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First report of a postharvest fruit rot in apple caused by *Athelia* sp. in Brazil

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Abstract

A fungus with white mycelia grows on apple fruits and bins stored in cold temperature for a long period in Brazil and presence of the mycelia externally with the internal rot in apples was observed. The objectives of our work are to identify this fungus and verify its pathogenicity. The internal transcribed spacer (ITS) region was sequenced and the pathogenicity test was performed on apples of Gala and Fuji cultivars. The fungal colonies were formed only incubate around 10ºC and colony growth stopped in high temperatures. Blast results of ITS sequence with Genbank confirmed that this fungus is *Athelia* species and formed disease in both apple cultivars. This is the first report of *Athelia* sp. causing post-harvest rot in apples in Brazil. The results of this work are fundamental to select a correct management strategy aiming to reduce losses by post-harvest rots.

Keywords – *Atheliaceae* – Basidiomycetes – *Fibularhizoctonia psychrophile* – *Malus domestica*

Introduction

Brazil has been excelled in apple cultivation, producing a little over 1 million tons of fruit annually (Abpm 2019). The Santa Catarina state is the largest producer, with a total production estimated at 535 thousand tons from a cultivated area of 13.6 thousand hectares in 2020/2021 harvest (Epagri 2021).

The sustainability of Brazilian apple production is directly linked to the recognition and adequate management of the main diseases that affect the crop. In recent years, several new diseases have been identified in Brazil, such as branch cankers caused by *Dickeya dadantii* (Ogoshi et al. 2019a) and *Pseudomonas syringae* (Araujo et al. 2020), and new diseases are emerging, demanding major efforts to correctly identify the etiologic agent and adopt the most appropriate management measures.

In October 2020, apple fruits were received at Caçador Experimental Station-EPAGRI to

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identify a possible fungus that was causing internal rot in fruits stored in a cold chamber in long period (8-9 months). The fruits presented externally white mycelia (Fig. 1A) and rot internally (Fig. 1C, D). In the bins, where the fruits were stored, this mycelial growth was also observed (Fig. 1B).

Technicians and growers reported that this fungus begins to appear in October, when it quickly spreads on the fruit surface and in the bins. In addition, this fungus develops both in the storage conditions of Gala and Fuji cultivar, but damage internally is more frequent in Fuji. There was a correlation above 80% with the presence of the mycelium in the region of the calyx with rot internally (Fig. 1D) and these symptoms and signs were more frequent in fruits produced in São Joaquim/Santa Catarina region.

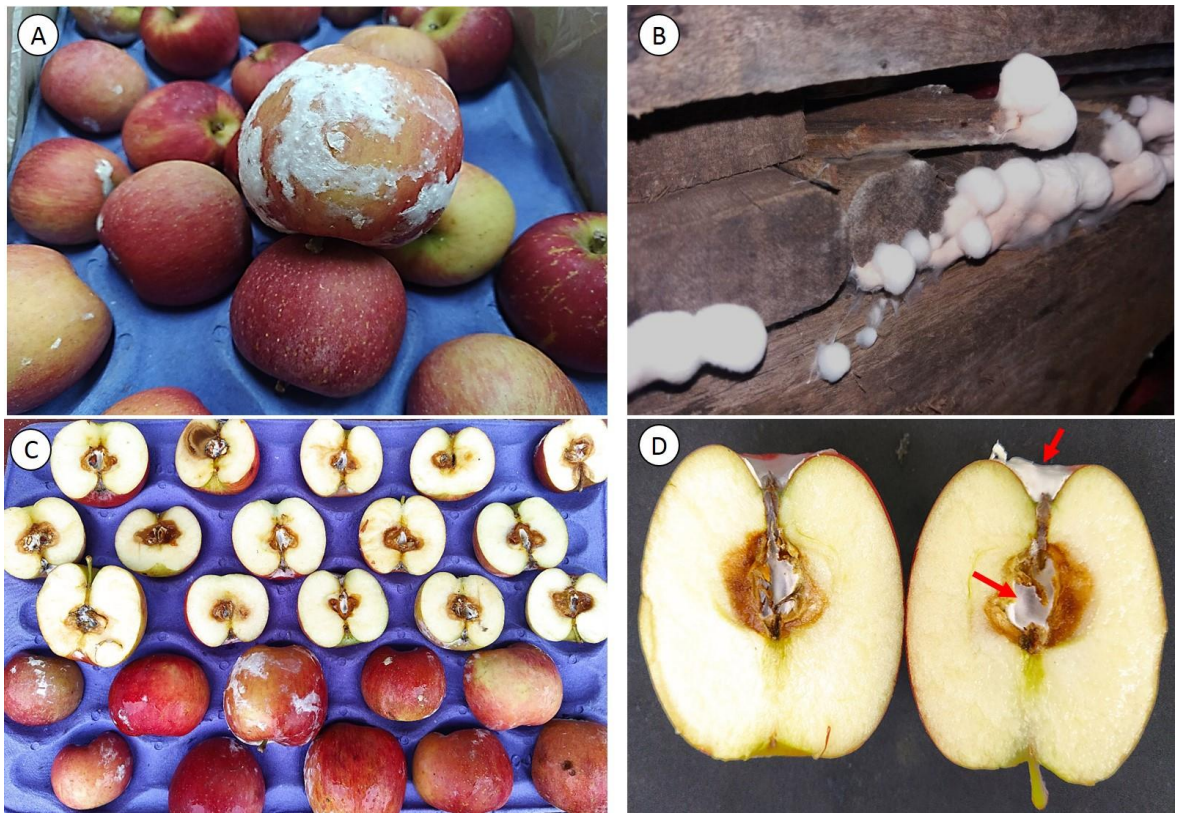

Fig. 1 – A Apple fruits with the presence of white mycelia. B Fungus colonizing the bins. C Fruits with white mycelium and internal rot. D Presence of mycelia in the calyx region and inside the rotted fruits.

Another point that drew attention was that bins located just below the evaporators and the humidification nozzles in the cold chambers had a higher fungus incidence, demonstrating a possible relationship between high humidity in the environment and the presence of this fungus. In addition, in cold chambers with a high fungus incidence, there has been an increase in the release of various volatile compounds. Therefore, the objective of this work was to identify this fungus and to verify its pathogenicity.

Materials & Methods

Fungi isolation

Direct isolation was carried out from the mycelia in malt extract agar. Further, pieces of the fruits were cut in the transition lesion of healthy and dead tissue, and passed in 70% alcohol for 30 seconds, 0.5% sodium hypochlorite for 1 minute and twice in sterile distilled water. After drying, they were transferred to malt extract agar and incubated in biological oxygen demand (BOD) incubator at 25ºC and 10ºC for 15 days.

Pathogenicity test

To check whether the fungus was capable of causing disease in apple fruits, and to fulfill Koch's postulate, the pathogenicity test was carried out on Gala and Fuji cultivars of apple fruits. For this, small wounds were made with the aid of a 1 mm diameter stylus and mycelial fragments were inoculated. The fruits were stored in a box with moistened cotton forming a humidity chamber and in BOD incubator with a temperature of 10ºC. The experimental design was completely randomized with six replications.

DNA extraction, PCR amplification and sequencing

For DNA extraction, mycelial discs were added in flasks with malt extract broth, which were added in an shaker and incubated in BOD at 10ºC. After 20 days, the mycelia were filtered through gauze and macerated in liquid nitrogen. The DNA extraction was performed with the Pure Link® Genomic DNA mini Kit (Invitrogen, ThermoFischer Scientific, USA) following the manufacturer's protocol. After the extraction, an electrophoresis run was performed on 2% agarose gel, in TBE 1X buffer (Tris – HCl 0.89M; Boric Acid 0.89M; EDTA 0.02M) to confirm that extraction was successful.

The ITS1-5.8S-ITS2 regions of the rDNA were amplified using the ITS1/ITS4 primers (White et al. 1990). For the PCR reaction, 2.5 µL of buffer (200 mM Tris-HCl, pH 8.4 - 500 mM KCl, 1x concentrate), 0.5 µL of dNTPs (2.5 mM), 2, 5 µL of each primer, 0.5 µL of MgCl2 (50 mM), 0.35 µL Taq DNA polymerase (5 U µL -1), 3.5 µL DNA (10 ng µL -1) and 12.65 µL of sterilized ultrapure water. The reaction mixture was placed in a thermocycler (Loccus TC9639). The amplification cycles were: 2 minutes initial denaturation at 95ºC, followed by 35 cycles, consisting of a 45 second denaturation stage at 95ºC, an annealing step, 45 seconds at 55ºC, and an extension step 50 seconds at 72ºC, and a final extension of 72ºC for 5 minutes.

Phylogenetic analyses

The DNA sequence obtained was analyzed in Chromas software and compared with the sequences available on GenBank using the BLAST tool (Basic Local Alignment Search Tool). The sequences in GenBank with the highest percentage of identity with our sequence were selected and the phylogenetic trees were constructed. Our sequence was deposited in GenBank with accession number MZ129309.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 0,45000843 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 479 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Results

The colony characters of *in vitro* and *in vivo* were similar to each other. This growth occurred only when the plates were incubated at 10ºC (Fig. 2A), with no growth being observed at 25ºC. However, at 40 days after incubation, the formation of brown and circular sclerotia (Fig. 2B) occurred, a situation not observed in the fruits received in the laboratory.

In the pathogenicity test, the growth of the fungus on the fruits of both cultivars was observed at 20 days after inoculation. At 30 days after inoculation, there was an increase in the growth of the fungus on the surface of the fruits with the same characteristics observed in the packing houses both in the cultivar Fuji (Fig. 2C) and in the cultivar Gala (Fig. 2D). Direct re-isolation of the fungus was carried out and the same growth as the first isolation was observed, a fact that fulfills Koch's postulate.

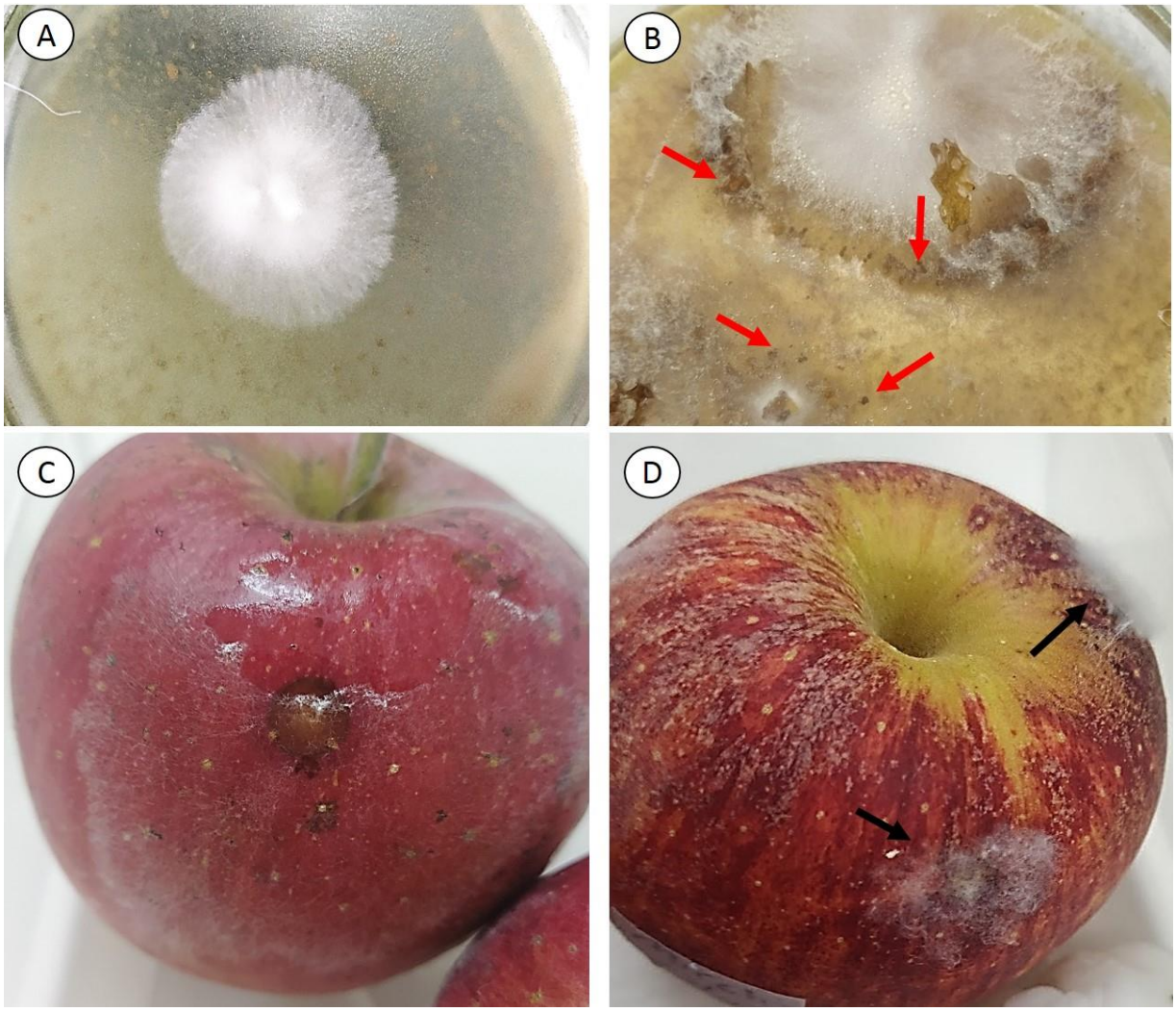

Fig. 2 – A Mycelial growth in malt extract agar at 10ºC for 20 days. B Production of sclerotia at 40 days after isolation. C Mycelial growth on the fruit surface of Fuji cultivar at 30 days after inoculation. D Mycelial growth on the fruit surface of Gala cultivar at 30 days after inoculation.

The MegaBlast results showed that our sequence showed 100%, 99.82% and 100% similarity with the sequences of the fungi *Athelia acrospora* (GenBank: MT305993.1), *Athelia epiphylla* (current name *Athelia arachnoidea*) (GenBank: LR694194.1) and *Fibularhizoctonia psychrophila* (syn. *Fibulorhizoctonia psychrophila*, teleomorph *Athelia* sp.) respectively. Some articles mistakenly wrote the name *Fibulorhizoctonia*, however, the correct name is *Fibularhizoctonia* G. Adams & B. Kropp, gen. nov. (Adams & Kropp 1996, Kirk et al. 2008, Index fungorum 2021, Species Fungorum 2021).

The phylogenetic tree with the ITS gene was constructed with another six sequences taken from the GenBank with proximity to our sequence. The *Athelia rolfsii* sequence was added with outgroup. Our isolate clustered with the other three isolates of the species *Athelia acrospora* (GenBank: MT305993.1), *Athelia epiphylla* (current name *Athelia arachnoidea*) (GenBank: LR694194) and *Fibularhizoctonia psychrophila* (Fig. 3).

Discussion

The results of our work demonstrate that the identified fungus is *Athelia* sp. This fungus belongs to *Atheliaceae*, *Atheliales*, *Agaricomycetida*, *Agaricomycetes*, *Agaricomycotina*, *Basidiomycota* (Index Fungorum 2021).

Some members of the *Atheliaceae* family are wood saprophytes, ectomycorrhizae, lichens, have symbiosis with termites, which can spread the fungus, and plant pathogens that cause rot in

fruits and vegetables when stored at low temperatures (Adams & Kropp 1996, Wenneker et al. 2017, Maekawa et al. 2020, Sulistyo et al. 2021).

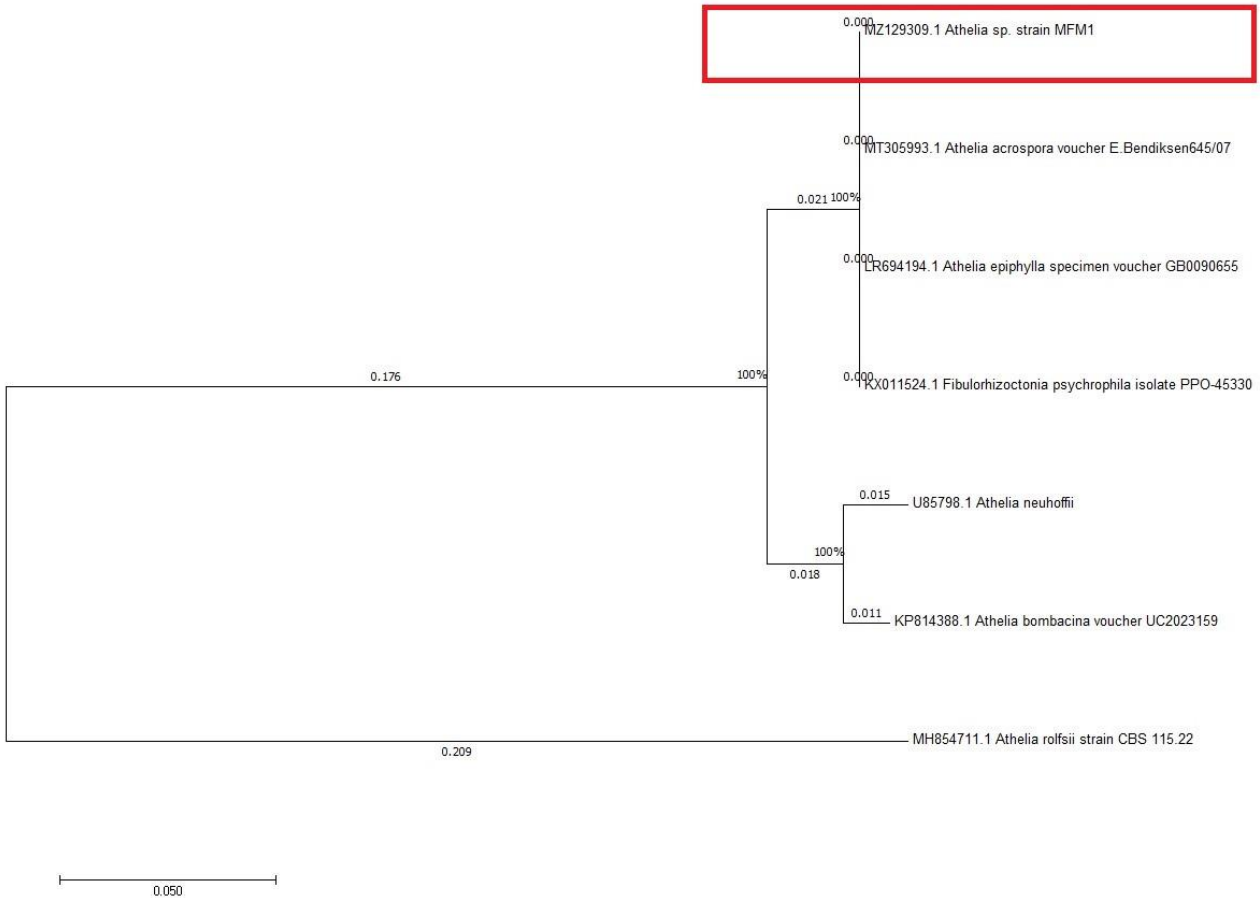

Fig. 3 – Neighbor-joining phylogenetic tree of *Athelia* sp. MFM1 isolate reconstructed from ITS sequences. The evolutionary distances were computed using the maximum composite likelihood method. We conducted the evolutionary analyses using MEGA 7 software. Branch points resulting from 10000 replicates.

Although *Athelia arachnoidea* (syn. *Athelia epiphylla*) has been grouped with 100% identity to our sequence and, we believe that our isolate is not this species, as *A. arachnoidea* can grow in a culture medium with an incubation temperature above 20ºC (De Vries et al. 2008). *Fibularhizoctonia psychrophila*, on the other hand, is closer to our isolate, as it is a psychrophilic fungi capable of growing between 0 to 20ºC and optimal mycelial growth between 9 to 12ºC, not having the ability to grow when incubated at 25ºC (De Vries et al. 2008, Wenneker et al. 2017).

Wenneker et al. (2017) demonstrated that *Fibularhizoctonia psychrophila* is the etiological agent of the lenticel spot in apple and pear fruits stored in long-term cold rooms in Netherlands. Their article shows images with a white mycelium in the fruits and bins similar to that observed by our work. The *Fibularhizoctonia psychrophila* isolates from the work by Wenneker et al. (2017) did not produce sclerotia in culture media, however, the *Fibularhizoctonia psychrophila* isolate from De Vries et al. (2008) produced sclerotia similar to our isolate.

Despite the evidence that our isolate is *Fibularhizoctonia psychrophila*, it was not possible to conclude only as sequencing of the ITS gene region. It is known that ITS region is highly conserved among the different species of fungi, but it varies between species of the same genus (White et al. 1990). However, as *Fibularhizoctonia psychrophila* is the anamorphic phase of *Athelia* sp. (De Vries et al. 2008) we can conclude that our isolate is from this genus, and new gene regions will be sequenced by our team to try to define the species involved in this new pathosystem in Brazil.

Regardless of the fact that it is not possible to define the species, our results are important for the apple production sector in Brazil, as there are reports of an increase in the appearance of this fungus in cold apple chambers stored in the long period, and there was still doubt as to whether was it really a phytopathogen or not.

It is known that some species of *Athelia* are decomposing agents of fallen leaves in forests (Adams & Kropp 1996). *A. bombacina* was effective antagonist to *Venturia inaequalis*, Apple Scab pathogen, when applied to leaves in laboratory, orchard and in prevent spring ascospore production with fall application, being possible to recovery the fungus on next season (Heye & Andrews 1983, Carisse et al. 2000). However, *A. bombacina* was recently reported causing postharvest fruit rot on pear in China (Jia et al. 2018). Therefore, orchard litter, such as roots, leaves and dead stems are potential sources of survival and inoculum *Athelia* sp. in apple orchards (Wenneker et al. 2017).

Considering, the decomposer nature of the fungus, we believe that the first source of the inoculum comes with the bins and spread to the apples during the storage period. For this reason, the bins might be sanitized properly before the cold chamber storage. Consequently, with the knowledge only the fungus genus, it is already possible to adopt a management strategy to prevent this pathogen from causing post-harvest losses in apple in Brazil. Since post-harvest rot can cause losses greater than 20% in the country depending on the year and the cultivar (Argenta et al. 2021) with an estimated loss of revenue of R$ 153 million per year (Ogoshi et al. 2019b). The results demand new studies to determine the first inoculum source to carry out management strategies.

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