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First report of root and crown rot caused by *Dactylonectria novozelandica* on strawberry in Uruguay

Primer reporte de *Dactylonectria novozelandica* causando necrosis de raíz y corona en frutilla en Uruguay

Primeiro relato de *Dactylonectria novozelandica* causando necrose de raiz e coroa em morangos no Uruguay

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Abstract

Since 2015, a high mortality rate of strawberry plants has been observed in the north of Uruguay related to crown and root diseases caused by a pathogens complex. In 2016, associated with the death of plants, *Cylindrocarpon* spp. was isolated with a frequency of 42.4 %, ranking third, preceded by *Neopestalotiopsis* sp. and *Fusarium* spp. Aiming to increase the knowledge of the pathogens that integrate the complex, three isolates of *Cylindrocarpon* spp. were characterized by molecular, morphological and pathogenicity analyses in strawberry plants. *Dactylonectria novozelandica* was identified as causal agent of crown and root necrosis of strawberry in Salto, Uruguay.

Keywords: *Cylindrocarpon* spp., pathogenicity test, Histone

Resumen

Desde 2015, se observó una alta mortandad de plantas de frutilla en el norte de Uruguay relacionada con enfermedades de corona y raíz causadas por un complejo de patógenos. En 2016, asociado con la muerte de plantas, *Cylindrocarpon* spp. se aisló con una frecuencia de 42,4 %, ocupando el tercer lugar, precedido por *Neopestalotiopsis* sp. y *Fusarium* spp. Con el objetivo de incrementar el conocimiento de los patógenos que integran el complejo, se caracterizaron tres aislados de *Cylindrocarpon* spp. mediante análisis moleculares, morfológicos y de patogenicidad en plantas de frutilla. Se identificó *Dactylonectria novozelandica* como agente causal de necrosis de corona y raíz de frutilla en Salto, Uruguay.

Palabras clave: *Cylindrocarpon* spp., prueba de patogenicidad, Histona

Resumo

A partir do ano 2015 um número significativo de mortes se observou em plantas de morango no norte do Uruguai devido a doenças da coroa e raiz causadas por um complexo de patógenos. Associado à morte, *Cylindrocarpon* spp foi isolado com uma frequência de 42,4% no 2016, ocupando o terceiro lugar de incidência depois de *Neopestalotiopsis* sp. e

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Fusarium spp. O objetivo do trabalho foi caracterizar três isolados de *Cylindrocarpon* spp por meio de análises moleculares, morfológicas e patogênicas em morangueiro. *Dactylonectria novozelandica* causou necorsis de cora e raiz em uma plantas de morango em Salto, Uruguai.

Palavras-chave: *Cylindrocarpon* spp., teste patogênico, Histona

1. Introduction

In Uruguay, strawberry crop is distributed in two main areas: Canelones and San Jose in the south, and Salto in the north. The value of this crop in farm systems lies in the intensive use of resources, workforce and capital, generating high profit margins per unit area[1].

An important number of death of plants has affected strawberry crop in the north of the country associated with root and crown diseases caused by a complex of pathogens that emerged in 2015. This mortality reached losses between 30-50 % in crops destined for fruit production. In addition, the loss was total in mother plants for nurseries[2]. Within the complex, there were identified *Neopetalotiopsis* sp. (81.8 %), *Fusarium* spp. (75.7 %), *Cylindrocarpon* spp. (42.4 %), *Rhizoctonia* spp. (24.2 %), among other genres with lower frequencies[3]. The species identified in Uruguay are *Colletotrichum fragariae*, *Verticillium albo-atrum*, *Rhizoctonia fragariae*, *Macrophomina phaseolina*[4], *Phytophthora cactorum*, *V. dahliae*[5] and *Neopestalotipsis clavispora*[6]. In addition, *Dactylonectria novozelandica* and *D. macrodidyma* were associated with the death of strawberry plants, however they were not reported as pathogens in other regions[3].

Pathogenicity and species of *Cylindrocarpon* are unknown in Uruguay. The aim of this work was to characterize isolations of *Cylindrocarpon* spp. by molecular and morphological analyses and test their pathogenicity in strawberry plants.

2. Materials and methods

2.1 Isolates of *Cylindrocarpon* spp.

Three isolates of *Cylindrocarpon* spp. were used from the collection of the Phytopathology Laboratory (School of Agronomy, Udelar). They were collected from naturally infected strawberry fruiting plants in 2018 in Salto (Table 1) and stored at −20 °C on filter paper inside sterilized envelopes. The isolates were randomly selected whilst still considering morphology, specifically the characteristics of the colony.

Table 1. Origin of the isolates of *Cylindrocarpon* spp. used

Isolate No.	Variety	Isolation zone
33	Clon T38.2	Root
92	Clon U20.4	Root
128	Clon Q67.3	Root

2.2 Molecular identification

Molecular identification was carried out using DNA extracted from three colonies of the fungi in potato dextrose agar (PDA) culture medium incubated for 13 days at 24 °C. The DNA concentration of the samples was determined using a Nanodrop 2000 Spectrophotometer Thermo Scientific, adjusting the concentration to 25 ng/µl. Genomic DNA was extracted using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA). The amplification was performed by PCR with the primers CylH3F (AGGTCCACTGGTGGCAAG) and CylH3R (AGCTGGATGTCCTTGGACTG), of a partial region of Histone (H3) gene[7]. The cycling conditions were: initial denaturation at 94 °C for 2 minutes, followed by 35 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds, and a final extension of 72 °C for 10 minutes. PCR amplification was carried out in a PTC-100 Peltier Thermal Cycler. The master mix of the reaction was composed of 10X buffer (50 mMKcl, 10 mM Tris HCl (pH 8)) 2.5 µl, nucleotides (DNTP) 0.8 µM, 0.5µl of each of the primers, DNA 1µl, DMSO 0.5 µl, and Taq polymerase (MgCl included) 0.2 µl was added, the final reaction volume was 25 µl reached with 19 µl MQ water. Amplification products were analyzed by electrophoresis using 2 µl of sample and 1 µl of DYE loading buffer loaded on 1 % agarose gel, a molecular weight marker of 100 bp (Gene Ruler 1 kb DNA Ladder Plus, Fermentas, Germany) and a negative control without DNA were also added. PCR products were sequenced using the service of Macrogen Inc., Korea (*www.macrogen.com*). The obtained sequences were manually corrected using the program MEGA version 5.1[8], and aligned with similar sequences obtained from GenBank with BLAST analysis to identify the species used.

2.3 Morphological identification

For the morphological characterization, it was proceeded according to what is described by Halleen and others[9]. One pure isolate plated on PDA culture medium incubated for 20 days at 24 °C under ultraviolet (UV) light was used. Fungal colony was characterized by color and texture, using the description by Domsch and others[10]. Microscopic observations were performed on an Olympus CX41 optical microscope at 40× magnification, registering the length and width of 50 conidia through the program DinoCapture 2.0. In addition, characteristics of the conidia were observed such as its shape and number of septa.

2.4 Pathogenicity tests

Three fungi were incubated in Petri dishes with PDA under UV light. 25 days later, the medium containing conidia were macerated, passing it several times through sterilized distilled water (SDW) with Drigalski spatula. The conidial suspension resulted from the maceration of one plate diluted in 25 ml of SDW, the concentration of the inoculum was adjusted to 10^6 conidia/ml. Pathogenicity assays were performed in 20 strawberry plants "INIA Guapa" with 69 days old. Each isolate was inoculated in five plants; roots were dipped for 30 minutes with the inoculum and planted in pots of one liter with Bas Van Buuren substrate, covered with nylon bags for 24 hours. The plants were held at 20±5 °C and 80 % relative humidity in a growth chamber with a 14-hour photoperiod. Five strawberry pots were used as control, with the same treatment, except that the roots were dipped into SDW without conidia. The plants were placed randomly and periodically watered with distilled water.

2.5 Re-isolations from symptomatic plants

Once the lesions developed, fungi were re-isolated from the inoculated plants. Symptomatic plants were washed under running water to remove the soil. Then five pieces from infected tissues from root and crown were cut and superficially disinfected with 70° alcohol for 1 minute, rinsed three times with SDW, and air-dried in a laminar flow hood. They were first plated on water-agar with 0.2 g of streptomycin sulfate, after 72-96 hours at 24 °C they were spiked onto PDA and incubated for 20 days. The re-isolated fungi were identified by DNA extraction and subsequent PCR amplification and sequencing.

3. Results

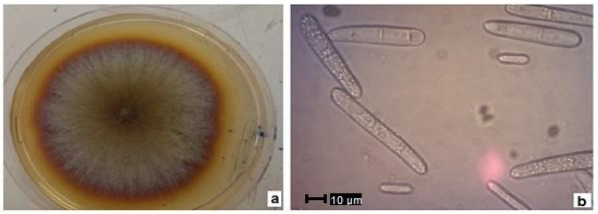
3.1 Molecular identification

BLAST analysis of three isolates deposited in GenBank (accession number: MT892668, MT892669 and MT892670, sequences of 33Cyl, 92Cyl and 128Cyl, respectively) showed 100 % identity with *D. novozelandica* (MK409914.1) for partial *his* gene.

3.2 Morphological identification

After 20 days, fungal colonies were orange to dark-brown and cottony aerial mycelium (Figure 1a). The fungus produced many conidia. Under a microscope, hyaline conidia with smooth, mostly straight walls with rounded ends (Figure 1b) of variable size between 27.3-45.8×4.1-7.3 µm were observed, presenting mostly 3 septa, coinciding with data reported[11].

Figure 1. a) Colony of *D. novozelandica* growing in PDA with 20 days of incubation. b) Conidia (40×)



3.3 Pathogenicity tests and re-isolations

Nine days after inoculation, all plants showed necrosis in the outer leaves (Figure 2). 137 days after inoculation, necrosis was observed in the root systems and incipient crown lesions (Figure 3). From the symptomatic plants, *D. novozelandica* was consistently re-isolated from symptomatic tissues in crown and root, while the plants without inoculation remained healthy and no fungus was recovered from their tissues.

Figure 2. Symptomatic outer leaves from cv "INIA Guapa" after inoculation with *D. novozelandica*

Figure 3. Symptomatic crown and root from cv "INIA Guapa" after inoculation with *D. novozelandica*

4. Discussion

Dactylonectria novozelandica has been reported as a vine pathogen in regions of America, Europe, and Oceania[11]. It was further reported as an avocado pathogen in Australia[12]. Although it was not reported as a strawberry pathogen, *D. novozelandica* was associated with the mortality of strawberry plants in the north of Uruguay. Previously in this region, *Cylindrocarpon* spp. (*Dactylonectria* spp.) was isolated from symptomatic plants with a high frequency —ranking third in terms of frequency[3].

In this work *D. novozelandica* is causing root and crown rot in strawberry as it was expected according to the background in Uruguay.

5. Conclusion

Dactylonectria novozelandica was identified molecularly and morphologically, and then its pathogenicity was tested. According to this, the present work is the first to determine the pathogenicity of *D. novozelandica* in strawberry. Therefore, this species is part of the pathogens complex that causes root and crown rot in Uruguayan strawberries.

This is the first report of *D. novozelandica* causing root and crown necrosis on strawberry in Uruguay.

Author contribution statement

VM: identification and pathogenicity tests, interpretation of results, article writing. GP: co-tutor of the work, interpretation of results, revising and editing. MA: pathogen isolation, collaboration with DNA extraction and pathogenicity tests. VE: interpretation of results, revising and editing. SPE: tutor of the work, interpretation of results, revising and editing.

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