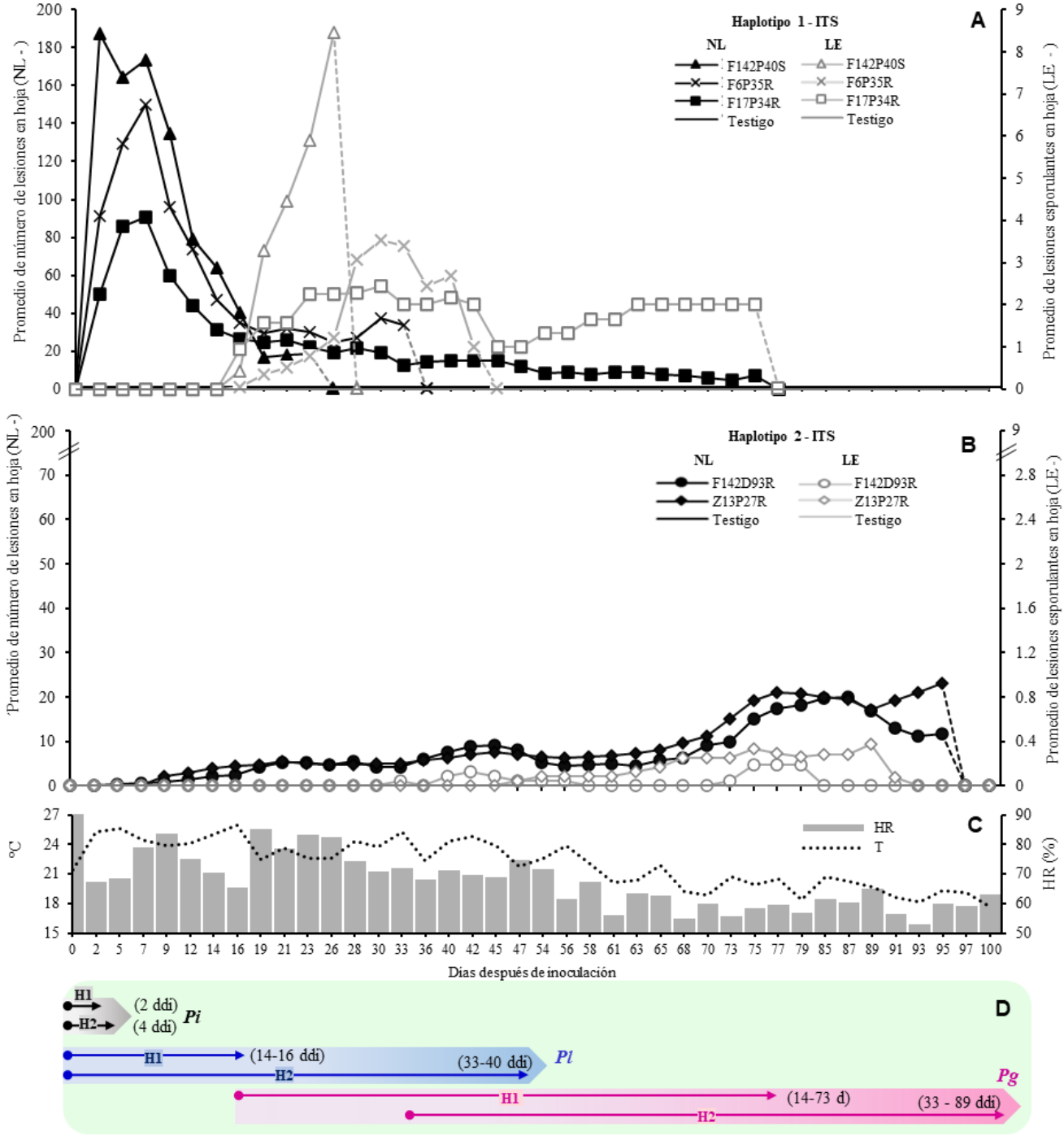

Figure 5. Dynamics of the number of lesions (NL) and sporulating lesions (LE) on strawberry leaves cv. San Andreas up to 95 days after spraying with five isolates of *N. rosae* obtained from strawberry (F) and blackberry (Z), **A**. Absolute average of NL and LE of three isolates of Haplotype 1 (H1). **B**. Absolute average of NL and LE in two isolates of Haplotype 2 (H2), **C**. Association of RH and T °C with the behavior of the fungus on tissue up to 100 days **D**. Estimation of incubation period (Pi), latency period (Pl), and generation period (Pg) by haplotype of *N. rosae*.

Chlorophyll units (CU) as an important monitoring attribute to determine stress due to biotic or abiotic factors during the three phenological stages: vegetative, reproductive, and maturation due to nitrogen loss, chlorosis associated with nutritional deficiencies, or the presence of pathogens (Vishwakarma, *et al*., 2023). In the experiment, the greatest loss of CU occurred in more pathogenic and aggressive isolates. These caused a reduction of 2.1 to 14.5 CU, compared to isolates of moderate or attenuated aggressiveness that showed CU < 2.1.

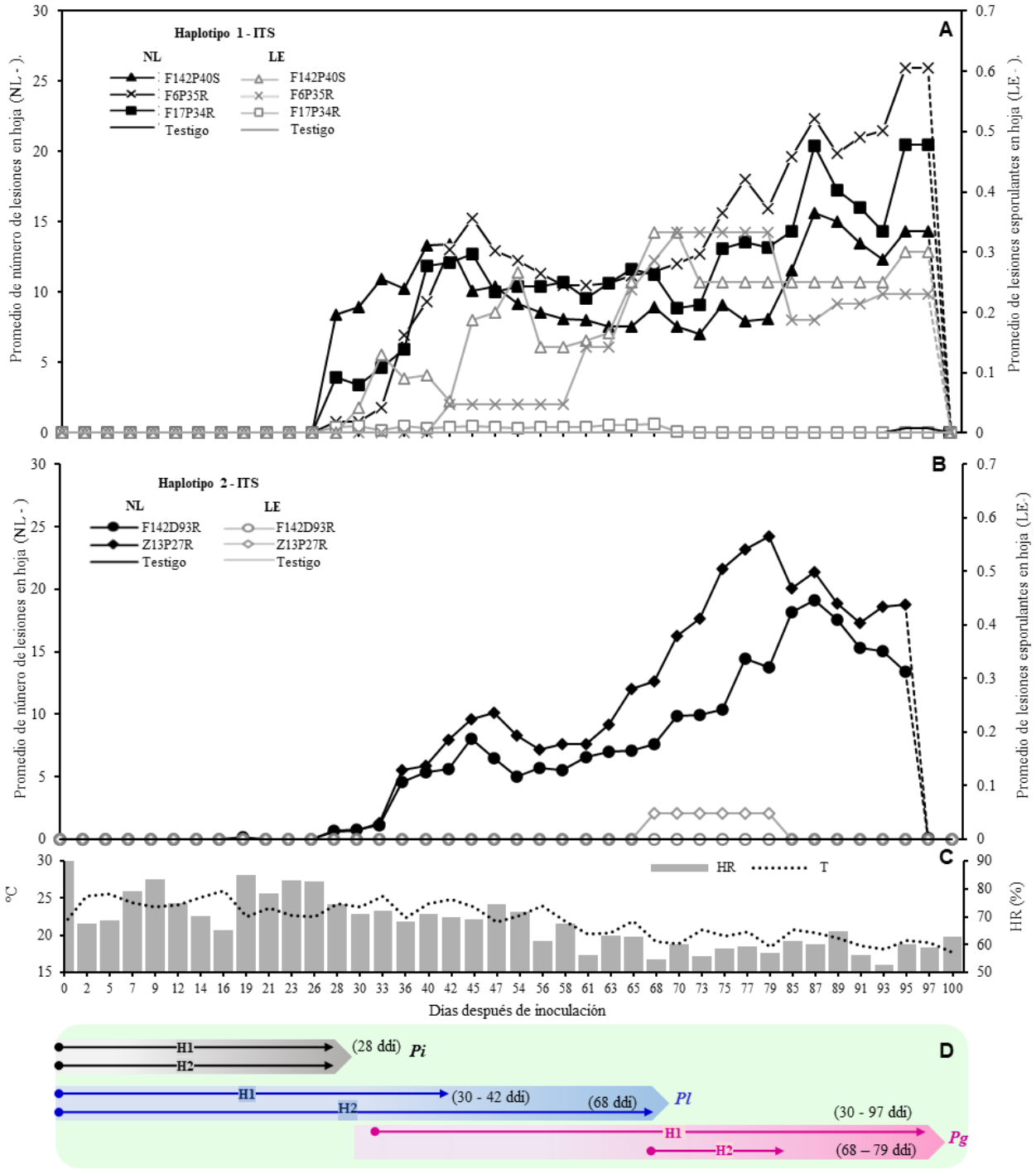

Figure 6. Dynamics of lesions (NL) and sporulating lesions (LE) on leaves up to 95 ddi per wound in the crown with five isolates of *N. rosae* obtained from strawberries (F) and blackberries (Z), **A**. Absolute average of NL and LE of three isolates of H1, **B**. Absolute average of NL and LE of two isolates of H2, **C**. Association of RH and T °C with the behavior of the fungus on tissue up to 100 days, **D**. Estimation of incubation period (Pi), latency period (Pl), and generation period (Pg) by *N. rosae* haplotype.

These results suggest a pathogenic effect of *N. rosae* isolates on chlorophyll concentration, evidenced by yellowing, chlorosis, and an increase in necrotic lesions in leaf tissue (Table 1). The pathogenic process of *N. rosae* promotes physiological alterations, causing losses in productivity. The decrease in UC may vary depending on the type of inoculation used on the tissue (de Souza Marques *et al.,* 2024).

Table 1. Average chlorophyll units (CU), plant height, and crown diameter in strawberry plants cv. San Andreas inoculated by spraying and wounding with five isolates of *N. rosae*.

			Incubation period 2 ddi			Latency period 14 ddi		
Inoculation	**Haplotype**	**ID**	**U.C**	**Height (cm)**	**Crown diameter (cm)**	**U.C**	**Height (cm)**	**Crown diameter (cm)**
Sprinkler	H1	F142P40S	43.8	16.5	0.9	29.3	14.4	1.1
		F6P35R	41.5	15.8	0.7	34.0	15.6	1.0
	H2	F17P34R	40.5	13.5	0.6	37.0	13.1	0.9
		F142D93R	41.1	15.8	0.8	40.2	16.2	1.0
		Z13P27R	40.0	15.3	0.8	38.0	15.3	1.0
	Control	.	40.8	13.7	0.9	40.0	15.3	1.1
Injury	H1	F142P40S	41.3	17.2	0.9	40.8	17.4	1.1
		F6P35R	49.6	16.8	0.9	43.6	16.8	1.1
	H2	F17P34R	41.3	15.8	0.8	39.2	15.3	1.1
		F142D93R	42.4	16.0	1.1	40.3	16.6	1.1
		Z13P27R	37.8	17.4	0.8	36.1	17.7	0.9
	Control	.	39.0	15.1	0.8	36.7	14.0	0.9

Reisolation and cultural characterization of *N. rosae*. A total of 374 reinisolations were performed based on pathogenicity tests. Cultures grown on PDA medium from leaf (100), stem (86), crown (96), and root (92) tissues showed average mycelial growth after inoculation (5-7 days). The colonies showed yellowish aerial mycelium and other whitish mycelium, rosette growth, and even wavy edges. All isolates produced black acervuli on the colony and immersed in the culture medium.

In general, the highest percentage of *Neopestalotiopsis rosae* CFU was recorded in the crown with 34%, followed by the root with 31% and, to a lesser extent, the leaf and stem with 21% and 14%, respectively. No CFU of the fungus were counted in the control. Interestingly, in the spray method where the inoculum was deposited on the aerial tissue, the highest concentration of inoculum was counted in the crown (CFU = 44) and root (CFU = 38). Comparatively, in wound inoculation, the highest inoculum load was also found in the crown (CFU = 83) and root (CFU = 88), and notably lower in leaves (CFU = 20) and stems (CFU = 29). This suggests parasitic specialization of the organism mainly in root, crown, and stem tissue, as has been documented for the genera *Pestalotiopsis, Pseudopestalotiopsis,* and *Neopestalotiopsis* (Maharachchikumbura *et al.,* 2011).

From a total of 92 preliminary re-isolations, 33 (17 from haplotype 1 and 16 from haplotype 2) were selected for cultural and molecular characterization, using criteria of isolate representativeness, inoculation method, and tissue type. In addition, morphological aspects of the colony were considered, including color, mycelium type, growth form, and conidiophore production. Cultural characterization lasted a total of 16 days, of which 12 days were to ensure homogeneous growth and 4 days for conidiophore development in medium. Growth in PDA medium, as described by Maharachchikumbura *et al*. (2014), confirmed phenotypic and genomic variability (Rodríguez-Gálvez *et al*., 2020). The changes were notable in colonial morphology; aerial mycelium with flat and radial growth, increased conidiophore production, and pigmentation of the culture medium to an orange color. Some *Neopestalotiopsis* species can produce metabolites that induce a change in the color of the culture medium (Jeewon *et al*., 2002). This may have been involved in this study.

Molecular characterization was corroborated by 550 bp band amplification. The sequencing results were compared with the sequences reported in the gene bank (NCBI). A percentage of identity and coverage >95% was considered. The ITS gene allowed the isolates to be identified at the species level. However, as it is a universal and conserved gene, it is less informative, represents low haplotype diversity, and restricts distribution. In addition, it results in poor resolution and taxon placement in the consensus maximum likelihood tree (Ramdial *et al.,* 2016). Thus, this gene is not associated with pathogenicity and aggressiveness. It is suggested that a study be conducted with other genes that allow robust genetic information to be obtained.

CONCLUSIONS

Through NL, LE, UC, ***Pi***, ***Pl***, and ***Pg***, significantly contrasting, the pathogenic variability of two ITS haplotypes of *N. rosae* prevalent in Michoacán obtained from strawberries and blackberries was demonstrated. A novel mechanistic understanding of pathogenesis is addressed, and *N. rosae* associated with blackberries with pathogenic capacity in strawberries, but with less aggressiveness, is reported for the first time. Sustainable regional management must consider the variable capacity of the fungus and its interaction with other pathogens with vascular capacity.

Limitations

This research was conducted with San Andreas strawberries and a limited number of fungal isolates. The pathogenic behavior of potential fungal isolates in other varieties may differ from the results found in this study.

Conflict of interest. This work was carried out without conflicts of interest.

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Contribution of the authors

EMH and **EMG**, sampling, experimental planning and execution, first draft of publication; **GMA**, conception, field sampling, analysis, and final writing. **GAS**, statistical analysis, sampling, **ALZ**, field logistics.

REFERENCES

Ceja-Torres L, Mora-Aguilera G and Mora-Aguilera A. 2014. Agronomical management influence on the spatiotemporal progress of strawberry dry wilt in Michoacan, Mexico. African Journal of Agricultural Research 9: 513-520. DOI: 10.5897/AJAR2014.8488

Ceja-Torres L, Mora-Aguilera G, Téliz D, Mora-Aguilera A, Sánchez-García P, *et al*. 2008. Fungi prevalence and etiology of strawberry dry wilt under different management systems. Agrociencia 42: 451-461. https://www.agrociencia-colpos.org/index.php/agrociencia/article/view/644/644

De Souza Marques M, Vitorino LC, Rosa M, Dário B, Silva FG, *et al*. 2024. Leaf physiology and histopathology of the interaction between the opportunistic phytopathogen *Fusarium equiseti* and *Gossypium hirsutum* plants. European Journal Plant Pathology 168: 329–349. Https://doi.org/10.1007/s10658-023-02759-z

Essa TA, Kamel SM, Ismail AM, and El-Ganainy SM. 2018. Characterization and chemical control of *Neopestalotiopsis rosae* the causal agent of strawberry root and crown rot in Egypt. Egyptian Journal of Phytopathology ID 221858557. https://doi.org/10.21608/EJP.2018.87411

Eurosemillas 2020. San Andreas, Principales Características. https://short.do/voJ6XO.

Garay-Serrano E, Cruz-Esteban S, Fernandez-Pavia S, Alvarado G and Gómez-Dorantes N. 2021. Pathogenic microorganisms infecting berries in Mexico. International Journal of Agriculture and Biology 25(5): 1007-1015. https://doi.org/10.17957/IJAB/15.1758

González F, Rebollar S, Hernández J, Morales L y Ramírez O. 2019. Situación actual y perspectivas de la producción de berries en México. Revista Mexicana de Agronegocios. Https://www.redalyc.org/journal/141/14161295012/14161295012.pdf

Jeewon R, Liew EC and Hyde KD. 2002. Phylogenetic relationships of Pestalotiopsis and allied genera inferred from ribosomal DNA sequences and morphological characters. Molecular Phylogenetics and Evolution 25(3): 378 - 392. https://doi.org/10.1016/s1055-7903(02)00422-0.

Krugh B, Bichham L and Miles D. 1994. The solid-state chlorophyll meter, a novel instrument for rapidly and accurately determining the chlorophyll concentrations in seedling leaves. Maize genetics cooperation. News Letter 68: 25 - 27.

Maharachchikumbura SSN, Guo LD, Chukeatirote E, Bahkali AH and Hyde KD. 2011. *Pestalotiopsis* morphology, phylogeny, biochemistry and diversity. Fungal Diversity 50:167-187. https://doi.org/10.1007/s13225-011-0125-x

Maharachchikumbura SSN, Hyde KD, Groenewald JZ, Xu J and Crous PW. 2014. *Pestalotiopsis* revisited. Studies in Mycology 79: 121 - 186. https://doi.org/10.1016/j.simyco.2014.09.005

Maharachchikumbura SSN, Guo LD, Cai L, Chukeatirote E, Wu WP, *et al*. 2012. A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterization of 14 new species. Fungal Diversity 56: 95 – 129. DOI 10.1007/s13225-012-0198-1

Lawrence DP, Brittain GD, Aglave B, and Sances FV. 2023. First Report of *Neopestalotiopsis rosae* causing crown and root rot of strawberry in California. Plant Disease 107: 566. https://doi.org/10.1094/PDIS-04-22-0871-PDN

Luan YS, Shang ZT, Su Q, Feng L, and An LJ 2008. First report of a *Pestalotiopsis* sp. causing leaf spot of blueberry in China. Plant Disease 92:171. doi: 10.1094/PDIS-92-1-0171A

Manzo SG, Guzmán GS, Rodriguez GM, James A y Orozco SM. 2005. Biología de *Mycosphaerella fijiensis* Morelet y su interacción con *Musa* spp. Revista Mexicana de Fitopatología 23:122 - 131.

Mendoza-Ramos C, Mora-Aguilera G, Coria-Contreras JJ, Santana-Peñaloza B, Acevedo-Sánchez G, Martínez-Bustamante VI, Gutierrez-Espinosa MA and Rubio-Cortés R. 2021. *Fusarium* spp. and inoculum load estimation associated to comercial *Agave tequilana* offsets at different regional epidemic inductivity levels. Mexican Journal of Phytopathology 39(1): 1 - 18. https://doi.org/10.18781/R.MEX.FIT.2006-8

Morales-Mora LA, Andrade-Hoyos P, Valencia-de Ita MA, Romero-Arenas O, Silva-Rojas HV, *et al*. 2020. Characterization of strawberry associated fungi and in vitro antagonistic effect of *Trichoderma harzianum*. Mexican Journal of Phytopathology 38: 434-449. https://doi.org/10.18781/r.mex.fit.2005-7

Morales-Mora LA, Martínez-Salgado SJ, De Ita MAV, Andrade-Hoyos P, Silva-Rojas HV, *et al*. 2019. First report of leaf spot and anthracnosis caused by *Pestalotiopsis* sp. on strawberry in Puebla, Mexico. Plant Disease https://doi.org/10.1094/PDIS-05-19-1010-PDN

Montes-Hernández CE, Acevedo-Sánchez G, y Mora-Aguilera G. 2024a. Patogenicidad, agresividad y carga de inóculo de dos haplotipos-ITS de *Neopestalotiopsis rosae* en fresa (*Fragaria* x *ananassa*) cv. San Andreas. Revista Mexicana de Fitopatología 42s:105 pp. https://prourls.link/Ahp06z

Montes-Hernández CE, Campos-Cruces AA, Acevedo-Sánchez G y Mora-Aguilera G. 2024b. Patogenicidad, agresividad y carga de inóculo de dos haplotipos-ITS de *Neopestalotiopsis rosae* en zarzamora (*Rubus* sp.) cv. Madeline. Revista Mexicana de Fitopatología 42s: 106 pp. https://prourls.link/I9nrtt

Montes-Hernández CE, Morales-Cortés ME, Campos-Cruces AA, Mendoza-Ramos C, Martínez-Bustamante VI, *et al*. 2021. Caracterización morfológica de *Neopestalotiopis* sp. en cinco medios de cultivo. Revista Mexicana de Fitopatología 39s: 106 pp. https://www.rmf.smf.org.mx/suplemento/suplemento392021/VS392021_2.pdf

Olivares-Rodriguez G, Angeles-Núñez JG, Mondragón-Rojas F, Rivas-Valencia P, *et al*. 2024. Control *in vitro* de *Neopestalotiopsis* sp. aislada de fresa empleando *Trichoderma* y fungicidas comerciales. Revista Mexicana de Fitopatología 42(4): 52. https://doi.org/10.18781/R.MEX.FIT.2024-28

Ramdial H, Latchoo RK, Hosen FN and Rampersad SN. 2016. Phylogeny and haplotype analysis of fungi within the *Fusarium incarnatum-equiseti* species complex. Phytopathology 107: 109 - 120. https://doi.org/10.1094/PHYTO-05-16-0209-R

Rebollar-Alviter A, Silva-Rojas H, Fuentes-Aragón D, Acosta-González U, Martinez-Ruiz M. *et al*., 2020. Una enfermedad fúngica emergente de la fresa asociada con la pudrición de la raíz, la pudrición de la corona y la mancha foliar causada por *Neopestalotiopsis rosae* en México. Plant Disease 104 (8). https://doi.org/10.1094/PDIS-11-19-2493-SC.

Rodríguez-Gálvez E, Hilário S, Lopes A and Alves A. 2020. Diversity and pathogenicity of *Lasiodiplodia* and *Neopestalotiopsis* species associated with stem blight and dieback of blueberry plants in Peru. European Journal of Plant Pathology 157: 89-102. Https://doi.org/10.1007/s10658-020-01983-1

Rotondo F, Klass TL, Scott K, McCarteney M, Jacobs MJ, *et al*.2023. First Report of *Neopestalotiopsis* disease in Ohio caused by an emerging and novel species of *Neopestalotiopsis* on strawberry. Plant Disease 107:940(3). Https://doi.org/10.1094/PDIS-02-22-0400-PDN

Sun Q, Harishchandra D, Jia J, Zuo Q, Zhang G, *et al*. 2021. Role of *Neopestalotiopsis rosae* in causing root rot of strawberry in Beijing, China. Crop Protection 147: 105710. https://doi.org/10.1016/j.cropro.2021.105710

DGSIAP. 2025. Anuario Estadístico de la Producción Agrícola. Consulta, diciembre 2025. https://nube.agricultura.gob.mx/cierre_agricola/

Vishwakarma M, Kulhare PS and Tagore GS. 2023. Estimation of chlorophyll using SPAD. International Journal of Environment and Climate Change. 13: 1901-1912. 10.9734/IJECC/2023/v13i113348.

White T J, Bruns T, Lee S and Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322 pp. *In*: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego. USA. Https://nature.berkeley.edu/brunslab/papers/white1990.pdf

Wu HY, Tsai CY, Wu YM, Ariyawansa HA, Chung CL, *et al*. 2020. First report of *Neopestalotiopsis rosae* causing leaf bling and crown rot on strawberry in Taiwan. Plant Disease. Https://doi.org/10.1094/PDIS-05-20-1045-PDN

Wu HY, Tsai CY, Wu YM, Ariyawansa HA, Chung CL, *et al*. 2020. First report of *Neopestalotiopsis rosae* causing leaf bling and crown rot on strawberry in Taiwan. Plan Disease e-Xtra. https://doi.org/10.1094/PDIS-05-20-1045-PDN