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Morpho-cultural, molecular characterization and identification of the pathogen of the burning of leaf and cashew fruit

Caracterização morfo-cultural, molecular e identificação do patógeno da queima da folha e do fruto do cajueiro

Morfocultural, caracterización molecular e identificación del patógeno de la quema de hojas y el fruto del anacardo

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Abstract
The cashew tree (*Anacardium occidentale* L.) is one of the most important fruit trees cultivated in the semi-arid regions of tropical climate in the world. However, this crop is affected by numerous diseases throughout its production cycle. The burning of the leaf and fruit of cashew is a relatively new disease in Mozambique and is characterized by causing necrotic lesions in fruits and leaves. Thus, the present work was carried out with the objective of isolating and making the morphological and molecular characterization of the fungus responsible for burning the leaf and fruit of the cashew tree and determining its taxonomic identity. The study was based on laboratory tests with the aid of the optical microscope and polymerase chain reaction (PCR). In PDA, cultures of spongy dark coloration were often isolated and distinguished; abundant orange and white and, spongy, typified as *Neofusicoccum s*pp., *Colletotrichum s*pp. and *Pestalotiopsis s*pp., respectively. Molecularly, the results confirmed that at least these genera of fungi are involved in the disease of leaf burning and cashew fruit. No *Cryptosporiopsis s*pp. isolates were isolated. However, it is recommended to evaluate the interactive/synergistic effect of the different isolates obtained from samples with symptoms of leaf burning and cashew fruit in the different stages of host tissue development.
Keywords: Cashew; Burning of the leaf and the fruit of the cashew tree; Mozambique.

Resumo
O cajueiro (*Anacardium occidentale* L.) é uma das mais importantes frutíferas cultivadas nas regiões semiáridas de clima tropical do mundo. Porém, esta cultura é afectada por inúmeras doenças ao longo de todo o seu ciclo produtivo. A queima da folha e do fruto do cajueiro é uma doença relativamente nova em Mocambique e caracteriza-se por causar lesões necróticas em frutos e folhas. Assim, realizou-se o presente trabalho com o objectivo de isolar e fazer a caracterização morfológica e molecular do fungo responsável pela queima da folha e do fruto do cajueiro e determinar a sua identidade taxonómica. O estudo baseou-se em testes laboratoriais com auxílio do microscópio óptico e da

Reação em Cadeia da Polimerase (PCR). Em PDA, foram frequentemente isoladas e distinguidas culturas de coloração escura esponjosa; alaranjadas e brancas abundantes e, esponjosas, tipificadas como sendo de *Neofusicoccum* spp., *Colletotrichum* spp. e *Pestalotiopsis* spp., respectivamente. Molecularmente, os resultados confirmaram que pelo menos esses géneros de fungos estão envolvidos na doença da queima da folha e do fruto do cajueiro. Nenhum isolado de *Cryptosporiopsis* spp. foi isolado. No entanto, recomenda-se fazer a avaliação do efeito interativo/sinergético dos diferentes isolados obtidos de amostras com sintomas de queima da folha e do fruto do cajueiro nas diferentes fases de desenvolvimento do tecido hospedeiro.
Palavras-chave: Cajueiro; Queima da folha e do fruto do cajueiro; Moçambique.

Resumen
El anacardo (*Anacardium occidentale* L.) es uno de los árboles frutales más importantes cultivados en las regiones semiáridas de clima tropical en el mundo. Sin embargo, este cultivo se ve afectado por numerosas enfermedades a lo largo de su ciclo de producción. La quema de la hoja y el fruto del anacardo es una enfermedad relativamente nueva en Mocambique y se caracteriza por causar lesiones necróticas en frutas y hojas. Así, el presente trabajo se realizó con el objetivo de aislar y realizar la caracterización morfológica y molecular del hongo responsable de quemar la hoja y el fruto del anacardo y determinar su identidad taxonómica. El estudio se basó en pruebas de laboratorio con la ayuda del microscopio óptico y la reacción en cadena de la polimerasa (PCR). En PDA, las culturas de color esponjoso oscuro a menudo se aislaron y distinguieron; abundante y esponjoso naranja y blanco, tipificado como *Neofusicoccum* spp., *Colletotrichum* spp. y *Pestalotiopsis* spp., respectivamente. Molecularmente, los resultados confirmaron que al menos estos géneros de hongos están involucrados en la enfermedad de la quema de hojas y la fruta de anacardo. No hay aislados de *Cryptosporiopsis* spp. ha sido aislado. Sin embargo, se recomienda evaluar el efecto interactivo/sinérgico de los diferentes aislados obtenidos de muestras con síntomas de quema de hojas y anacardos en las diferentes etapas del desarrollo del tejido huésped.
Palabras clave: Anacardo; Quema de la hoja y el fruto del anacardo; Mozambique.

1. Introduction

The cashew tree (*Anacardium occidentale* L.) is a plant cultivated in almost all tropical countries and has as its center of origin from the northeast of Brazil (Viana *et al.*, 2020; Vivek *et al*., 2013). Currently, it is spread in South America, Central America, Africa, Asia where India, Brazil, Mozambique, Tanzania, Kenya and Côte d'Ivoire stand out as the main producers (Araújo, 2013).

Although cashew tree is considered a rustic plant, this anacardiaceous can be affected by several phytosanitary problems (Freire *et al*., 2002), mainly caused by fungi, which highly compromise its development (Sobrinho *et al.*, 2021; INCAJU, 2007; Cardoso *et al*., 2005).

The present study aimed to characterize culturally, morphologically and molecularly the fungi responsible for the burning symptoms of the leaf and fruit of the cashew tree. Specifically, the objectives were: (1) isolate and to describe culturally and morphologically the fungi responsible for the symptoms described and (2) describe molecularly and determine the taxonomic identity of the fungi responsible for the symptoms of leaf burning and cashew fruit.

2. Methodology

2.1 Pathogen isolation and pathogenicity test

In Inhambane and Gaza provinces, a total of 20 samples of cashew leaves and fruits were harvested. For each collection site, the observational examination of the symptoms was made, followed by the packaging of the same in individual paper bags, coded and stored in a colman in order to transfer to the laboratory (Ramos, 2021; Uaciquete *et al.*, 2013).

For the isolation of the peptide, the symptomatic organs (Figure 1A) were sanitized in running water and then cut into small fragments. Posteriorly, the fragments were disinfected in a 1% sodium hypochlorite solution for 30 seconds followed by immersion in distilled and sterilized water to remove excess disinfectant (Diniz *et al.*, 2021; Lima *et al*., 2013; Serra and Coelho, 2007).

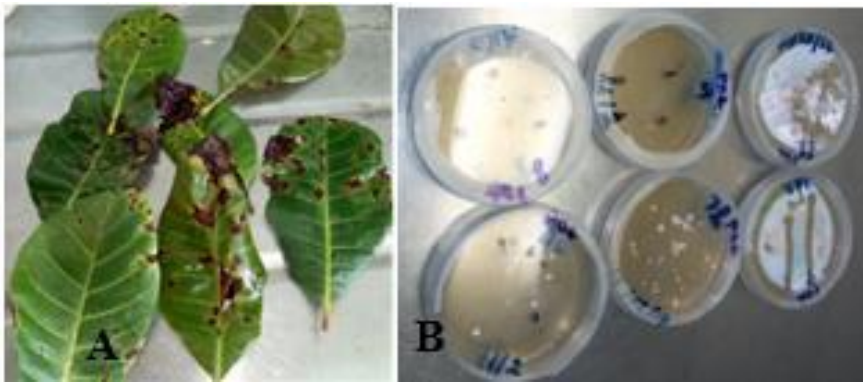
After disinfection, the leaf fragments were arranged on sterile filter paper for drying, later cut and transferred to Petri

dishes with 40 ml of PDA medium, which was prepared and sterilized as described by Majune *et al*. (2019).

The medium was placed in sterilized Petri dishes and left to cool (Carvalho, 2008). Then, the plates with PDA were stored in the refrigerator at 4ºC (Fernandez, 1993). After inoculated they were sealed with parafilm (Figure 1B) and kept on an incubation shelf at a temperature of 25 ± 2 °C (Dominic *et al*., 2014; Cordeiro *et al*., 2007).

After 48 h of incubation, the discs were transferred to new plates containing the PDA medium (Figueirêdo, 2005). The plates were incubated for a period of seven days (pure culture) under the conditions described by Dominic *et al*. (2014) and Majune *et al*. (2019).

Figure 1. Samples and plaques used in the isolation of fungi associated with leaf and fruit burning. (A) Sample of cashew leaves with lesions. (C) Petri dishes sealed with leaf fragments from the infected material.



Source: Authors.

After the isolates were obtained, the pathogenicity test was made, para the effect, it was carried out before the preparation of the suspension and calculation of the concentration of the inoculum, obeying the modified procedures of Fernandez (1993), Carollo and Santos Filho (2016). The concentration of the spore suspension was adjusted in sterile and distilled water until the concentration of 106 spores/ml was obtained (Fernandez, 1993; Carollo and Santos Filho, 2016).

The inoculum suspension was sprayed on cashew seedlings at a temperature of 25-28°C. They were wrapped in transparent plastic bags with moist cotton in the first 24 hours (Figure 2), to provide a humid environment of about 90% relative humidity (Majune *et al*., 2019).

Figure 2. Seedlings in wet chamber by means of transparent plastics.

Source: Authors.

2.2 Morpho-cultural, molecular characterization and identification of pathogens

The fungal isolates obtained were characterized culturally and morphologically in PDA plates based on the method defined by Domicic *et al.* (2014) e Ramos (2021), which includes among other characteristics, the form of growth, staining of the isolates, types of conidia, diameter of the colonies in the culture medium, presence and number of appendages in the conidia. The morphological structures of the fungi were observed under optical microscope and por last, with the aid of taxonomic keys, morphological identification of isolated fungi was made (Majune *et al*., 2019).

For molecular characterization, five samples were analyzed using the Polymerase Chain Reaction (PCR) test. The first three were pure cultures resulting from the isolation of the fungus in PDA medium from leaves with symptoms of burning of the leaves and fruits of the cashew tree while the other two were isolated directly from samples consisting of infected leaves.

Thus, the isolates A (A04), B (A01) and C (A02) were sampled for PCR; Sheet 1 (A03); and 2 (A05). Finally, for molecular characterization, genomic DNA was extraction from the fungus using extraction kits, specific for leaf fungi, depending on the type of sample (Lutz *et al*., 2013).

2.2.1 DNA extraction on leaves with symptoms

For leaf isolates, the Genomic DNA extraction kit DNeasy® Plant Mini Kit was used, according to the manufacturer's instructions. After the extraction and purification steps, DNA was quantified in a Nanodrop spectrophotometer. At the same time, the efficiency of the extraction process was certified, and a genomic DNA race was carried out in 1% agarose gel (Ramos, 2021; Mulandane *et al*., 2018).

2.2.2 DNA extraction in isolates on plates

The genomic DNA of the colonies (mycelium) of fungal isolates was extracted using the ZR Soil Microbe DNA MiniPrepTM extraction kit. For DNA extraction, the manufacturer's protocol was followed. For visualization and running in general, the procedure described in section 2.2.1 was followed above.

2.2.3 DNA amplification in PCR

The molecular detection of fungi was made through PCR test, which consisted of amplification, in vitro, of the ITS1 and ITS4 regions of the dna of fungi. A GeneAmp PCR System 9700 thermocycler® was used. The amplification protocol was modified by Zhu *et al*. (2012); Mills *et al*. (1992); Majune *et al.* (2019) and Uaciquete (2013).

In this process, a final volume of 25 ml was added 2.5 µl of Taq buffer (10x), making a final concentration of 1x; 1.0 µl of dNTP-mix at 10 mM (final concentration = 0.4 mM); 0.5 µl of each primer (final concentration = 0.5 µM); 0.3 µl of Taq Polymerase at 5U/µl (final quantity = 1.5U) and 5 µl of eatted DNA. Then water was added to obtain a final volume of 25 µl. The conditions for the execution of the reactions were: 94°C for 3 minutes; 40 cycles of 94°C for 45 seconds, 58°C 60 seconds and 72°C for 90 seconds and a final elongation step at 72°C for 10 minutes.

The primers used were ITS-1 5'–TCCGTAGGTGAACCTGCGG-3' (forward) and ITS-4 5'-TCCTCCGCTTATTGATATGC-3' (reverse), whose sequences were removed from White et al. (1990), produced at Inqaba Biotechnical Industries (Pty) Ltd and the expected fragment had approximately 350-400 base pairs (pb).

To visualize the PCR result, the samples were run in 1% agarose gel (Mulandane *et al.*, 2018). After the race, according to the results obtained, 5 PCR product samples were selected, which were then carefully packed, labeled preserved in a refrigerated colman and sent for sequencing in the laboratory of Inqaba Biotechnical Industries (Pty) Ltd in South Africa.

2.2.4 Identification of pathogens and phylogenetic analysis

Sequencing was done by Inqaba Biotechnical Industries (Pty), raw sequences were cleaned and assembled using Geneious v10.2.6 (Biomatters, Ltd, New Zealand). Each sequence was identified using BLAST (Altschul *et al*., 1990; Morgulis, 2008). Reference sequences were downloaded from NCBI (NCBI, 2013). Mozambican sequences were aligned against reference sequences using MUSCLE (Ramos, 2021; Edgar, 2004) and a maximum likelihood tree was constructed isin PhyML v. 3.3.20180214 (within Geneious) with Bootstrap of 1000 repetitions and General Time Reversal nucleotide substitution model (Tavaré, 1986). The ITS sequence from Phialea strobilina (EF596821) was used as an outgroup for the alignment and phylogenetic tree.

3. Results and Discussion

3.1 Isolation, morphocultural carachterization of the pathogen and pathogenicity test

In the PDA, the pure cultures of the isolates showed to be heterogeneous in terms of color, aspect, shape and rate of mycelial growth. Thus, it was possible to distinguish three morphological types from the isolates as described below:

(a) Isolated dark color, with spongy appearance, velvety, abundant (Figure 3 Top, A), with rapid growth compared with other isolates of the same lesion. At the beginning of the growth, it was presented, with dark gray color, velvety. Conidia were aseptated hyaline, short with a half flattened central part, without appendages (Figure 3 Bottom, A).

The characteristics described are similar to those of the isolate and conidia of *Neofusicoccum s*p., of the family Botryosphaeriaceae, isolated in the vine culture (Garrido and Gava, 2014). In this crop, fungi cause descending rot disease, necrosis in leaves and plant death (Garrido and Gava, 2014). In Brazil, the fungus *Neofusicoccum s*p. was isolated in cashew crop with symptoms of necrosis and death of leaves over time (Cardoso *et al*., 2018; Coutinho *et al*., 2018),

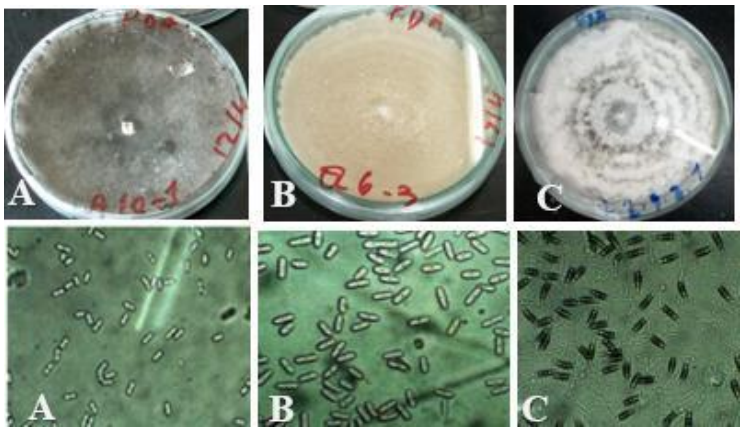
(b) Grayish white isolates (Figure 3 Top, B) at the beginning with small rings and orange dots, which with age became creamy and orange. These isolates had a smooth appearance, moderate density. The conidia (Figure 3 Bottom, B) were hyaline, aseptate, without appendages, rectums, fusiform, with tapered, elongated and other oblong apexes, with rounded apexes.

Results obtained by Menezes (2006), Cordeiro *et al*. (2007), Tozze Júnior *et al.* (2015), proved that the characteristics described above are typical of the genus Colletotrichum that causes anthracnose in cashew trees. However, the occurrence of variability in the color of the isolates and in the characteristics of conidia was also verified in cocoa, mango, cashew, strawberry, peach and coffee isolates (Mafacioli et *al*., 2006; Floodplain, 1995).

(c) Isolates characterized by white coloration, vigorous mycelium, cotton aspect and abundant sporulation. At the beginning of growth, it was smoothly, sticky, but with age it forms rings, little velvety, dark coloration at the base and white at the top (Figure 3 Top, C). The mycelium was septate, branched and light brown; fusiform conidia, straight to slightly curved, septate with two to four atrophic appendages (Figure 3 Bottom, C).

The description of these isolates is typical of the characteristics of the causative agent of Pestalotiosis (Serra and Coelho, 2007; Kruschewsky, 2010) can thus be affirmed that there is agreement with our work regarding the cultural morpho identification of peptides. In sequencies, the *isolates of Colletotrichum s*pp. and *Pestalotiopsis s*pp., were often found from the same symptoms characteristic of leaf burning and cashew fruit (Intini and Sijaona, 1983; Uaciquete, 2013) which suggests a co-occurring of these pathogens in symptoms of burning.

Figure 3. TOP: Pure cultures of different isolates. (A) Smooth, spongy and abundant dark coloring. (B) Cream and orange coloring on the base. (C) white color, vigorous, cotton-like, with rings, dark at the base and white at the top. Bottom: Structures observed in the optical microscope at the magnification of 40X. (A) Aseptate hyaline conidia, short with medium flattened central part (B) Long aseptate hyaline conidia. (C) Light brown hyphae and septate conidia.



Source: Authors.

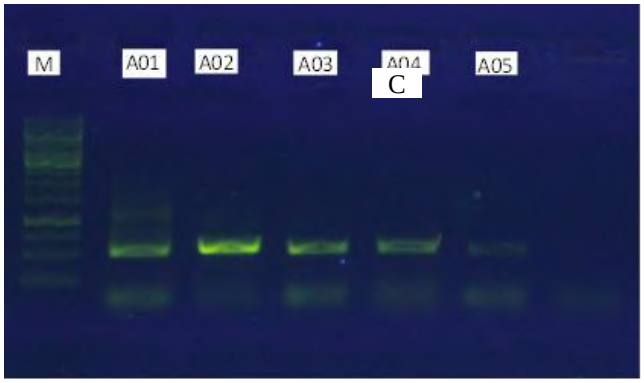
The pathogenicity test resulted in the reproduction of symptoms from the third day after inoculation. Leaf lesions were like those observed in the field of sampling. The control (leaves and seedlings sprinkled with water only), showed no symptoms during the test.

Reproduction of typical symptoms in leaves inoculated with fungal isolates shows the infectivity of the isolates. Therefore, using the PDA medium, 3 pathogens were constantly re-isolated and cultured from symptoms resulting from inoculation, thus fulfilling the Koch Postulates (Micheriff, 2001).

3.2 Molecular characterization and identification of the peptide

At the molecular level it was observed that Primer ITS1 and ITS4 successfully amplified genomic DNA from samples A (A04), B (A01) and C (A02); Sheet 1 (A03); and 2 (A05). The bands of PCR products, with size between 300 - 400 bp, were observed as shown in Figure 4. No band was observed in the negative control.

Figure 4. Amplification of samples of plate isolates and cashew leaves by PCR using ITS1 and ITS4 primers. Where M is the molecular weight marker of DNA, Samples A01, A02, A03, A04 and A05. Legend: A04 Isolated A; A01 - Isolate B; A02 - Isolate C; A03 Sheet 1; and A05 - Sheet 2; C - Negative control.



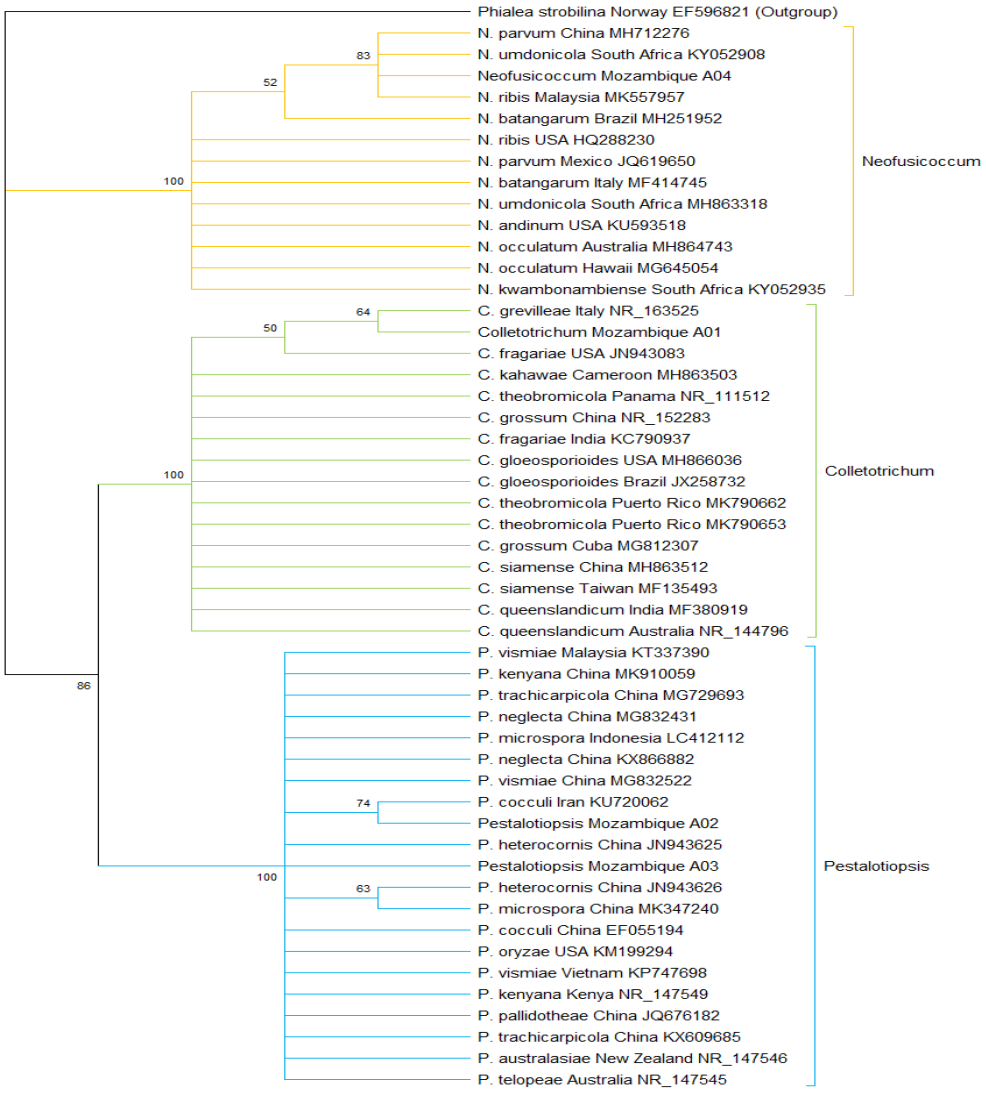
Source: Authors.

Of the five samples sent for sequencing (nucleotide sequences), in only four samples' fungi were molecularly identified up to the gender level through BLAST (Altschul *et al*., 1990; Morgulis, 2008), among them: Pestalotiopsis, Colletotrichum and Neofusicoccum. These genera were also identified in a similar study conducted in Tanzania (Majune *et al*. 2019).

In this, a phylogenetic tree was created, composed of nucleotide sequences of Mozambican isolates and sequences lowered in Genbank (NCBI, 2013) that demonstrated similarity with pathogenic fungi of fruit trees, including cashew tree (Figure 5). Blast nucleotide analysis showed that 50 species have maximum nucleotide identities of 99.63-100% of cashew disease under study.

The sequences of Mozambican isolates have percentage values above 50% reliability in phylogenic relationships (Bootstrap values index) with sequences downloaded in Genbank (REF), as illustrated in Figure 5.

Figure 5: Phylogenetic tree of its1 and ITS 4 genes isolated from Mozambican fungi of the genera: Pestalotiopsis, Colletotrichum and Neofusicoccum extracted from the NCBI. In each nodule is indicated the Bootstrap index (as a percentage) where only values above 50% are considered reliable.



Source: NCBI.

The phylogenetic tree shows that *Pestalotiopsis* from Mozambique (sample A02) has 100% identity with genbank fungal species of the genus Pestalotiopsis and in 74% grouped with *Pestalotiopsis cocculi* Iran. The A03 sample shows 100% rate of Bootstrap values in reaction to fungi of the GenBank of the genus Pestalotiopsis.

From the fungi isolates of this study, the genus Neofusicoccum, belonging to the family Botryosphaeriaceae, was also identified and occurs mostly associated with tropical fruit trees (Coutinho, 2016), causing deaths, resinosis, fruit rot and cancers in several woody hosts (Cardoso *et al*., 2018).

Neofusicoccum from Mozambique (sample A04) presents according to the phylogenetic tree data 83% of clustering *with Neofusicoccum Parvum* China, *Neofusicoccum Umdonicola* South Africa and *Neofusicoccum Ribis* Malaysia. However, this genus has 99.63 – 100% identity with the fungal species of GenBank.

In cashew crop in Brazil there are records of isolation *of the species Neofusicoccum batangarum*, *Neofusicoccum kwambonambiense*, *Neofusicoccum sp*., associated with symptoms of "dieback" (progressive death or death by definhation), cancer in stems and necrosis in the leaves of cashew plants (Cardoso *et al*., 2018; Coutinho *et al*., 2018), as well as associated with symptoms of leaf burning and cashew fruit (Majune *et al*., 2019).

Neofusicoccum parvum and *Neofusicoccum umdomicola* are species that have previously been isolated in mangueira culture (Marques *et al*., 2013). However, since the anacardiaceae family hose is the same as the cashew tree, these fungal species may probably occur in plants of the family, for example in cashew trees, which justifies the detention of these species in the Genbank BLAST, with phylogenic relationships with *Neofusicoccum* Mozambique (A04) in the present study.

The identity of *Colletotrichum* Mozambique (A01) according to sequencing data had ranged from 99.44 – 100% with species tracked in Genbank of the same genus. However, in the phylogenetic tree, a percentage of grouping of 64% is represented in relation to *Colletotrichum grevilleae* Italy.

Among the species described in the phylogenetic tree, in Mozambique had already recorded the occurrence in the cashew crop of *Colletotrichum gloeosporioides*, *Colletotrichum sp*., *Colletotrichum siamense*, *Pestalotiopsis sp*. *Fusarium sp*. associated with *Colletotrichum sp*. and *Phomopsis sp*. (Uaciquete, 2013; Masawe *et al*., 2015), in this context, the PCR technique used in this study confirms its identity.

Colletotrichum gloeosporioides was molecularly confirmed as a cause of anthracnose in Mozambique (Uaciquete, 2013), a fact that is supported by discoveries made by other authors in Brazil (Veloso *et al.*, 2022; Cardoso and Freire, 2002).

In a study on species of the genus Colletotrichum associated with cashew anthracnose in Mozambique, Masawe *et al.* (2015), states that cashew anthracnose is not caused by *Colletotrichum gloeosporioides*, but by a complex of *Colletotrichum* species. However, Cardoso and Freire (2003) also argue that the genus Colletotrichum beyond its complexity is very variable in morphology, pathogenicity and physiology.

The phenomenon described in which multiple species of the genus Colletotrichum are involved in cashew anthracnose, also occurs in other crops, such as avocado, pepper and strawberry (Uaciquete, 2013).

Following, Sijaona *et al*. (2005), Uaciquete (2013), Dominic *et al*. (2014) and Zhongrun and Masawe (2014), based on morphological characteristics of the isolates, report that the burning of the leaf and fruit of the cashew tree is caused by the fungus *Cryptosporiopsis* spp. However, molecular results of the present study, in samples with symptoms described by these authors did not find, the genus Cryptosporiopsis. However, Old *et al*. (2002), argues that species of the genus Cryptosporiopsis are predominantly stem pathogens of woody hosts in temperate regions.

Although there are no reports of the occurrence *Pestalotiopsis heterocomis* in cashew culture in Mozambique, results on studies of diseases that attack cashew crop in Burkina Faso reveal that this fungus causes leaf spots and with high frequencies in cashew crop (Wonni *et al.*, 2017). Meanwhile, Jeewon *et al.* (2004) reports that fungi of the genus Pestalotiopsis

are widely distributed, occurring in soils, branches, seeds, fruits and leaves, and there are currently about 234 species described (Kruschewsky, 2010).

According to the results obtained in this study, it can be said that the genus Colletotrichum, when associated with Pestalotiopsis and Neofusicoccum, constitute a complex that causes the burning of the leaf and fruit of the cashew tree.

4. Conclusion

The types of isolates identified in the present study showed to be heterogeneous in terms of color, appearance of isolates in PDA medium and conidia variable in terms of color, size form and presence of appendages. The results of this study show that in Mozambique, the burning of leaf and cashew fruit is associated with a complex of fungi of the genera Pestalotiopsis, Neofusicoccum and Colletotrichum. In this study, no isolates of *Cryptosporiopsis* spp. were found, which has been referred to as the causative agent of leaf and fruit burning.

In this study, only the PDA was used for isolation. However, the use of other culture media may lead to the identification and characterization of other pathogens.

It is recommended to evaluate the interactive/synergistic effect of the different isolates obtained in samples with symptoms of leaf burning and cashew fruit in the different stages of host development and with particular phase to improve post-inoculation incubation conditions since moisture in leaf blade and host cult can be determinant for symptom growth, diversification of the time and the geography of sampling.

It is also suggested that in future studies we study the relationships of pathogens and the environment, testing control measures in the perspective of dissemination of the results to cashew producers.

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