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Sanitary analysis, transmissibility and pathogenicity of fungi associated with cashew nuts

Jaíza Francisca Ribeiro Chagas[1], Solange Aparecida Ságio[1], Evelynne Urzêdo Leão[1], Aloísio Freitas Chagas Júnior[1], Marcos Vinicius Giongo[2], Raimundo Wagner de Sousa Aguiar[1], Rodrigo Ribeiro Fidelis[1] and Gil Rodrigues dos Santos[1]*

[1]Departamento de Produção Vegetal, Universidade Federal do Tocantins, Caixa Postal 66, 77402-970 - Gurupi, TO, Brasil.
[2]Departamento de Ciências Florestais e Ambientais, Universidade Federal do Tocantins, Caixa Postal 66, 77402-970 - Gurupi, TO, Brasil.

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There are few reports of transmission of fungus associated with cashew nuts (*Anacardium occidentale* L.) in Brazil and worldwide. Thus, the aim of this study was to evaluate the incidence and severity of anthracnose on leaves and fruits (nuts) of cashew, nuts sanitary analysis, nuts-seedling transmission and pathogenicity of fungi associated with cashew nuts. To this end, leaves and nuts of cashew collected in the cities of Gurupi and Formoso do Araguaia, Tocantins state, Brazil, were used. The anthracnose average incidence and disease index were evaluated by observing the typical symptoms of the disease. The nuts were subjected to sanity analysis, by the filter paper method and transmission test via nuts for the seedlings. High anthracnose incidence and severity were observed in leaves and cashews nuts. *Aspergillus* sp. *Cladosporium* sp. and *Colletotrichum gloeosporioides* showed high incidence in all nuts samples analyzed. There was high anthracnose transmission by the nuts for the seedlings. Only *C. gloeosporioides* was pathogenic to cashew seedlings. Higher germination rates were observed with lower levels of lesions on the external surface. The fungal association with cashews nuts damaged generally normal seedlings development and quality.

Key words: *Anacardium occidentale*, *Colletotrichum gloeosporioides*, anthracnose, sanitary analysis.

INTRODUCTION

The cashew (*Anacardium occidentale* L.) is a tropical native species from Brazil spreading almost all-Brazilian territory (Coutinho et al., 2016). Constitutes an income source for the population and has great economic and

*Corresponding author. E-mail: gilrsan@uft.edu.br.

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social importance, especially in the Northeastern region. Although most of the cashew production is located in the country coastal region, a large increase in production area is occurring in Semiarid and Cerrados (Brazillian savanna) regions, probably due to favorable conditions for a higher fruits (nuts) and pseudo fruits (flower peduncle) quality than in any other country region. The cashew propagation is carried out through nuts (seeds) or vegetative parts of plant, the latter being the most recommended, as it ensures obtaining more uniform fields with desirable characteristics and more productive (Dendena and Corsi, 2014). The planting through nuts is still widely used in several regions. However, the use of contaminated and/or infected nuts by pathogens can result in lower plant population and lower production and pseudo fruits quality because they are vehicles of many pathogens that serve as initial inoculum and can cause epidemics in the orchard.

Anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. [anamorphic phase *Glomerella cingulata* (Stoneman) Sp. Schrenk], is the most widespread and destructive disease of cashews in Brazil. In most plants, it is mainly transmitted through seeds, but also through plant infected parts. Symptoms may appear on leaves, fruits and seedlings, occurring in all plant development stages and can cause seedlings necrosis and death (Dendena and Corsi, 2014). The fungus may attack during all the months in the year, but cause greater damaged in the rainy season and when issuing news hoots. The disease management was carried out using sanitary practices and chemical control (Figueirêdo et al., 2012). The use of resistant clones is also should be adopted, however, all commercially available clones are susceptible to disease (Figueirêdo et al., 2012).

Healthy seed must have a high germination rate and be free of seed-borne pathogens and healthy seeds are recognized as an important input in any agricultural production system. However, there is a little information available about the transmission of fungi through the cashew nuts in Brazil and worldwide, which makes this study of great importance. Based on the exposed, the aim of this study was to evaluate the incidence and severity of anthracnose on leaves and nuts of cashew nuts, sanitary analyzes, cashew nuts - seedlings transmission and pathogenicity of fungi associated with cashew nuts.

MATERIALS AND METHODS

Sample collection

The study was carried out from August, 2013 to October, 2014. An amount of leaves and nuts were collected from a total of 20 cashew trees, previously selected, from two cities of Northern region Brazil, Gurupi (11° 43' S; 49° 04' S) and Formoso do Araguaia (11° 47'45''S; 49° 31'52''W), State of Tocantins. After the collection, the nuts were mixed for performing the tests.

Pathogen identification

Cashew leaves fragments with anthracnose symptoms were subjected to sterilization by immersion in 50% alcohol solution for 30 s, 1% sodium hypochlorite for 40 s and three washes in sterile distilled water. After, the leaf fragments were placed on Petri dishes containing potato dextrose agar (PDA) culture medium, and maintained in a growth chamber at 25°C ± 2°C for seven days. After incubation, the fungi identification was carried out by analyzing the fungal structures, with the aid of an light microscope. The observed structures were compared with structures described in the literature (Barnett and Hunter, 1972).

The identified *Colletotrichum* sp. isolates were grown on PDA medium and incubated for seven days for further molecular identification at specie level. For DNA extraction, discs containing the mycelia were excised and inoculated into 60 mL of liquid medium Yeast extract (10 g yeast extract, 10 g dextrose, 1 L distilled water) with antibiotic (Cloranfenicol - 200mg/L). For mycelial growth, they were kept under agitation (120 g) for seven days at 27 °C. The mycelium was separated from the liquid medium by vacuum filtration. After washing with sterile distilled water the harvested mycelium was ground to powder in liquid nitrogen, with a pestle and a mortar. DNA was extracted from the powdered mycelium using a CTAB method adapted from Zolan and Pukilla (1986). Briefly, about 0.5 g of this powder mycelium was then transferred to a micro centrifuge tube, and 1 mL of preheated (65ºC) extraction buffer (2% p/v de CTAB; 2.5% p/v de PVP; 2M de NaCl; 100 mM Tris-HCl (pH 8.0), 25mM EDTA (pH 8.0), 2% (v/v) ß-mercaptoetanol) were added. The powder was mixed with the buffer, vortexed briefly, and stored at 60°C for 40 min and then centrifugate at 11,000 *g* for 10 min. After separation of the tissue from the aqueous phase, an equal volume of chloroform/isoamyl alcohol (24:1) was added. The RNA was degraded by treatment with RNase A (50 mg/mL) for 30 min at 37°C. The DNA was precipitated by adding 2.5 volumes of absolute ethanol and pelleted by centrifugation for 15 min at 12,000 *g*. The pellet was washed with 70% ethanol and resuspended in ultrapure water. The DNA concentration and purity were measured using a spectrophotometer.

The extracted DNA was subjected to the polymerase chain reaction (PCR) using a forward primer CaLac-f (5'-GAAGATCTCGGCACCATCAT-3') and a reverse primer CgLac-r (5'- AACAACAGGGACCAGGTCAG-3') to amplify the laccase gene of *C. gloeosporioides* (Shi et al., 2008). Each PCR reaction contained 2.5 ng of DNA, 13 µL of sterile water, 10 mM of Tris-HCl, 2 mM of $MgCl_2$, 0,1 mM of dNTP's, 1.25U Taq DNA Polymerase (Ludwig Biotec, Brazil) and 0.4 µM of each primer (final volume = 25 µL). This solution was submitted in thermal cycler with the following parameters: 4 min at 94°C, 40 cycles of 1 min at 60°C, 2 min at 72°C, and a final extension of 5 min at 72°C. After amplification the PCR products were subjected to electrophoresis on a 1.5 % agarose gel stained with Blue Green Loading dye (Promega, United States).

Incidence and anthracnose severity

Fifty cashews leaves and nuts were randomly obtained from 16 cashews tree to quantify the anthracnose incidence and severity. A completely randomized experimental design was used with 11

replicates. Average incidence of anthracnose on leaves and nuts was expressed in percentage using: % incidence = $[(\sum x/n) \times 100]$. Where x = number of infected leaves or fruits and n = total number of leaves or fruits observed. In the assessment of anthracnose average severity on leaves and nuts, we used rating scale 0-4 adapted from Santos et al. (2005), where 0 = no symptoms; 1 = symptoms at 10% of the affected area; 2 = 11 to 25% of the affected area; 3 = 26 to 50% of the affected area; and 4 = more than 50% of the affected area. Later, the disease index (DI) was determined using the McKinney index formula (1923) as shown: DI=$[\sum F(n) \times 100]/(TNL \times MN)$]. Where: ΣF(n) = the sum of notes frequency; TNL= total number of examined leaves; e MN = maximum scale note.

Sanitary analysis of cashew nuts

For this assay, the blotter test method was used, following the description in the Rules for Seed Testing – RAS (Brasil, 2009). The experimental design was completely randomized, with 16 replications. Initially, the nuts were washed in water and subjected to sterilization in 1% hypochlorite, 70% ethanol and distilled water. The nuts were placed in a transparent box (gerbox), with 25 nuts on each box, on two layers of germitest paper previously autoclaved and moistened with sterile water. The boxes containing the nuts were kept in incubation chamber at 25°C ± 2°C for seven days, with alternate light. After incubation, the fungi identification was carried out on the nuts as described above. The values of results were expressed in percentage of incidence of each fungus. When it was not possible the fungus genera classification using the structures present, the hyphae of these fungi were collected and placed on PDA medium, for later identification. For confirmation of Koch's principles, the fungi found in the nuts were isolated on PDA medium. To test the pathogenicity of these fungi to cashew plants, the conidia were inoculated by spraying at a concentration of 1 × 10^5 conidia mL using a manual sprayer in cashew seedlings at 40 days after emergence. Then the seedlings are stored for 24 h in a moist chamber until disease symptoms onset.

Cashew nut – seedling transmission

For the cashew nut – seedling transmission assay, 50 nuts in three replications, totalizing 150 cashew nuts were sowed without disinfestations, on plastic trays containing autoclaved sand. After the sowing, the trays were transferred to a greenhouse with temperature of 30°C ± 5°C. Daily irrigation was performed with the aid of a watering pot. The seedlings evaluation was made 15 days after the emergence by observation of the characteristic symptoms. The fungi transmission was assessed by considering the percentage of seedlings with anthracnose symptoms on leaves, steam and cotyledon. It is also evaluated the percentage of germinated and non-germinated nuts and the percentage of dead seedlings. To identify the fungi species in the seedlings lesions, the injured fragments of the primary root, stem, cotyledon and leaf were removed and submitted to the isolation process as described already.

Effect of anthracnose severity on the cashew nuts in the germination

The nuts were selection based on external surface visual observations and separated according to damage level using a rating scale adapted from Santos et al. (2005). We used a completely randomized design with three replicates; the treatments consisted of five different severity levels and each replicate was constituted by 50 cashew nuts. The sowing was made on polypropylene trays, with autoclaved sand as substrate. After sowing, the substrate was moistened and the trays were kept in a greenhouse with temperature of 30°C ± 5°C. The assessment was carried out over 30 to 50 days after the seedlings emergence. It's considered as germinated nuts, those that gave rise to normal seedlings with all the essential structures (Brasil, 2009). The transmission was observed in normal and not normal seedlings by count seedling with lesions on leaves. The pathogen identification was carried out as described above.

Statistical analysis

The data of anthracnose averange incidence, disease index and the relationship between infection severity and anthracnose transmission were subjected to analysis of variance (ANOVA) and differences between means were determined according to Tukey's test, at 5% probability. The data expressed as percentage were transformed into arcsin √x/100 for statistical purposes, when necessary. The graphics were plotted using the Sigma Plot (version 10.0) software and the statistical procedures were held with Sisvar (version 5.3) software.

RESULTS AND DISCUSSION

Pathogen identification

All isolates from cashew leaves with anthracnose symptoms were identified, based on morphological aspects, such as conidial morphology and presence or absence of setae, with similar characteristics to those described for species belong to the genera *Colletotrichum* sp. However, because definitive identification of *Colletotrichum* species based on morphology is difficult due to overlapping ranges of conidial and colony characteristics and the fact that variation in morphology is accepted for isolates within species, a number of molecular methods have been used to characterize species of *Colletotrichum* (Uaciquete et al., 2013).

Based on the analysis of laccase gene with the specific primers, this study was able to confirm that the cashew isolate were identified as *C. gloeosporioides.* The primer tested in our study generated amplification products of 505 bp (Figure 1) corresponding to *C. gloeosporioides* laccase gene described by Shi et al. (2008). Morphological and molecular characteristics performed in this study confirmed *C. gloeosporioides* as the causal agent of cashew anthracnose. These finds corroborate with the described by and Uaciquete et al. (2013) that *C. gloeosporioides* is commonly reported as the causal agent of anthracnose in cashew trees. The identification and confirmation of the *Colletotrichum* species pathogenic to cashews is crucial to the development of more efficient control strategies, besides contributing for a better

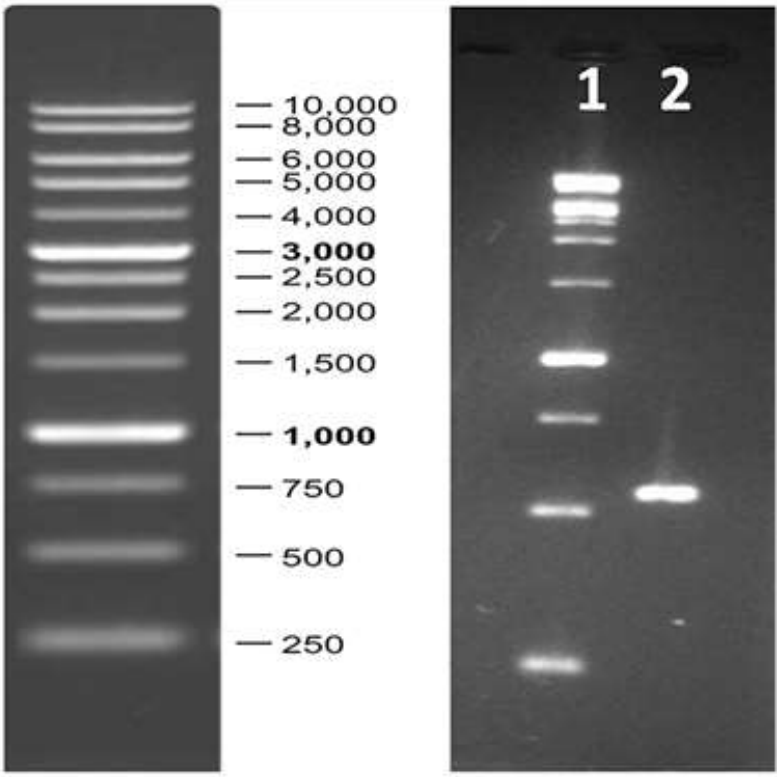

Figure 1. The amplification product obtained by PCR using the oligonucleotides CgLac-f and Calac-r specific to *Colletotrichum gloeosporioides*. Lane 1: molecular marker 1Kb; Lane 2: Cashew Isolated.

understanding of the anthracnose disease epidemiology.

Incidence and anthracnose severity

There was a high anthracnose incidence on the leaves (68.1%) in this study (Figure 2A). The anthracnose incidence on the leaves differed statistically (Tukey, P≤0.05) to the anthracnose incidence in nuts (31.1%). Distinct of the observed in anthracnose incidence, disease index was higher in the nuts than in the leaves, 34.7% and 31%, respectively (Figure 2B). Anthracnose of cashew is a disease known in several regions where it grows cashew, being its incidence always related to the region climatic conditions. The climatic conditions of the Brazilian Northern region are favorable to the growth and proper development of cashew tree. However, high temperatures and high relative humidity (around 85%) found here provide better pathogen development and increasing severity on leaves, stems and fruits, as observed in these study. Long-term exposure to disease promoting or predisposing factors can increase disease development in perennial hosts (Frare et al., 2016), as cashews trees. Host nutritional status is also an important abiotic factor that may be related to increased disease in orchards without proper maintenance, such as where the study was conducted.

Transport of fungi associated with cashew nuts

The results showed a high incidence of *Aspergillus* sp. (49.71%) in all tested samples (Figure 3). The incidence of the genus *Cladosporium* sp. (17.14%), *Penicillium* sp. (11.42%), *Pestalotia* sp. (0.5%) and *C. gloeosporioides* (26.85%) were also observed. Among all fungi found in the cashew nuts only *C. gloeosporioides* was pathogenic to cashew seedlings.

The fungi genus *Aspergillus*, *Cladosporium* and *Penicillium* found in large quantities in the assessed cashew nuts are involved in the decay process of the nuts at sowing stage, by interfering directly in germination rate and seed vigor. These fungi may attack different kinds of seeds, once they are able to survive on several

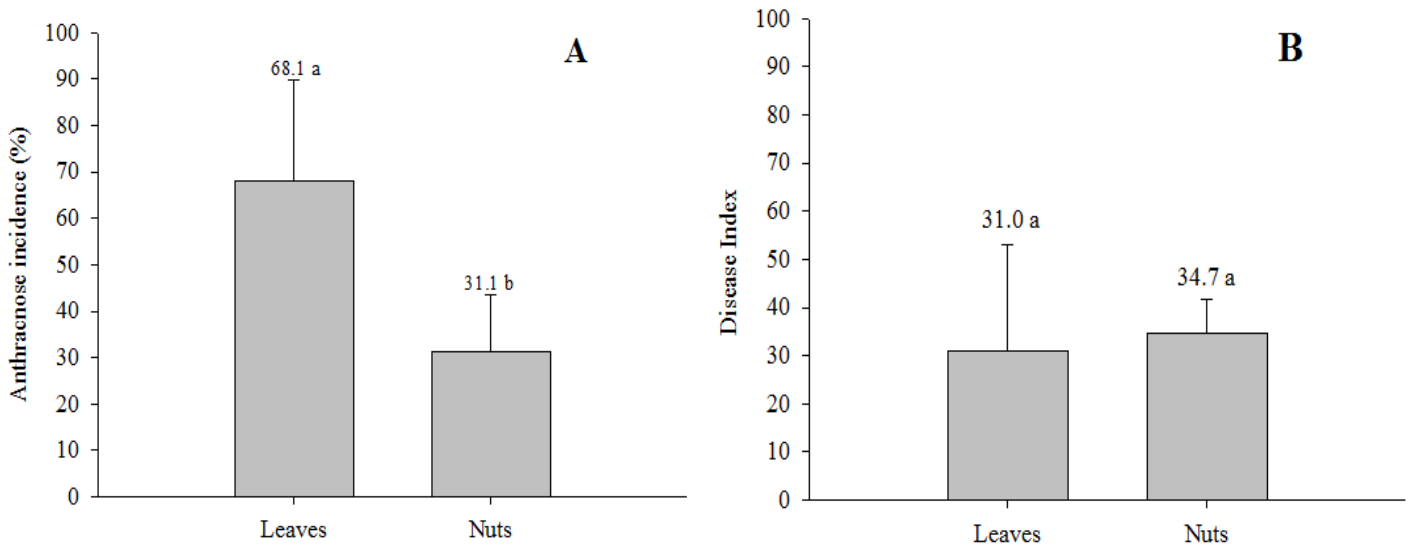

Figure 2. Anthracnose average incidence (A) and disease index (B) of cashew leaves and nuts (*Anacardium occidentale* L.). Percentage of anthracnose incidence data transformed in arcsin √x/100. Means by the same letter are not significantly different (Tukey, P≥0.05).

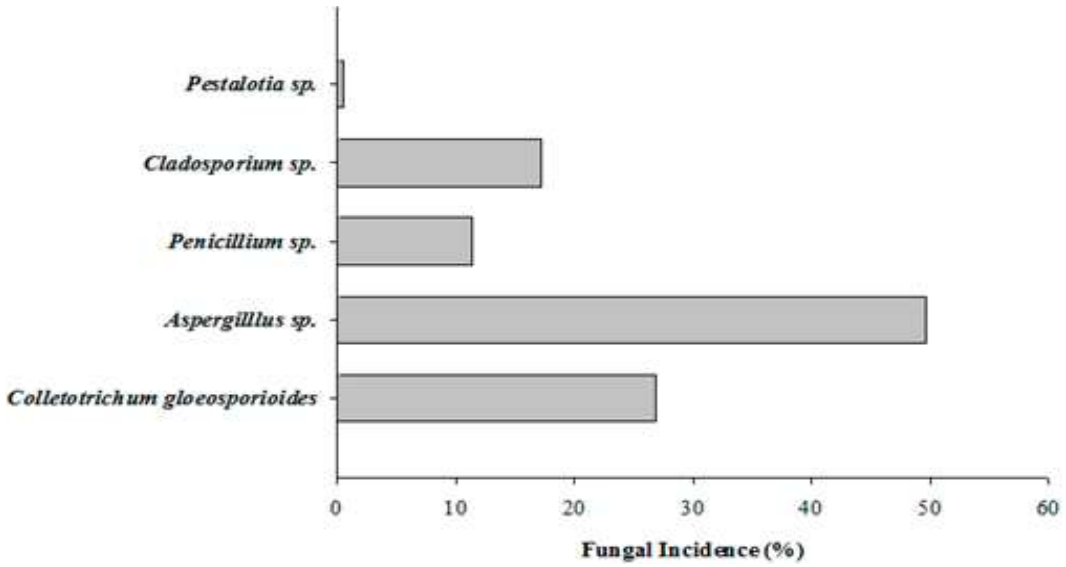

Figure 3. Fungal incidence associated with cashew nuts (*Anacardium ocidentale* L.) assessed by the sanitary test.

substrates, and are resistant to a variety of environmental conditions (Sharma and Kulshrestha, 2015). The high incidence of these fungi makes necessary the use of some control method, since it affects directly the final product quality, particularly cashew nuts intended for human feeding. It must be alert to contaminants fungi, *Aspergillus* sp. and *Penicillium* sp., which always occurs in higher percentages, besides being potentially mycotoxin-producing. Thus, they pose a potential hazard to consumers' health. The presence of some metabolites and traces of aflatoxin in cashew kernels has been demonstrated (Milhome et al., 2014). It is estimated that about 10% of the annual production of cashew nuts is unsuitable kernels for industrial processing and for

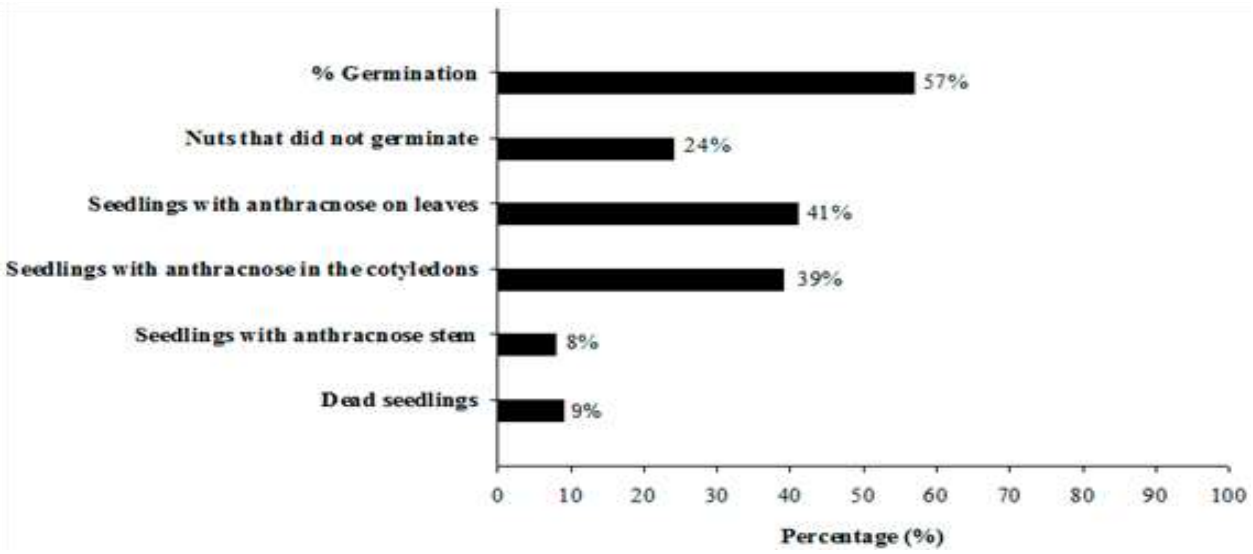

Figure 4. Percentage (%) of cashew seedling (*Anacardium occidentale* L.) with anthracnose symptoms, % dead seedlings and % germination in the transmission assay.

human feeding, due to the fungi presence, mostly (Adeigbe et al., 2015). Despite the well-known antibiotic properties of the cashew nut testa and shell (Schulze-Kaysers et al., 2015), a wide spectrum of fungi can survive saprophytically on cashew seeds. Following seed germination, young tissues may be susceptible to some of these fungi. The attack caused by fungi present in nuts on the cotyledons, causes seedling nutrients loss and healthy tissues destruction, resulting in a not normal seedlings development, with wrinkled and small size leaves and twisted stems.

The fungus *C. gloeosporioides* is commonly reported as causing rot in nuts (Sarkar, 2016), being found in the present study with an incidence of over 50% in the evaluated samples. However, the infection seeds not ensure the pathogen transmission, because in addition to the host, the factors linked to the environment and the pathogen must be considered. The fungus can survive in dead tissue and in the soil and can be spread by rain splash, wind, insects, etc. Nonetheless, when the pathogens find healthy tissue it can cause severe injury in favorable environmental conditions, and greatly decrease of nuts and peduncle production (Dendena and Corsi, 2014).

Cashew nut – seedling transmission

There are several factors limiting the efficiency of pathogen transmission by seeds. Each pathogen has specific requirements of temperature, humidity, oxygen, quantity of nutrients, mechanical injury, seed age and the presence of antagonistic microorganisms on the seed (Rego et al., 2012). In this study, anthracnose transmission of cashew nuts for the seedlings was verified through the percentage of seedlings with disease symptoms (Figure 4). In cotyledons was observed anthracnose incidence on 39% of seedlings and leaves symptoms were observed in 41% of the seedlings. For stem symptoms, there were few plants with anthracnose symptoms (8%). A similar result was found by Lopez and Lucas (2002) under laboratory conditions, they noted that 33% of seedlings emerging from non-treated cashew seeds had necrotic lesions on cotyledons, hypocotyls or epicotyl. *C. gloesporiodides* was detected in red pepper (*Capsicum annuum* L.) seeds and is associated with anthracnose in this crop. This pathogen can be transmitted from the endosperm tissue for hypocotyl and rootlets and is located generally on the surface of seeds, in the endosperm and embryo (Ali et al., 2016). The percentage of germinated nuts, with and without disease symptoms it was low (57%) and the quantity of nuts which do not germinate was 24%, while the number of dead seedlings was 9% (Figure 4). Seeds pathogen carriers may result in failures germination, seedling death or development of low-quality plants, thereby compromising production (Coutinho et al., 2016), as observed in this study.

Effect of anthracnose severity on the cashew nuts in the germination

There was a higher germination rates in nuts with lower

Table 1. Relationship between the infection severity in cashew nuts (*Anacardium occidentale* L.) and transmission of cashew anthracnose by *Colletotrichum gloeosporioides*.

Samples	Severity level[1]	% of emergency[2,3]	% seedlings with anthracnose[2,3]
	0 = (no symptoms)	78.5[a]	4[b]
	1 = (1-5.9% of LA)	62.5[a]	31[ab]
Cashew nuts	2 = (6-25.9% of LA)	79.5[a]	45.4[ab]
	3 = (26-50% of LA)	77[a]	47[ab]
	4 = (> 50% of LA)	57[a]	62.5[a]
CV (%)		16.25	27.04

[1]LA - lesion area. [2]Means by the same letter are not significantly different (Tukey, P≥0.05). [3]Data transformed arcsin√x/100.

lesions levels on the outside surface (Table 1). The nuts germination started at 19 days after sowing; however, it was found that there was a delay in some nuts and uneven germination. Nuts with a high degree of severity (note 3 and 4) gave greater number of plants with leaf spot and anthracnose symptoms. In a humid chamber, the presence of *C. gloeosporioides* in the injuries was found. The transmission in this case may be conditioned to the severity of the seed fungal infection, or be related to the lesion size and the amount present in the seed inoculum. Silva et al., (2013) using selected seeds with different levels of *C. lindemuthianum* incidence observed variation from 70 to 100% in the transmission rate of seeds with incidence of 1 to 5% the pathogen.

Germination, while above 50%, demonstrates the damaging effect of the fungi on the vigor of the nuts and possibly the onset of leaf spots. In nuts with higher levels of severity were observed higher rates of not normal seedlings, beside the highest number of nuts that did not complete germination, and seedlings that died after emerge. These effects may be related to the fact that most affected nuts having lower reserve level to assist the early seedling development, besides the high incidence of fungi that probably causes more damage to seedlings. It was also found that despite of the good external appearance in some nuts, not always indicate the absence of pathogens, since many of them may be in a latent state waiting for adequate moisture conditions for manifesting.

The difficulty of obtaining healthy seedlings, due to the number of pathogens that are associated with cashew nuts, might be one of the causes of low germination and reduced development of seedlings coming from seeds. To Marques et al., (2013) the dwarf cashews seed germination begins on the 10th day after sowing and continues until the 25th day, however normally 80% germination occurs between the 12th and the 20th day after sowing. Different from previously reported, the low vigor and the nuts germination delay observed in this study, may also be attributed to genetic uneven of the nuts, as these come from a heterogeneous population and uneven, which may have contributed to the results.

The results of this study showed that fungal associated to cashew nuts significantly damage the normal development and quality of seedlings, as well as serve as the initial inoculum for future disease outbreaks.

Conclusion

The presence of *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *C. gloeosporioides* were observed in most of the analyzed nuts. The pathogen *C. gloeosporioides* can be transmitted efficiently to cashew seedlings via nuts. Cashew nuts with a high degree of anthracnose severity lead the emergence of a larger number of symptomatic seedlings.

Conflict of Interests

The authors have not declared any conflict of interests.

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