

REGULAR ARTICLE

Outbreaks of crown rot in *Fragaria x ananassa* caused by *Neopestalotiopsis mesopotamica* in Ecuador

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ABSTRACT

Strawberry crown rot is one of the most important diseases in Ecuador, with an incidence of up to 70% in the field. In the world, the strawberry crown rot is caused by different etiological agents, which makes efficient control of the disease difficult. In this study, 12 samples of strawberry plants were collected, at different stages of disease development, to determine the causative agent in Ecuador. The fungal isolates, consistent with the isolation, were identified and characterized using molecular and morphological approaches, and finally, Koch's postulate was carried out. Based on the form of growth, mycelial color, conidial morphology, and molecular characteristics (sequencing of the ITS and EF-1 α region), the pathogen associated with the symptoms showed 99% similarity with the species *Neopestalotiopsis mesopotamica*. The result of Koch's postulates showed symptoms similar to those observed in field plants, in addition, the same inoculated isolate was recovered from artificially inoculated tissues. This is the first report of this fungus causing strawberry crown rot in Ecuador and in the world.

Keywords: Crown rot; *Fragaria x ananassa*; *Neopestalotiopsis mesopotamica*

INTRODUCTION

The strawberry (*Fragaria x ananassa* Duchesne) is one of the most cultivated and consumed fruits in the world, with an estimated global production of more than 8 million tons (FAOSTAT, 2017). World strawberry production is led by Asia (45.6%), America (26%), Europe (21.2%), and Africa and Oceania occupying the fourth and fifth place with 6, 5% and 0.7 % respectively. Between 2014 and 2018, Ecuador produced 7.708 t of fruit in an area of 500 ha (FAOSTAT, 2017), and only the province of Chimborazo has 136.38 ha of planted strawberries, distributed in Riobamba (82.28 ha), Chambo (22.75 ha), Guano (18.86 ha), Pallatanga (11.48 ha) and Penipe (1.01 ha) (Gobierno Autónomo Descentralizado de la Provincia de Chimborazo, 2019).

Several diseases have affected strawberry production in Ecuador, among them, crown rot is one of the most important, as it affects the leaves, fruits and crowns of the

plants, and when uncontrolled, can lead to losses of up to 50% in production (Rebollar-Alviter et al. 2020). Symptoms of the disease are initially observed in the leaves, which have margins reddish-brown, reduced in size, resulting in withering. The inner part of the crown's tissues shows brown or reddish necrosis (Wu et al. 2021; Rebollar-Alviter et al. 2020).

Many fungal species have been associated with the disease in recent years, however, species belonging to the genus *Neopestalotiopsis* have been gaining prominence as emerging pathogens in strawberries (Rebollar-Alviter et al. 2020). The species *Neopestalotiopsis clavispora* has been considered as a causal agent of strawberry crown rot in Korea (Park et al. 2019), Uruguay (Machín et al. 2019), Spain (Chamorro et al. 2016), Argentina (Obregón et al. 2018), and Italy (Gilardi et al. 2019), while the species *Neopestalotiopsis rosea* is considered the causative agent in Egypt (Essa et al. 2018), Mexico (Rebollar-Alviter et al. 2020) and Taiwan (Wu et al. 2021).

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In recent years, epidemic outbreaks of the disease have been observed in the cultivar 'Albion', in commercial fields located in the county of Yaruquíes, in Chimborazo. The main symptoms observed were necrotic areas and discoloration of the roots and crown, resulting in the death of the plant (Fig. 1A). Due to the high incidence of crown rot in the strawberry crop and also the lack of information on the causative agent of the disease in Ecuador, the objective of this research was to isolate, characterize and identify the etiological agent of the disease, so that measures of efficient control, leading to increased strawberry production in this region.

MATERIALS AND METHODS

Collection of samples

The collections were carried out in April 2019, in commercial strawberry fields (cv. 'Albion') located in the municipality of Yaruquíes, province of Chimborazo, Ecuador (lat 1°40'49 "S, lon 78°40'30 " W). Twelve plants with symptoms of crown rot (brown leaves, withered foliage, reddish-brown coloration of the canopy's vascular tissues) were collected randomly from three different fields. The collected plants stored in plastic bags and kept on ice until the Plant Pathology Laboratory of the Faculty of Natural Resources of the Escuela Superior Politécnica de Chimborazo, for further processing and analysis.

Pathogen isolation

The symptoms in each sample collected were characterized visually with the aid of stereoscopic microscope COMECTA - IVYMEN 5313309(Hong Kong, China), and the aspects considered for isolation were degree of crown necrosis and color. From each of the symptoms, the causal agent was isolated using the methodology described by Alfenas and Mafia (2016) for fungi. After growth, monoconidial cultures of each isolate were obtained, and these were preserved in water and maintained at a temperature of 10 °C.

Morphological characterization

For morphological characterization, 5 mm discs of monoconidial cultures obtained were transferred to plates containing Spezieller Nährstoffarmer Agar (SNA) culture medium, maintained at 25 °C in alternating periods of light and dark for 10 days. Microcultures were made in SNA culture medium, following the methodology described by Alfenas and Mafia (2016). After 10 days, 50 conidia were analyzed using a LEICA DM 150 light microscope (Tokyo, Japan) with an 40X objective, considering the characteristics mentioned by Maharachchikumbura et al., (2014), as the shape and dimension (μm) of the conidia, number of septa, and number of appendages of the apical and basal part of the conidia. The colonies were evaluated for the shape and color present on the edge and reverse of the plates, using three Petri dishes per isolate.

DNA extraction, PCR and sequencing

Molecular identification was performed at the Plant Pathology Laboratory of the Escuela Superior Politécnica de Chimborazo. The fungal DNAs were extracted from fresh mycelium grown in PDA medium, using the Promega Wizard® kit, according to the manufacturer's recommendations. The DNA was quantified with a BioPhotometer Plus 6132 spectrophotometer (Eppendorf, Hamburg, Germany) and adjusted to a final concentration of 25 $\text{ng}\cdot\mu\text{L}^{-1}$. The polymerase chain reaction (PCR) was performed with the specific primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') described by White et al. (1990), which amplify the ITS1, 5.8S and ITS2 region of the rDNA, and with the EF-1 α primers: EF1-728F: (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R: (5'-TACTTGAAGGAACCCTTACC-3') that amplify the elongation factor 1-alpha (Carbone and Kohn 1999).

The PCR reaction was performed to a final volume of 25 μL , using the DreamTaq DNA Polymerase enzyme kit (Thermo

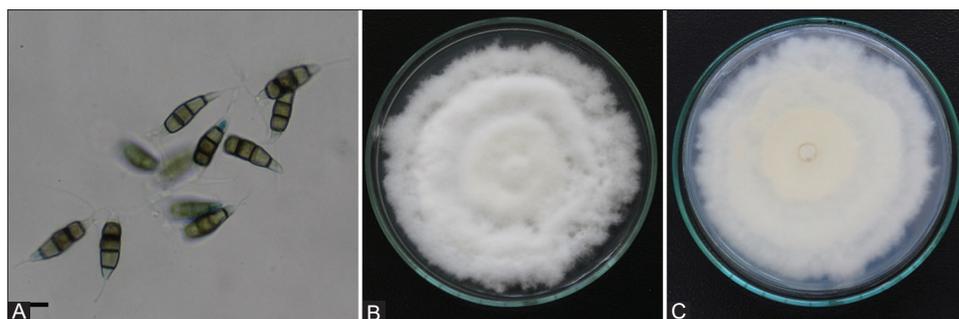


Fig 1. Morphological and cultural characteristics of *Neopestalotiopsis mesopotamica* isolated from necrosis present in the crown of strawberry plants cv. 'Albion'. A: Conidia of *N. mesopotamica* grown in SNA culture medium; B-C: Colony of *N. mesopotamica* grown in PDA culture medium

Fisher Scientific, USA), following the manufacturer's recommendations. The negative control of the PCR reaction was done by replacing the volume of fungal DNA in the reaction with deionized water. The amplification reaction was carried out using the following thermal profile: initial pre-denaturation at 96 °C for 2 min, followed by 35 cycles of: denaturation at 96 °C for 1 min, annealing for 1 min (ITS: 56 °C, EF-1 α : 54 °C), extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The PCR products were confirmed by agarose gel electrophoresis (1%), compared to a 100 bp GeneRuler molecular weight marker from Fermentas (Massachusetts, USA). The PCR products were purified with Exonuclease I from Fermentas (Massachusetts, USA). The purified PCR products were sent for sequencing to Macrogen (Seoul, Korea). Sequences in both directions were obtained for each sample generated from fluorescent cycles using a model sequencer 3730XL (Applied Biosystems, Foster City, California, USA). The electropherograms generated from Sanger sequencing were analyzed with the program SeqAssem (Hepperle 2004) and the arrangement of the nucleotides in ambiguous positions was corrected by comparing the forward and reverse sequences for the assembly of the contigs.

Molecular identification

The obtained sequences were contrasted with sequences present in GenBank database, using the BLAST search tool (Basic Local Alignment Search Tool). Reference strings available on GenBank, and those generated in the works of Maharachchikumbura *et al.* (2014), were selected and aligned with the sequences of this study using the Muscle® software implemented in the MEGA7 Program (Kumar *et al.* 2016), and the concatenated alignment (ITS and *tef-1*) was generated with the Sequence Matrix software version 1.7. 8 (Vaidya *et al.* 2011). Phylogenetic analyzes were based on Maximum Likelihood (ML) and were performed with RAxML v. 8.2.12. (Stamatakis 2014) and the parameters used were: fast bootstrap analysis (with 1000 repetitions) and search for the best ML tree score. All phylogenies were performed using the CIPRES Science Gateway online interface (Miller *et al.* 2010). The resulting trees were visualized in the FigTree software v1.4.3 (<http://tree.bio.ed.ac.uk/software/>) and edited in the graphics program Inkscape 0.92.3 (<http://inkscape.org>). The sequences obtained in this study were deposited in the Genbank (accession numbers: MT705715, MT705716, MT654501, MT654502 and MT654499 (ITS); MT812982 and MT812983 (TEF1- α)).

Koch's postulates

The determination of the infection capacity of the fungal isolates obtained was carried out in a greenhouse (24 °C \pm 3 °C, relative humidity of 70%), through the inoculation of the pathogen in healthy strawberry plants cv. 'Albion' with 2 months old. For the inoculation of the pathogen, a

spore suspension was prepared (1x10⁶ spores.mL⁻¹) of the representative isolate H27. For a better representation of the symptoms, three different methods of inoculation were performed, injection of the crown through a sterile syringe (1 mL of the suspension of the fungal inoculum in the upper part of the crown); cuts in the root system followed by immersion of the roots in the inoculum suspension (2,000 mL of inoculum for 15 min); deposit of the inoculum suspension directly on the substrate (50 mL of inoculum at 1 cm from the stem). For each inoculation method, 30 plants were used, and the control treatment was done using the same inoculation methods, replacing the inoculum with sterile distilled water. Symptom assessment was performed daily, and the experiment was performed twice. Koch's postulates were confirmed by reproducing the symptoms initially observed in the field and by re- isolating the inoculated fungi and comparing their structures with those initially obtained.

RESULTS

Isolation and morphological characterization

From the isolations made from different plant parts and symptomatic plants of strawberry cv 'Albion', in the province of Chimborazo, isolates with similar morphological characteristics were constantly obtained, and of these, 5 monoconidial isolates (H27, H28, H33, H37 and H97) were chosen for the morphological characterization. The isolates grown in SNA culture medium showed white mycelium, with an aspect cottony (Fig. 1B-C).

Microscopic observation of the reproductive structures of the isolates allowed us to determine that they correspond to the same organism. The isolates formed fusiform conidia in four ellipsoid septa (average 22.5 to 32.5 μ m x 7.5 to 12.0). The apical and basal cells were conical and hyaline, while the cell before the basal cell was lighter than the third and fourth cells. The conidia had a basal appendix and two to three filiform appendages (Fig. 1A), similar to the description for the genus *Neopestalotiopsis* (Maharachchikumbura *et al.* 2014).

Molecular identification

The genomic DNA of the isolates was extracted and the target sequences of the ITS and EF1-1 α genes were amplified and sequenced. Molecular identification based on the ITS gene was performed for all isolates, while for the EF1-1 α gene and for the concatenated analysis (ITS and EF1-1 α), two representative isolates, H27 and H28, were selected. The BLAST search showed more than 98% identity between the sequences of the isolates in this study and the species *Neopestalotiopsis mesopotamica* (CBS 299.74). The maximum likelihood phylogenetic analysis based on the ITS region (Fig. 2A) was not able to delimit the species to which the

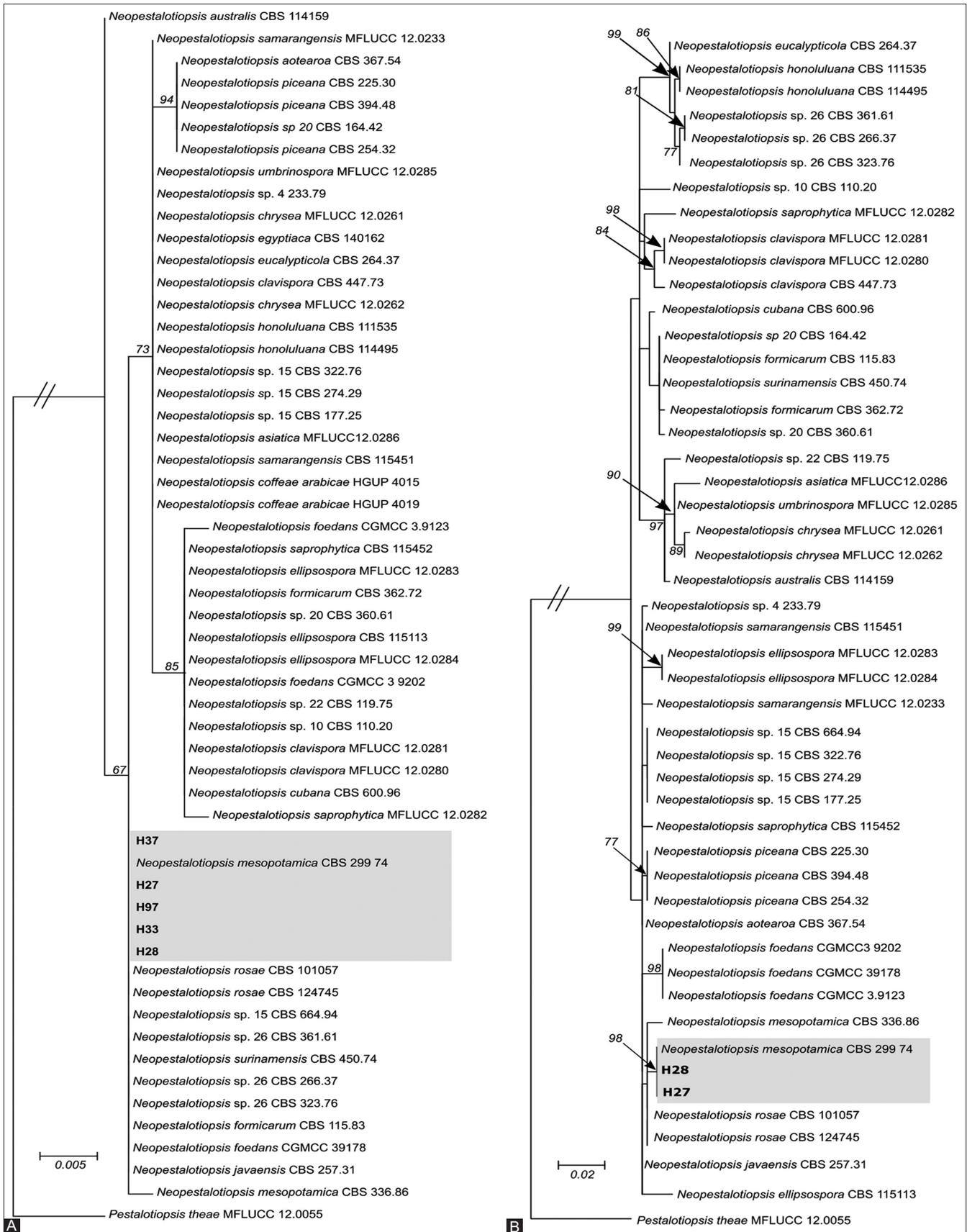


Fig 2. RAxML tree of *Neopestalotiopsis* species based on a dataset of ITS (A) and TEF1- α (B). The tree was rooted with *Pseudopestalotiopsis theae* (MFLUCC 12.0055). Numbers in the nodes represent the *bootstrap* values. The scale bar refers to the number of substitutions per site. Isolates obtained in this study are highlighted in bold

isolates obtained in this study belong, and the isolates were grouped in clade with 7 species (*N. rosea*, *N. foedans*, *N. formicarum*, *N. javaensis*, *N. mesopotamica*, *N. sp. 15* and *N. sp. 26*). The phylogenetic inference performed by the maximum likelihood method based on the EF1-11 α gene (Fig. 2B) and the concatenated analysis (Fig. 3) revealed that isolates H27 and H28 belong to the species *Neopestalotiopsis mesopotamica* Maharachch., KD Hyde and Crous (2014), forming a well-defined clade with high statistical support (98% bootstrap support), distinct from other species of *Neopestalotiopsis* (Fig. 3).

Koch postulates

After inoculating of *Neopestalotiopsis* spp. in 2-month-old strawberries, daily assessments of symptoms were made. At the level of the canopy of the plant, an initial reddish-orange darkening of the interior of the crown tissues was observed and subsequently all the internal tissues of the crown became reddish-brown (Figure 4, Fig. 5F-H). At the bottom of the leaflets, wilting was observed (Fig. 4E), with necrotic spots on the margins and at the apex of young leaves. Subsequently, this necrosis progressed until the leaves dried completely and caused a general collapse of the plant (Fig. 4B-C, Fig. 5F).

When inoculating the strawberry crown using the root cutting method followed by immersion in spore suspension *N. mesopotamica*, it was observed that 50% of the inoculated plants withered at 40 days after inoculation, and the first symptoms were observed at 30 days. However, by inoculating the crown at crown injection method (Fig. 5A), *N. mesopotamica* caused wilt symptoms in 100% of the inoculated plants at 35 days after inoculation, and the first symptoms were observed at 5 days. Inoculation with the inoculum suspension dump method on the substrate (Fig. 5B), caused wilt symptoms in 70% of the inoculated plants at 55 days after inoculation, and the first symptoms were observed at 35 days. Plants inoculated with sterile distilled water did not show symptoms in any of the treatments (Fig. 5 C-E). In all the inoculation methods used, the symptoms presented were similar (Fig. 5E-G). Koch's postulates were repeated twice and the same pathogen was constantly isolated from symptomatic tissues of the disease.

DISCUSSION

Through this study, the etiology of the fungus that causes crown rot was established in strawberries fields in Ecuador, with emphasis on the province of Chimborazo. At the field level, a diversity of symptoms was observed in the crowns, associated with this pathogen. As the disease progressed, marked changes in the color and morphology of the lesions that the fungus causes in the tissues of the crown were observed.

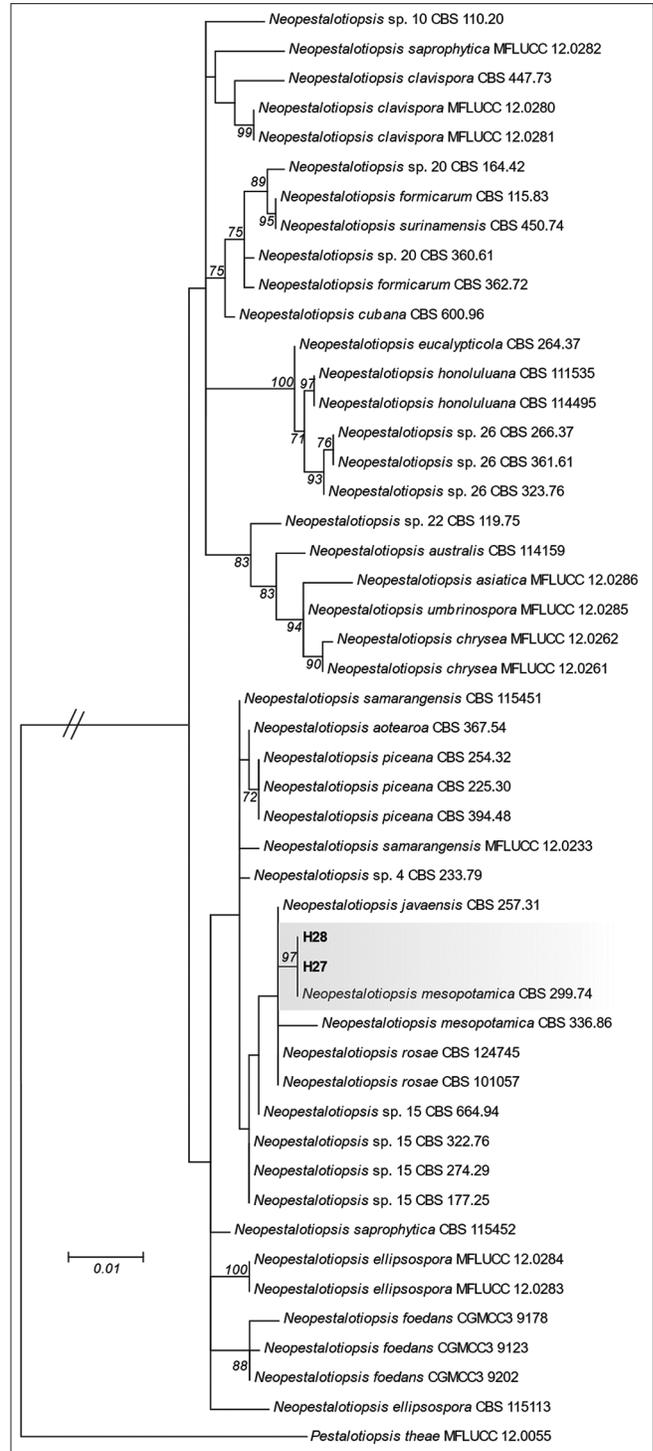


Fig 3. RAxML tree of *Neopestalotiopsis* species based on the concatenated dataset of ITS and EF1- α . The tree was rooted with *Pseudopestalotiopsis theae* (MFLUCC 12.0055). Numbers in the nodes represent the bootstrap values. The scale bar refers to the number of substitutions per site. Isolates obtained in this study are highlight in bold.

The cultural and morphological characteristics observed in the isolates of *Neopestalotiopsis* spp. were similar to those described by (Dung et al. 2016; Chamorro et al. 2016; Essa et al. 2018; Sigillo et al. 2020), who first reported *Neopestalotiopsis* spp. as a causal agent of strawberry crown

rot in different regions around the world. In recent years, several species of *Neopestalotiopsis* have been reported affecting strawberry production (Farr and Rossman 2020), thus showing that *Neopestalotiopsis* is an emerging pathogen in strawberry cultivation.

Maharachchikumbura et al. (2014) describe the cultural and morphological characteristics of *Neopestalotiopsis* spp. that coincide with those found in this research. Morphological comparisons and molecular analyzes identified isolates *Neopestalotiopsis mesopotamica* associated with crown rot in strawberry plants in Ecuador. *Neopestalotiopsis mesopotamica* has already been linked to tomato fruit rot (Ayoubi and

Pari 2016), but to our knowledge, this is the first report of *N. mesopotamica* causing strawberry crown rot in Ecuador. In other South American countries where the disease has already been reported, such as Argentina and Uruguay, the main pathogen associated with strawberry crown rot is *N. clavispora* (Obregón et al. 2018; Machín et al. 2019), while in central America, the species *N. rosea* is the most mentioned, causing the disease (Rebollar-Alviter et al. 2020).

The pathogenicity of the species was confirmed by the different inoculation methods used and by the visualized symptoms. All inoculated plants showed symptoms similar to those observed in the field. Injuries to the roots and crown before inoculation of the pathogen, have shown to have a positive effect on the acceleration of the infectious process, as well as the appearance of symptoms in relation to the inoculation in which the inoculum was placed directly on the substrate. Studies with other fungi phylogenetically close to *Neopestalotiopsis* spp. as *Pestalotiopsis* showed that, in some cases, injuries are necessary for the infection to start or accelerate, in some cases, it was observed that plants without injuries remained asymptomatic (Sousa et al. 2004). Rodrigues et al. (2014) when studying the infectious process of *P. longisetula* in strawberry leaves showed that there was an appressorium formation and no evidence of direct penetration of the germ tube in the host. This result illustrates the need for a previous opening for the pathogen to enter. In other groups of fungi, such as Botryosphaeriaceae, the behavior of infecting only previously injured areas of the plant has already been observed (Machado et al. 2014).



Fig 4. Symptoms caused by *Neopestalotiopsis mesopotamica* on strawberry leaves cv. 'Albion'. A: symptoms occurring at random in strawberry plantations; B-C: necrotic areas and discoloration of roots and crown resulting in plant death; D: beginning of discoloration of the leaves between the veins, becoming reddish-brown; E: advance of discoloration followed by wilting of the leaves.

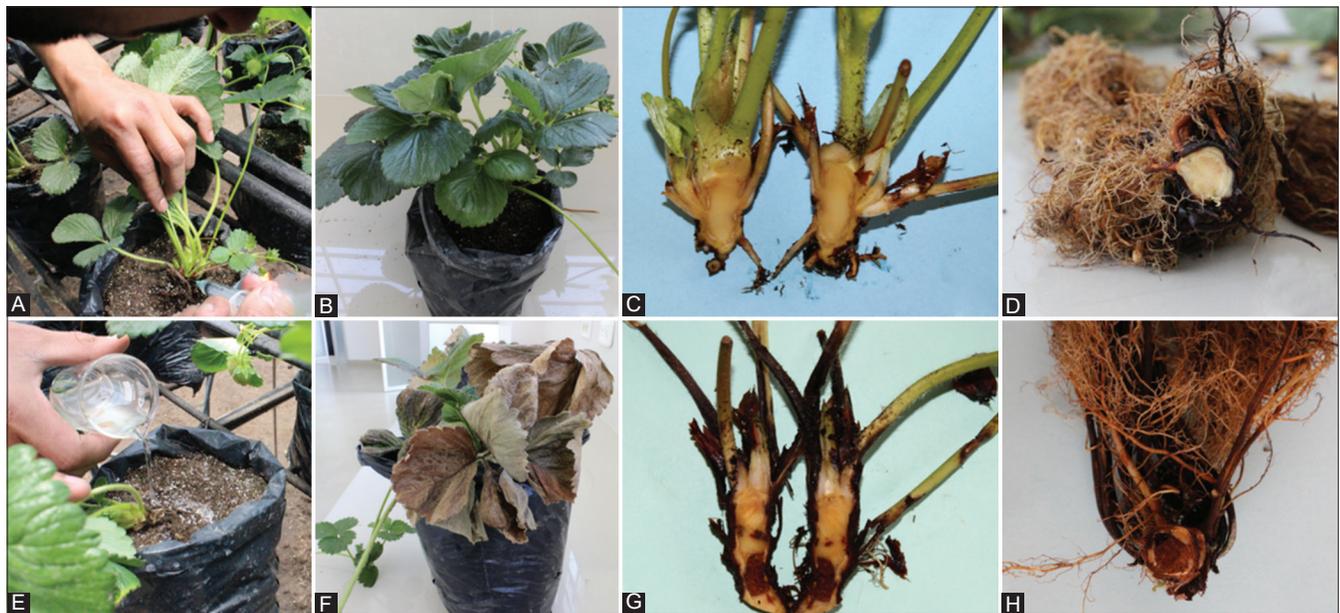


Fig 5. Inoculation methods *Neopestalotiopsis mesopotamica* and representation of the symptoms obtained in the execution of Koch's postulates. A: injection of the inoculum suspension; B: deposit of the solution on the substrate; C-E: Strawberry control plants not inoculated with the pathogen; F: Strawberry plants with necrosis, wilting and discoloration; G: longitudinal section of the crown showing necrotic areas; H: cross section of the crown showing necrotic areas.

This study provides new knowledge about a new disease emerging in strawberry cultivation, with a high destructive potential. Thus, further additional investigations are important to ensure the continuity of strawberry cultivation at different scales in Ecuador.

CONCLUSIONS

Through morphological, molecular and pathogenicity analyzes, the etiologic agent of crown rot in strawberry plants (*Fragaria x ananassa*) in Ecuador, with an emphasis on the province of Chimborazo, was identified as *Neopestalotiopsis mesopotamica*. For our understanding, this is the first report of *N. mesopotamica* causing strawberry crown rot in the country. The report of this disease in this new geographic region and its correct identification are essential for an adequate management of the disease, aiming to avoid its spread of the pathogen to other areas of strawberry production.

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Authors' contributions

Conceptualization, Hamilton O. Intriago-Reyna, Fernando J. Rivas-Figueroa and Pablo I. Álvarez-Romero; **Methodology**, Hamilton O. Intriago-Reyna, Fernando J. Rivas-Figueroa, Álvaro M. Rivera-Casignia, Ana F. T. A. Ferreira e Ferreira and Pablo I. Álvarez-Romero; **Investigation**, Hamilton O. Intriago-Reyna., Fernando J. Rivas-Figueroa, Álvaro M. Rivera-Casignia, Ana F. T. A. Ferreira e Ferreira and Pablo I. Álvarez-Romero; **Formal analysis**; Hamilton O. Intriago-Reyna., Fernando J. Rivas-Figueroa, Álvaro M. Rivera-Casignia, Ana F. T. A. Ferreira e Ferreira and Pablo I. Álvarez-Romero; **Data curation**, Ana F. T. A. Ferreira e Ferreira and Pablo I. Álvarez-Romero; **Writing, review and editing**, Hamilton O. Intriago-Reyna., Fernando J. Rivas-Figueroa, Álvaro M. Rivera-Casignia, Ana F. T. A. Ferreira e Ferreira and Pablo I. Álvarez-Romero. All authors have read and agreed to the published version of the manuscript.

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