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Ceratobasidium sp. AG-A, ROOT PATHOGEN OF *Calibrachoa hybrida*

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ABSTRACT

Calibrachoa (*Calibrachoa hybrida*, Solanaceae) is an annual flowering ornamental plant, which is widely used in landscape design. In October 2019, 5% of the plants grown in a greenhouse in Buenos Aires, Argentina, showed a sudden wilt characterized by chlorosis, loss of turgor, and root rot. The objective of this study was to identify the causal agent of the disease. Five morphologically identical isolates developed from the roots of wilting plants. One of the isolates was selected and identified using morphological and molecular markers, while its pathogenicity was confirmed by Koch's postulates. The obtained results allow concluding that *Ceratobasidium* sp. AG-A (anamorph *Rhizoctonia* sp.) is the causal agent of the disease. To the best of the authors' knowledge, this is the first report of *Ceratobasidium* sp. as pathogen of calibrachoa in the world.

Key words: ornamental plants, *Rhizoctonia*, fungus, wilt, rot.

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INTRODUCTION

Floriculture is an important branch of horticulture, which deals with flowers and ornamental plants and aims at satisfying people's aesthetic needs (Kuzichev and Kuzicheva, 2016). Consumption is on the rise, mainly in emerging markets like Eastern Europe, Russia, China, India, East Asia and, to a lesser extent, in the traditional markets of Western Europe, North America, and Japan (ITC, 2022). Flowering plants are used within floriculture in either the cut-flower industry, where harvested flowers are the final product, or the flowering pot plant industry, where plants are sold for home or urban landscaping purposes (Chandler and Brugliera, 2011). Calibrachoas (*Calibrachoa hybrida*, Solanaceae) belong to the group of flowering pot plants and are characterized by a profuse production of brightly colored flowers during spring and summer.

Modern plant breeding started with sedentary agriculture and the domestication of cereals, the first agricultural plants (Xu, 2010). In this sense, the relationship between people and ornamental plants dates to ancient times. Due to the increasing importance of ornamental plants as commodities, breeders create new and attractive varieties every year. Traits such as new colors, altered forms, enhanced fragrance, and increased longevity are in high demand by the consumers, who are continually seeking for novel products (Vainstein, 2002).

All the hybrids of calibrachoa offered in the market are native to South America. In fact, the different species of this genus grow naturally in Brazil and Argentina (Stehmann and Greppi, 2011; Fregonezi et al., 2012; Greppi et al., 2013). Three national cultivars (Overá Fucsia INTA, Pampa Salmón INTA, INTA 06575) are currently cultivated, while other three cultivars are to be registered in Argentina. Besides, INTA 06575 is grown in the USA and Japan as Garden Rose Superbells.

Crop surveys conducted on a regular basis in these ornamental crops identified *Sclerotinia sclerotiorum* (Borrelli et al., 2020) and *Fusarium oxysporum* (Borrelli et al., 2021) as calibrachoa soilborne pathogens. In October 2019, plants of calibrachoa INTA 06575 grown in a polyethylene greenhouse in Hurlingham (Buenos Aires, Argentina) showed sudden wilt (Fig. 1A). The aim of this study was to identify the causal agent of the disease.

MATERIALS AND METHODS

Disease assessment

The crops were monitored to establish disease progression, incidence and cultivars affected by wilt. For that purpose, the number of symptomatic plants was recorded, and the development of aerial symptoms was monitored.

At the onset of disease, five symptomatic plants were collected from the greenhouse benches. The pots were removed, the substrate was eliminated, and the roots were washed under running tap water. After that, the crowns and roots were observed under an Olympus SZX9 dissecting microscope.

Pathogen isolation and characterization

Five to ten mm long pieces of the diseased roots were surface disinfested by immersion in 70% ethanol for one minute, and bleach (20 g Cl L^{-1} water) for another minute. Subsequently, they were rinsed with sterilized distilled water, blotted dry on sterile filter paper, and cultured at 22 °C on 4% potato dextrose agar (PDA) medium for five days. The emerging colonies were sub-cultured on fresh PDA medium at 22 ºC. The pure isolates were maintained in slants at 4 °C.

The color and growth of the colonies were recorded; and the vegetative structures of the isolate were observed under an Olympus BX50 microscope. The cells nuclei were stained with Safranin O, according to the procedure developed by Bandoni (1979) to observe and count the number of nuclei per young vegetative hyphal cell. For subsequent studies, one isolate was selected among those with similar growth characteristics on PDA medium.

To better clarify the identity of the pathogen, a molecular characterization of the isolate was performed amplifying and sequencing part of the ITS rDNA region using primers ITS1 and ITS4 (White et al., 1990). The sequence obtained in this study was submitted to GenBank (accession number OL471501) and a nucleotide BLAST comparison with published sequences at NCBI was made (http://www.ncbi.nlm.nih.gov/).

For pathogenicity tests, forty potted plants of calibrachoa INTA 06575 were produced by asexual propagation. When the plants were 2 months old, twenty of them were inoculated by placing 0.5 cm^2 PDA plugs obtained from the pure isolate on PDA, near the stem bases (ten out of those twenty plants were punctured at the stem bases, prior to inoculation). The rest of the plants (ten wounded and ten unharmed controls) were treated with sterile PDA plugs. Each plant was placed in a transparent polyethylene bag

and placed on the lab bench. Air temperature was set at 24 °C during the assays. The bags were removed after 72 h, and the plants remained in the greenhouse for observation. The method described for the isolation of the fungus was used to re-isolate the pathogen from the roots of inoculated plants.

RESULTS AND DISCUSSION

Disease assessment

Disease incidence was estimated at 5%. The leaves showed slight chlorosis, and then moderate chlorosis, followed by loss of turgor. Permanent wilting resulted in death of most of the plants.

The roots of wilting plants exhibited a brown discoloration (Fig. 1B). The outer cortexes and epidermis sloughed off readily, leaving the vascular cylinders (Fig. 1C). Round to elliptical sunken lesions appeared on taproots (Fig. 1D), especially near the substrate level. No symptoms were observed on the base of the stems. Only cultivar INTA 06575 showed symptoms among all the cultivars in the greenhouse.

Pathogen isolation and characterization

Five rapidly-growing morphologically similar fungal colonies developed from diseased roots after five days. They were initially whitish and turned light brown, reaching the edges of the plates in 5 days (Fig. 2A). The hyphae were brown, 5.5 µm (3.1-8.1) wide, branched at right angles, constricted at the branching point, with septa close the branch (Fig. 2B). These characteristics were consistent with the description of the genus *Rhizoctonia* DC (Sneh et

Fig. 1. Wilt of *Calibrachoa hybrida* INTA 06575. A) Wilting (left) and healthy (right) plants (5 cm Scale bar); B) Discolored roots (5 cm Scale bar); C) Root with cortex and epidermis disintegration, and exposed vascular system (3 mm Scale bar); D) Sunken lesions on roots (0.4 mm Scale bar).

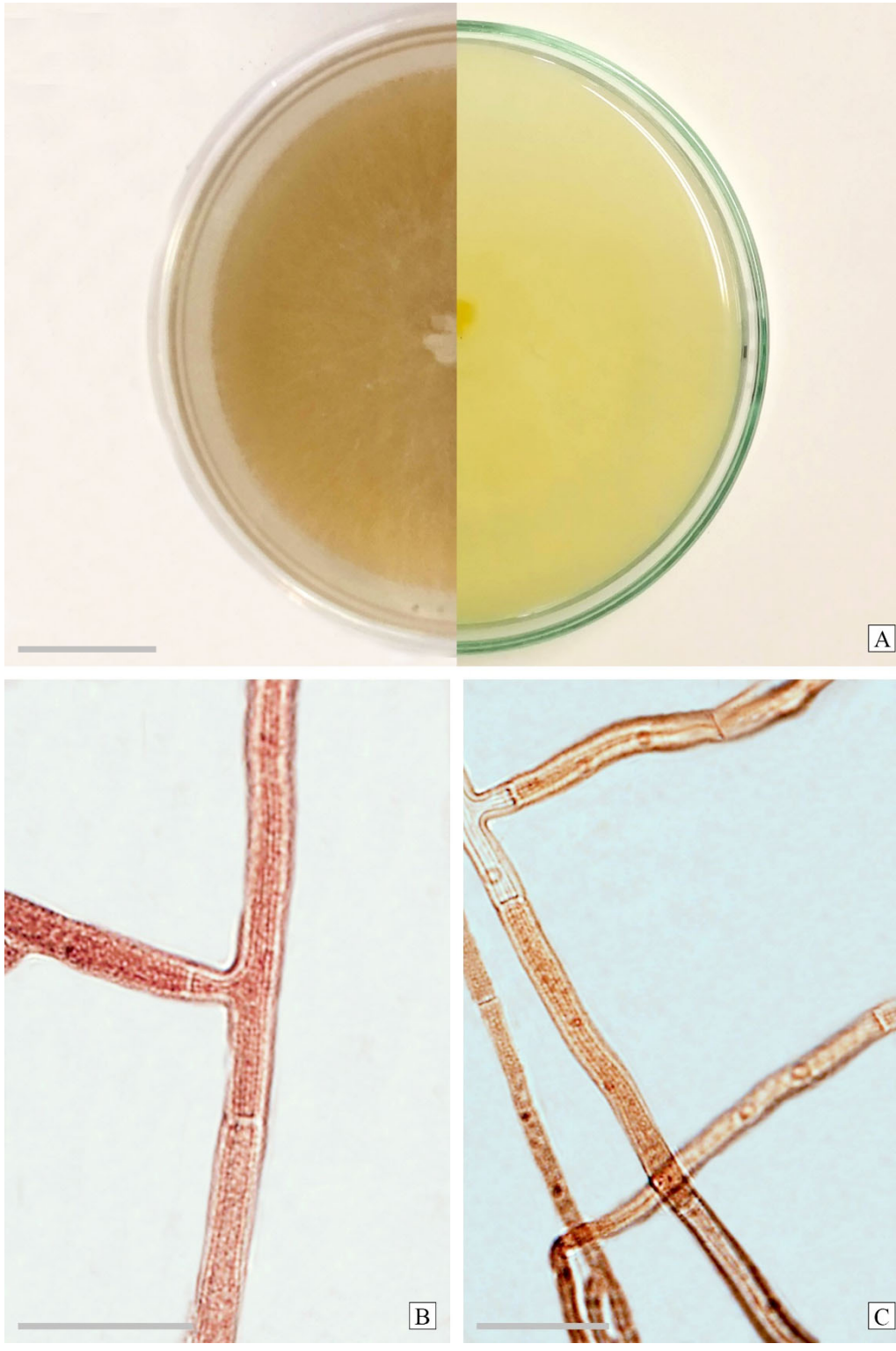

Fig. 2. Cultures of *Ceratobasidium* sp. AG-A. A) Top and bottom views of five-day growth on PDA (2 cm Scale bar); B) Hyphae showing typical constrictions at the branching point (20 µm Scale bar); C) Binucleate hyphal cells (20 µm Scale bar).

al., 1991). The hyphae showed two nuclei per cell (Fig. 2C). Neither clamp connections nor sclerotia were evident. Basidia were not produced. The method of transferring the strains from high to low nutrient media (Murray, 1984) could be used in further additional studies, attempting to induce sexual reproduction. The isolate selected as representative was coded as INTA-IF-568 and deposited in the BAFCcult as 6001. The nucleotide BLAST comparison with published sequences at NCBI showed 99.83% of similarity with sequences of *Ceratobasidium* sp. AG-A (ex. KM017962, KF176587, LC549193).

The genus *Ceratobasidium* D.P. Rogers (syn. *Pellicularia* Cooke) belongs to the family Ceratobasidiaceae, phylum Basidiomycota (Index Fungorum, 2022). *Ceratobasidium* and *Tulasnella* are teleomorphs of binucleate *Rhizoctonia* (BNR) (Sharon et al., 2007; 2008). The staining method by Bandoni (1979) was used and the nuclei present in hyphal cells were counted properly.

According to the average number of nuclei per cell, the isolate INTA-IF-568 is a BNR. Even though hyphal fusions were not studied in this work, molecular identifications placed the isolate in the anastomosis group AG-A, one of the AGs assigned to BNR. It would be interesting to determine if AGs different from AG-A are capable of infecting calibrachoas in order to increase knowledge of host range and distribution of the pathogen. The identification of the anastomosis group to which isolate INTA-IF-568 belongs is useful, especially for the chemical control of the disease, as AGs can differ in fungicide sensitivity (Muzhinji et al., 2018).

Six days after inoculation, control plants remained asymptomatic. At the same time, all the inoculated plants showed symptoms like those initially observed, which were more severe in plants with injured stem bases (Fig. 3A). All wilting plants died within 10 days (Fig. 3B) due to root rot. The pathogen was recovered from the symptomatic roots. In this way, Koch's postulates were fulfilled. To the best of our knowledge, this study is the first report of *Ceratobasidium* sp. AG-A causing wilt of calibrachoa. The loss of turgor of the diseased plants is consequent with the necrosis caused by the pathogen on the roots, and thus the disease can be called calibrachoa wilt.

There are fifty-one records of *Ceratobasidium* sp. causing disease on thirty-nine plant species in the world (Farr and Rossman, 2022). The clade AG-A is pathogenic on potato (Solanaceae) (Miles et al., 2013), soybean, pea, snap bean (Fabaceae) (Yang et al., 2005), pack choi and red cabbage (Brassicaceae) (Yang et al., 2005; Erper et al., 2021), sugar beet (Amaranthaceae) (Miles et al., 2013), and strawberry (Rosaceae) Fang et al., 2013. The present study adds calibrachoa to the list of solanaceous hosts of the pathogen.

Numerous crop plants are susceptible to more than one soilborne fungal pathogen (Malcolm et al., 2013). *Ceratobasidium* sp., *Sclerotinia sclerotiorum* and *Fusarium oxysporum* are known to infect calibrachoa. As many other soilborne diseases, those reported in calibrachoa are characterized by basal rot, leaf chlorosis and

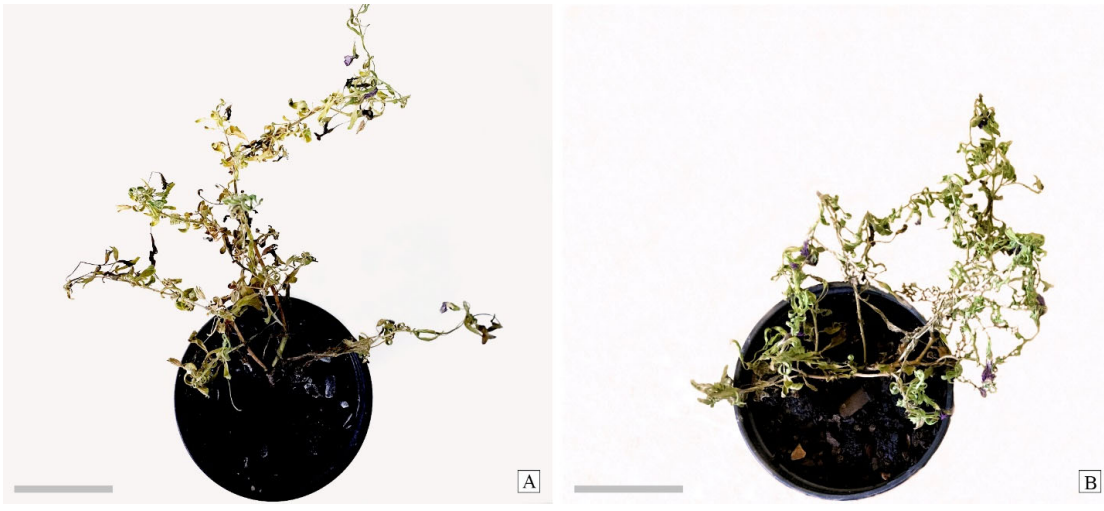

Fig. 3. Pathogenicity test, day 6. A) Injured inoculated Calibrachoa INTA 06575 showing foliage necrosis (5 cm Scale bar); B) Unharmed inoculated plant, with intense loss of turgor (5 cm Scale bar).

wilt. The secondary symptoms are so similar that make the diseases indistinguishable from each other unless the pathogens are isolated. However, differences can be observed below the substrate level since *S. sclerotiorum* only causes crown rot, while *Ceratobasidium* sp. and *F. oxysporum* disintegrate the roots. Regarding the incubation period, it differs among these three host pathogen interactions. In fact, leaf symptoms were evident at 5 days for *S. sclerotiorum* (Borrelli et al., 2020), 6 days for *Ceratobasidium* sp., and 6 days for *F. oxysporum* (unpublished), while plant death occurred at 10 days for *Ceratobasidium* sp., 13 days for *S. sclerotiorum* (Borrelli et al., 2020) and 16 days for *F. oxysporum* (unpublished).

Knowledge of the presence of pathogens is essential for the development and implementation of proper disease management strategies. It is important to note that *Ceratobasidium* sp. and other soilborne fungi can cause the loss of entire plants, resulting in a great economic impact on intensive crop production.

From the grower's point of view, agronomic performance of ornamental plants is highly important (Vainstein, 2002). Genotypes of interest to plant breeders include plant shape, flower production, and the response to the limiting pests. Calibrachoa soilborne pathogenic fungi are among them. Once identified, the categorization of cultivar differential response to basal rots is essential to develop proper disease management practices as well as to guide plant breeding. Any effort to improve plant health and quality will certainly have an impact on horticultural crop production.

CONCLUSIONS

- *Ceratobasidium* sp. AG-A causes wilt of *Calibrachoa hybrida.*
- Root rot is the primary symptom of the disease.
- This is the first report of the disease in the world.

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