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SHORT COMMUNICATION

Additional species of *Aspergillus* causing bole rot disease in *Agave sisalana*

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ABSTRACT

The production of sisal in Bahia, Brazil, has been declining due to the occurrence of a disease know as bole rot. *Aspergillus niger* was regarded as the only causal agent. In this study *A. brasiliensis* and *A. tubingensis*, in addition to *Aspergillus niger*, identified on the basis of morphological and molecular analyses, were shown to cause bole rot of sisal. Their pathogenicity was confirmed but their significance for the epidemiology of the disease in the field remains unclear.
Key words: β-tubulin, pathogenicity, phylogenetic analysis.

Sisal (*Agave sisalana*) is originated in the Yucatan peninsula, Mexico and was brought to Brazil in the 1900's for fibre production. This crop was introduced into the semi-arid region of the Brazilian Northeast, where it is grown in approximately 263,472 ha (IBGE, 2012). Bahia State is the main producer, contributing with 94% of the national production, generating 94,000,000 USD per year of revenue. Sisal is a crop that depends mainly on familiar labour and employs 700,000 workers in one of the poorest regions of the country (Silva et al., 2008). Since the last decade, sisal production has been declining due to a disease known as bole rot. Infected plants exhibit wilting and yellowing of leaves and reddening of the stem or bole and base of the leaves followed by death (Sá, 2009). The fungus *Aspergillus niger* was identified by morphological features as the agent of the disease (Coutinho et al., 2006). Several species of *Aspergillus* are morphologically indistinguishable, especially the ones in section *Nigri* (the black Aspergilli) and the *Aspergillus niger* complex (Giraud et al., 2007). The following species belong to the *A. niger* complex: *A. niger, A. tubingensis, A. luchuensis, A. brasiliensis, A. costaricaensis, A. lacticoffeatus, A. piperis, A. vadensis*, *A. eucalypticola*, *A. welwitschiae*, and *A. neoniger* (Perrone, 2007; Varga et al., 2011; Hong et al., 2013). The objective of this study was to isolate, identify and test the pathogenicity of Aspergilli isolated from sisal plants and soil.

Aspergillus isolates were obtained from soil and from diseased plants in the sisal-growing region of Bahia. Soil dilutions or surface-sterilized fragments from sisal stems were plated on potato dextrose agar (PDA) with 6% NaCl, that is semi-selective to *Aspergillus* (Berjak, 1984). The DNA of the 23 isolates used in this study was isolated by the protocol described by Doyle & Doyle (1990). Random Amplified Polymorphic DNA (RAPD) analysis was done with primers A1, A6 and OPA4 according to Abed (2008). The program FreeTree (Hampl et al., 2001) was used to construct the dendrogram using the distances of Jaccard and the UPGMA method. Seven isolates of different groups were selected from the RAPD analysis for molecular identification by sequencing a fragment of 520 pb of the β-tubulin gene. Primers Bt2a and Bt2b (Glass & Donaldson, 1995) were used for amplification and sequencing, which were done according to standard protocols using an ABI 310 sequencer. Sequences were deposited in public databases and the accession numbers are given in Figure 1. Alignment of the obtained sequences and reference sequences from public databases was performed using the program MAFFT version 6.0 (Katoh et al., 2002). The phylogenetic tree was constructed with the maximum likelihood method implemented in the program MEGA version 5.0, with the Kimura-2 parameter nucleotide substitution model and bootstrap analysis with 1000 resamplings. Pathogenicity tests were done by inoculating each of the seven isolates shown underlined in the phylogenetic tree (Figure 1B) in five 4-month old sisal plantlets grown in polyethylene bags filled with 1 kg of soil under greenhouse conditions. Spore suspensions were prepared by growing the isolates on PDA for 10 days at 25ºC and harvesting the spores with a Drigalsky rod. The concentration of the suspensions was adjusted to 10^7 conidia/ml in a haemocytometer. The stem of the plantlets was wounded with two 1-mm diameter needles

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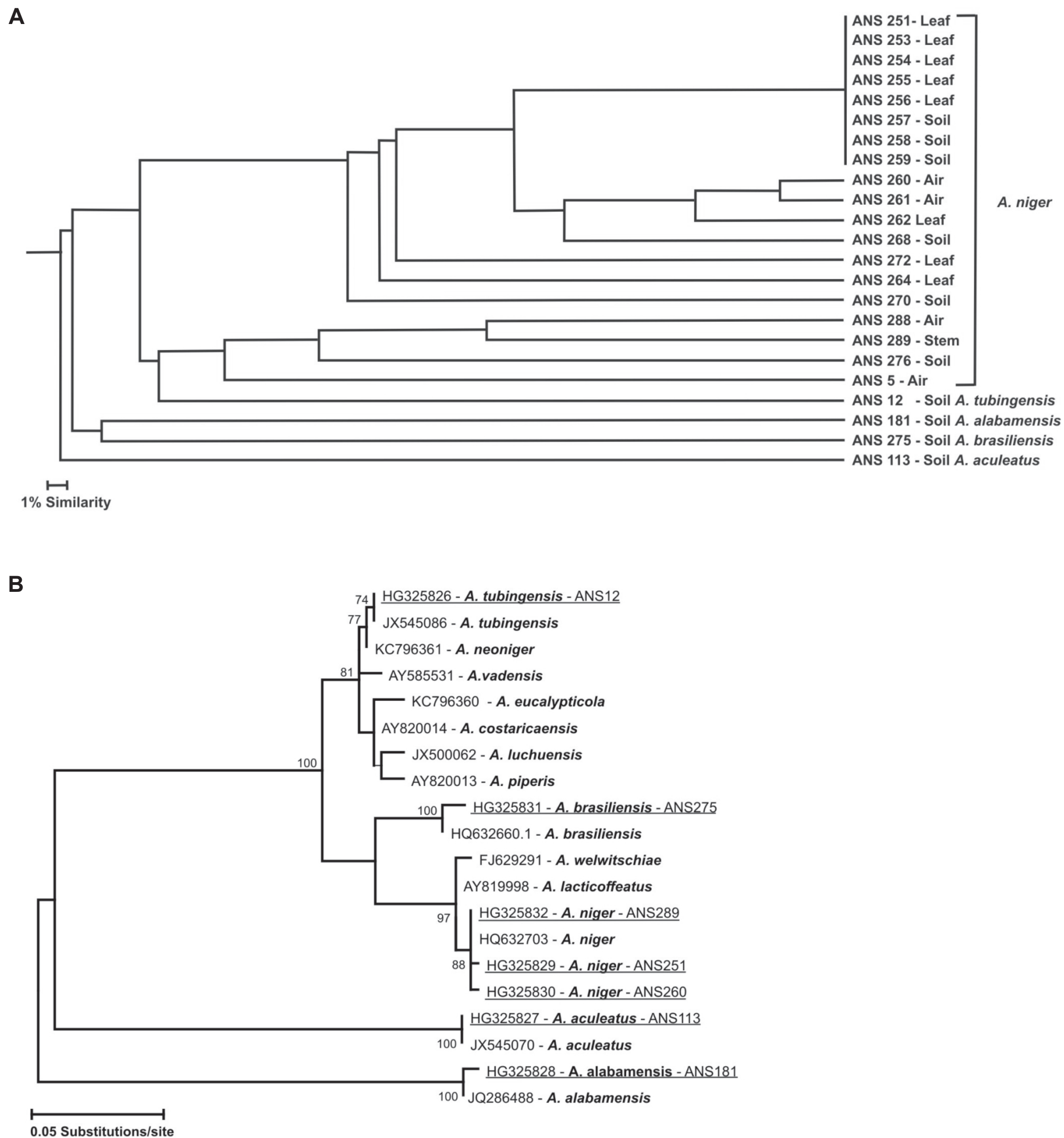

FIGURE 1 - *Aspergillus* isolates obtained from the sisal growing region of Bahia state. **A**. Dendrogram generated with the UPGMA method with the distances calculated with Jaccard's coefficient. Data from three RAPD primers and 23 isolates of *Aspergillus*; **B**. Maximum likelihood phylogenetic tree of selected isolates of *Aspergillus* inferred with 500-bp aligned sequences of the β-tubulin gene. Acession numbers are shown followed by species name. Sequences underlined were obtained from isolates used in this study. Sequences without an underline mark were obtained from public databases for comparison purposes. Bootstrap values higher than 70% are shown on the appropriate nodes.

fixed to a wooden base. The needles were 2 cm apart and produced 2 cm deep wounds. Each plantlet was inoculated with 100 µl of each spore suspension. Controls were treated with sterile distilled water. The experiment was installed in a randomized design with five replicates per treatment. Thirty days later, plantlets were removed from the bags and split open to observe the incidence of the disease. This whole experiment was repeated at least three times for each isolate. When symptoms were present, fragments of the diseased plantlet were surface-sterilized and plated on PDA to confirm the identity of the isolates. Furthermore, DNA of the isolates recovered was re-sequenced to compare with the sequences obtained for the isolates originally inoculated.

From the 23 isolates obtained in association with sisal plants, one was from a diseased stem, eight from leaves, 10 from soil, and four from the air. RAPD analysis showed that

the isolates were grouped into 16 different genetic groups with 100% similarity (Figure 1A). From these 16 groups, only *Aspergillus alabamensis* (ANS 181) and *A. aculeatus* (ANS 113) could be distinguished from the others in terms of morphology. Sequence analysis showed the following species in association with sisal: *A. niger*, *A. aculeatus*, *A. alabamensis*, *A. tubingensis* and *A. brasiliensis* (Figure 1B). The identity of the sequences varied from 99 to 100% when they were compared with other sequences from the databases. Pathogenicity tests showed that only *A. niger*, *A. tubingensis* and *A. brasiliensis* were pathogenic to sisal (Figure 2). The incidence of the disease was 100% for *A. niger*, 60% for *A. tubingensis* and 80% for *A. brasilensis*. The re-isolations and sequencing of the Aspergilli from lesions of diseased plants confirmed the pathogenicity of *A. niger*, *A. tubingensis* and *A. brasiliensis* as agents of sisal bole rot disease.

Little scientific information is available on sisal in general and specifically on the diseases that affect this plant. Only two earlier reports, Coutinho et al. (2006) and Soares et al. (2006) show that *A. niger* is the agent of sisal bole rot disease. We show here for the first time that besides *A. niger*, *A. tubingensis* and *A. brasiliensis* are able to cause the disease in sisal. Although species of *Aspergillus* are known as saprophytes or opportunistic pathogens in peanuts, onions and *Welwitschia mirabilis* (Moraes et al., 1997; Nunes et al., 1997; Hong et al., 2013), they cause this devastating disease in sisal, with incidence in the field varying from 5% to 65% (Coutinho et al., 2006; Abreu et al., 2007). The species *A. tubingensis* and *A. brasiliensis* were isolated from soil and the real significance of these isolates in the epidemiology of the disease remains to be determined. *A. alabamensis* was found in great numbers in soil and was not able to cause disease in sisal. Our observations indicate an inverse relationship in densities of *A. niger* and *A. alabamensis*, suggesting that the latter could act as a natural antagonist. Unfortunately, there is one study showing that *A. alabamensis* may colonize the

FIGURE 2 - Pathogenicity of *Aspergillus* to sisal. **A**. Sisal plantlet with advanced symptoms of the disease; **B**. Plantlet inoculated with *Aspergillus niger* on the left and control at the right; **C**. Section of the stem of a diseased plantlet showing the red color characteristic of the disease; **D**. Section of the stem of an uninoculated plantlet.

lungs of patients with cystic fibrosis (Balajee et al., 2009). This report adds two new potential agents of sisal bole rot disease in the sisal-growing region of Bahia state, Brazil.

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