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Molecular identification of fungal species associated with leaf lesions of marama bean seedlings in Namibia

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During one of our greenhouse experiments on the marama bean plant (*Tylosema esculentum*) at the University of Namibia, various signs of necrosis and spotting were observed on leaf tissues of the newly developed seedlings. In this study, we hereby describe findings of the investigations undertaken to attempt and determine the possible causative agent(s) of such plant leaf infections. In brief, infected seedling leaves were first surface-sterilized and then used in form of leaf-discs (2 x 2 mm), to inoculate potato dextrose agar (PDA). Alongside this, control leaves were also similarly treated and overlaid onto PDA. While fungal growths were observed on all infected leaf-discs, no growth was observed on control discs. For each of the various fungal strains growing on the infected leaf discs, pure cultures were obtained by repeatedly sub-culturing the strains onto PDA. Subsequently, single spore cultures were also aseptically isolated from each of the obtained pure cultures and further developed into mycelia through inoculation and incubation in potato dextrose broth (PDB). All in all, a total of eight single spore cultures were obtained from the overall inoculations and sub-culturing. Subsequently, the total genomic DNAs of each of the obtained single spore cultures were isolated followed by amplification of their internal transcribed spacer (ITS) regions. The amplified ITS regions were then sequenced followed by a comparison of their nucleotide patterns with the GeneBank. Such a comparison then revealed the presence of a complex of several fungal isolates with eight commonly known species: *Penicillium brevicompactum, Epicoccum sorghi, Rhizopus stolonifer, Alternari solani, Fusarium equiseti, Penicillium olsonii, Fusarium chlamydosporum* and *Fusarium incarnatum*. These fungal species are commonly known to cause various diseases and infections in legumes and other agronomically important crops. Ideally, the presence of these fungal species in marama bean and their involvement in leaf tissue decay should be noted with great concern and interest since this plant has recently been proposed as a potential leguminous crop for possible adoption and utilization in domestication efforts.

Key words: Marama bean, *Tylosema esculentum*, fungi, Namibia, leaf infection, internal transcribed spacer (ITS).

INTRODUCTION

Marama bean is indigenous to the arid and semi-arid parts of Southern Africa. It is a staple food for the Khoisan and Bantu people in these areas. According to Jackson et al. (2010) and Chimwamurombe (2011), marama (morama) bean is as an excellent source of good quality protein (29-39%) and oil (24-48%) which is rich in cholesterol-free mono- and di-unsaturated fatty acids. Marama has further been found to be a good source

of micronutrients such as calcium, iron, zinc, phosphate, magnesium and B vitamins including folate. In addition, it is also reported to be a potential source of phytonutrients including phenolic compounds, trypsin inhibitors, phytates and oligosaccharides. These phytonutrients have been shown to contribute towards health and in particular, the prevention of non-communicable diseases such as cardiovascular ailments, diabetes, and some cancers. These attractive attributes of marama are the main factors driving for its possible adoption as a main stream domesticated crop in Namibia and Botswana. The bean has a very wide array of nutritional and health values with further potentials as a future cash crop for local communities.

It is against the backdrop of the agronomic potentials and values given above that vigilance on surveys for plant diseases caused by microbes such as fungi becomes very crucial and important. Fungi grow in nearly every ecological niche commonly as free living saprophytes, however, a few are on occasion found as accidental, often opportunistic, pathogens (Madigan and Martinko, 2006). Harmful effects of saprophytic fungi on human economy are often seen when food, timber and textiles are rotted. As parasites, fungi cause diseases in plants and animals (Webster and Weber, 2007). Great plant losses caused by natural factors can be suffered in natural ecosystems but by bringing crops together into fields, agriculture creates the ideal conditions necessary for the spread and perpetuation of plant diseases. Furthermore, the more selective farming is and the closer crops come to being true monocultures, the greater is the extent of agricultural losses due to any single agent like specific plant diseases (Moore et al., 2011).

Microbial diseases of plants of which 70-80% are fungal, cause 16% of annual crop losses worldwide (Oerke, 2006). In 2011, one in every eight crop plants on average, failed to yield because of fungal diseases (Moore et al., 2011). Many species of the genus *Cercospora* (Ascomycota) cause plant diseases, mostly of the leaf spot variety. Leaf spots are rounded blemishes occurring on the leaves of infected plants; a typical spot has a defined edge with a darker boarder central zone varying from yellow to brown (Khan et al., 2008). A disease in plants results only if the following three factors occur simultaneously: a susceptible host, a disease-causing organism (the pathogen) and a favorable environment for the disease; otherwise if one or more of these factors is not present, then the disease does not occur. The symptoms observed in a diseased plant depend on the effect of the pathogen on the physiological and biochemical processes of the plant. Photosynthesis is an essential function of plants and any pathogen that interferes or tampers around with it, will cause a disease that may appear as chlorosis and/or necrosis of the leaves and stems (Moore et al., 2011).

In order to determine the various and specific infections of plants by fungi, the routine usage of pure macro-molecular elements like DNA, RNA and protein isolated from single strain fungal cultures has become of huge and paramount relevance to the broader field of plant pathology. DNA sequencing of the internal transcribed spacer (ITS) and the large subunit (LSU) regions of rRNA, followed by comparative sequence analysis, has been the “gold standard” for molecular identification of most pathogenic fungi, and particularly culturable fungi. This strategy is fast and accurate, but is strongly dependent on sequence quality in existing reference databases (Tsui et al., 2011). In this study and during a greenhouse experiment at the University of Namibia, some signs of necrosis and spotting were observed on leaf tissues of marama seedlings and the leaves were immediately removed from the plants in order to investigate and determine the exact causative agent(s) of such a leaf decay effect. Herein, we outline and present the findings of such a typical investigation.

MATERIALS AND METHODS

Plant growth and development

A group of marama plants indigenous to Namibia were grown and developed at the University of Namibia under greenhouse conditions on the 26th of January, 2011. Seeds collected from three different locations: Aminius (AMI), Uitschott (UI) and Okomombonde (OKO) were first weighed and from each of the 3 locations, fifty (50) seeds were then randomly picked and their weights, radii and circumferences measured before being exposed to the pre-sowing treatment. Alongside these 150 experimental seeds, other 10 additional infection-free seeds (laboratory determined) were also included in the experiment to serve as controls.

In brief, all seeds were first placed into individual Petri dishes flooded with gibberellic acid-treated water (~0.1 µM) and then incubated at 4°C for 24 h in order to induce germination. These stratified seeds were then sown into sterilized potting soil (60% v/v peat-based soil and 40% v/v vermiculite) till germination. The generated seedlings were then maintained and routinely natured under controlled greenhouse conditions (average day/night temperatures: 23/16°C; range of day time irradiances: 150-650 $\mu mol \cdot s^{-1} \cdot m^{-2}$; average day/night humidities: 30/70%; range of daily CO_2 concentrations: 380-390 ppm) till some disease symptoms were visually observed on their leaf tissues on the 24th of May, 2011. Apparently, out of the total sown 150 experimental seeds, only 67 germinated (46.7%) while on the other hand, all control seeds did germinate (100%). Eventually, the routine naturing and monitoring process of all plants was finally terminated on the 8th of June, 2011 as most of them had, at this stage, died off.

Fungal isolations

Leaf tissues from 30 infected and 5 non-infected seedlings (2-3

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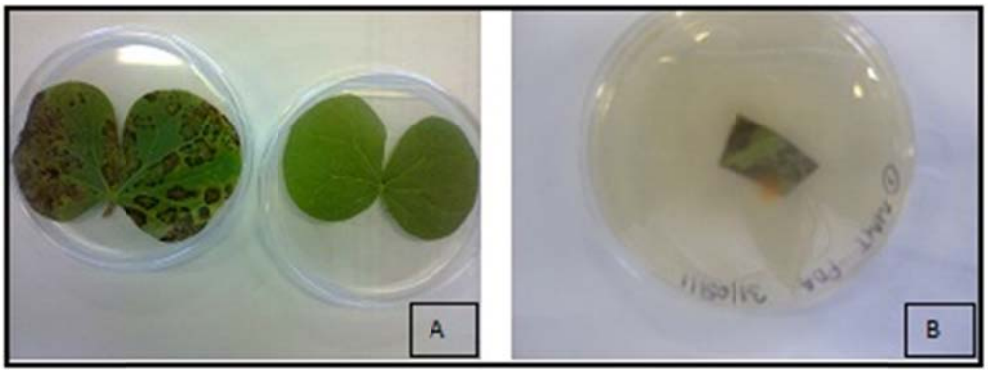

Figure 1. Seedling leaves used for the study of possible fungal co-infection in marama bean. (A) Petri dishes containing infected (left) and non-infected (right) marama seedling leaves. (B) A PDA Petri dish inoculated with a piece of surface sterilised infected marama leaf disc.

leaves per plant) were randomly collected and washed individually in running tap water before being moved to a laminar flow hood, where disc sections measuring approximately 2 x 2 mm were cut with a sterile scalpel blade. The disc sections were then surface-sterilized by dipping them into 0.5% sodium hypochlorite for 2 min, 70% ethanol for 2 min and rinsing in sterile distilled water followed by drying on a sterile filter paper. Concurrently, potato dextrose agar (PDA) was also prepared and subsequently sterilized by autoclaving at 121°C for 15 min. After sterilization, the media was allowed to cool down for 5 min at room temperature before being poured into sterile plastic Petri dishes and allowing for a further cooling to solidify the agar. The cut leaf sections were then individually placed into the PDA containing Petri dishes as shown in Figure 1.

The inoculated plates were subsequently incubated at room temperature (25°C) under white fluorescent light for 5 days. Isolation of pure cultures was then achieved by sub-culturing from hyphal edges and repeatedly inoculating onto fresh PDA media at 25°C up until the desired pure cultures were obtained. Control leaves (showing no symptoms) were also similarly treated but no fungal cultures were observed to grow on the inoculated media. From each of the isolated pure cultures, some single spore cultures were prepared by aseptically scraping the mycelium edges with a sterile needle under a microscope and then dissolving the scrapped edges into individual drops of sterile distilled water on clean microscope slides. The slides were left to air-dry and then aseptically viewed under a microscope at 100x objective. Magnification was then lowered to 60x in order to isolate single spores from each of the scraped and prepared mycelium edges with a sterile needle. The isolated spores were then aseptically placed onto sterile PDA media to grow as single spore cultures at 25°C. From each of the prepared single spore cultures, some 4 x 4 mm segments of the morphologically distinct mycelia were aseptically cut and sub-cultured into sterile conical flasks containing potato dextrose broth (PDB) at 25°C for 14 days or up until their mycelia had fully grown. The resulting mycelia were then harvested and filtered under gravity through Whatman paper in Buchner funnels. The filtered mycelia were then collected in Whatman paper and air-dried for two days in a HEPA filtration cabinet. The collected mycelia were once more dried at 60°C in an oven for 24 h and subsequently ground into a fine powder with a pestle and mortar for further downstream applications.

DNA isolation

Total genomic DNA was extracted from each of the prepared fungal mycelial powder using the Zymo Research, ZR Fungal DNA Mini prep kit and according to the manufactures instructions. The extracted DNA was then resolved onto a 1% agarose gel stained with 1 µg/mL ethidium bromide in tris-borate-EDTA (TBE) buffer and viewed under UV illumination to check the quality and concentration of the extracted product for subsequent PCR application.

Polymerase chain reaction

Polymerase chain reaction (PCR) amplifications were performed using a Thermo Scientific DreamTaq Green PCR Master Mix Kit in total reaction volumes of 25 µL using the following reconstitutions: 1X DreamTaq Green PCR Master mix, 400 nM forward primer (ITS 1 or 4), 400 nM reverse primer (ITS 4 or 5) and 1.0 ng DNA template. The ITS regions of the obtained fungal isolates were then amplified using the following primer sets: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′). The PCR profile on the thermo cycler was: Pre-denaturation (1 cycle): 95°C for 4 min; 35 cycles of denaturation: 95°C for 30 s, annealing: 55°C for 1 min, and extension: 72°C for 2 min; a final extension (1 cycle): 72°C for 5 min and samples were then held at 4°C. The PCR products were viewed on a 2% agarose gel in TBE buffer stained with 1 µg/mL ethidium bromide under UV illumination to check the sizes of the amplified products. The yielded amplicons were then cleaned up and concentrated for subsequent sequencing using a Zymo Research, DNA Clean & Concentrator-5. Sequencing was then carried out at Inqaba Biotechnology in Pretoria, South Africa.

Sequence analysis

The nucleotide sequences obtained by Inqaba Biotechnology were manually edited by eye from their determined chromatograms. In order to obtain the regions of overlaps, the forward and reverse sequences were aligned using complements of their sequences. All overlapping sequences were then trimmed and the resulting consensus sequences created in the Bio Edit Sequence Alignment Editor for each of the obtained fungal isolate. The consensus sequences were then used to create BLAST searches at the NCBI database using CLUSTAL X to align with organisms in the database and seeking the most likely species identities of the obtained fungal isolates.

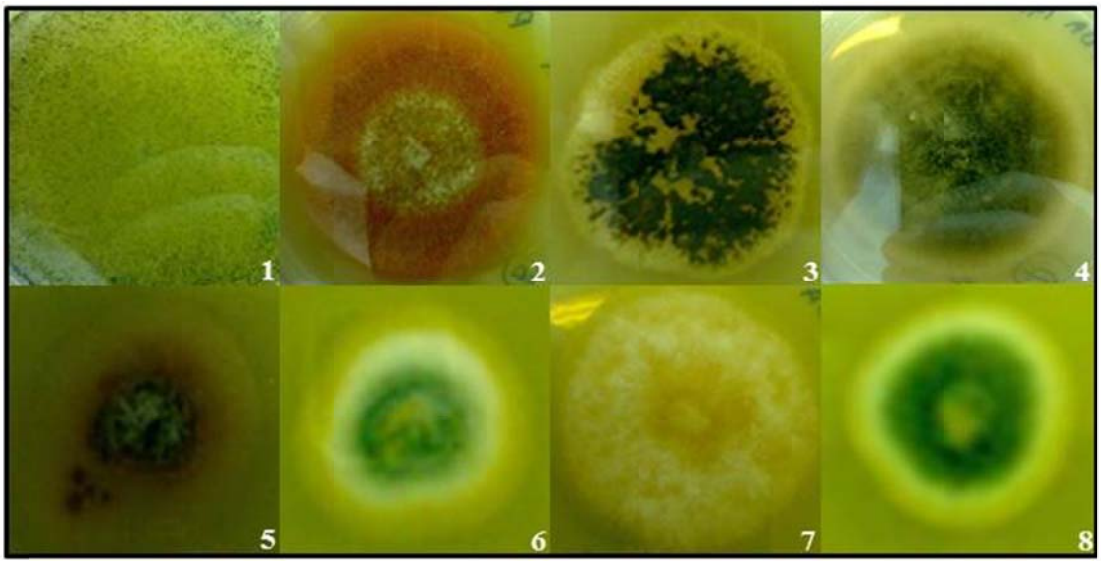

Figure 2. Isolation of pure fungal cultures from the infected marama leaf tissues. Desired fungal cultures were obtained by isolating hyphal edges from the infected leaf tissues followed by repeated sub-culturing onto new and fresh PDA. The numbers 1-8 represent the 8 morphologically distinct cultures that were successfully isolated in this study using this approach.

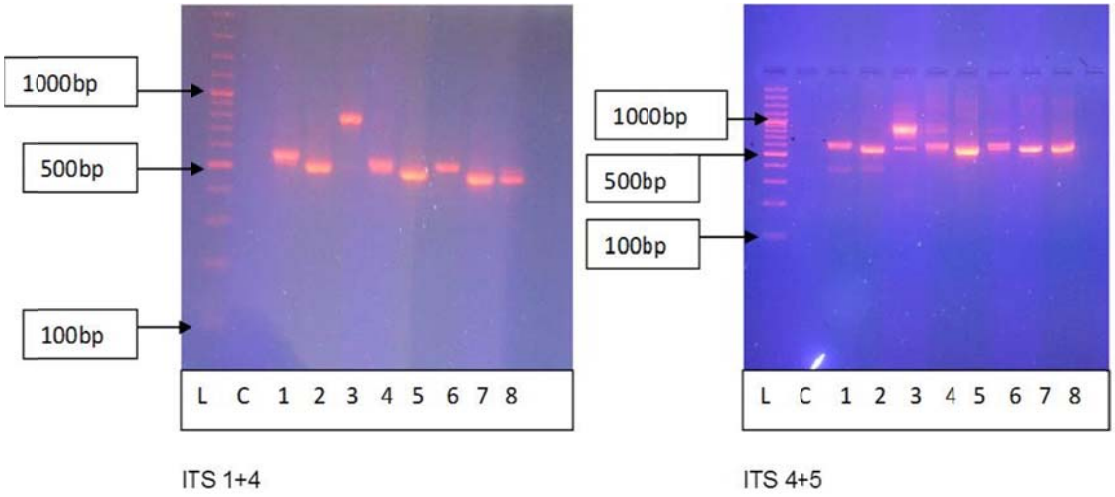

Figure 3. Electrophoresis gels of PCR amplification products of the ITS regions of fungal isolates found in marama leaf tissues. The left plane contains PCR fragments amplified with the ITS primer set 1 and 4, while the right plane contains PCR fragments amplified with the ITS primer set 4 and 5. In both planes, L represents the 1 kb DNA ladder, C represents the control sample while numbers 1-8 represent the 8 morphologically distinct fungal isolates that were cultured, isolated and assessed in this study.

RESULTS

Isolation of pure fungal cultures

From all the infected leaf tissues, a total of 8 morphologically distinct fungal isolates were isolated and cultured as shown in Figure 2. Conceivably, a moderately wide range of diverse fungi were found to be growing on the marama leaf tissue.

Polymerase chain reaction

From the PCR work undertaken, fungal ITS regions were successfully amplified for primer set ITS 1+4 (500-800 bp product) and primer set ITS 4+5 (750-830 bp product) and their associated nucleotide sequence data successfully obtained from Inqaba Biotechnology, Pretoria, South Africa. The electrophoretic results of the amplified ITS regions are shown in Figure 3 while the associated

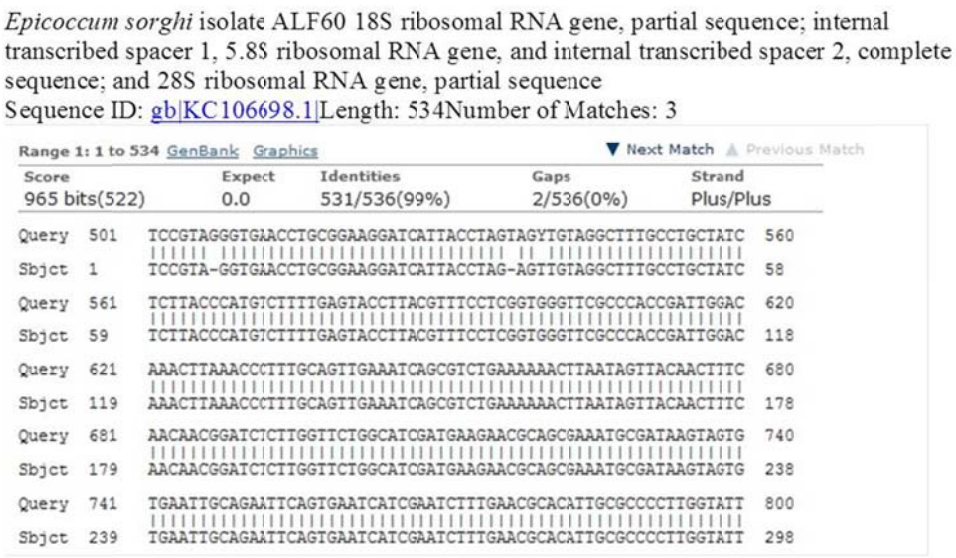

Figure 4. Sequence identity of the fungal isolates obtained from the infected marama leaf tissues. This is an identity representation of isolate 2 and as is determined by the BLAST search tool in the NCBI database. The identified isolate has a 531/536 similarity level to *Epicoccum sorghi*.

illustrative sequence information data for the amplified ITS regions is presented in Figure 4.

Illustrative sequence alignment data

Figure 4 illustrates the molecular identification of fungal isolates isolated from the infected marama leaf tissues using the BLAST search tool in the NCBI database. Sequences presented in the figure are for the fungal isolate 2.

The summarized results for the molecular identification of all the fungal isolates studied in this work are shown in Table 1. Identification was done using the BLAST search tool in the NCBI database and sequence analysis by the CLUSTAL X tool.

DISCUSSION

To the best of our knowledge, this is the first report on identifying fungal species that can affect marama leaf tissue and probably having a role as disease-causing pathogens and/or plant growth promoting endophytes. In this case, a moderately wide range of fungi (eight isolates) were isolated (Figure 2) and identified (Table 1) and therefore, the exact and specific disease-causing pathogen in this plant and at this point, could not be easily and clearly spelt out. Hence some further investigations using approaches like the Koch's postulate or other methods would need to be undertaken in order to exactly determine which of these fungal isolates is and/or are really pathogenic to the marama plant. Indeed, there is surely a possibility that one or more of these 8 isolates is and/or are the actual causal agent(s) of diseases in the marama plant while the other candidates could just be opportunistic or mutualistic partners that simply take advantage of the already established leaf infection.

Vega et al. (2006) in their previous study, found *Penicillium brevicompactum* to be present as an endophyte in coffee leaves collected in Colombia, Hawaii and at a local plant nursery in Maryland. Based on this premise, it is also highly possible that the *Penicillium* species isolated here could also have been present in the marama leaves as an endophyte. *Epicoccum* sp. and *Fusarium* sp. are the causal agents of sooty mold and leaf blight respectively in broad bean (French, 2006). The presence of *Epicoccum sorghi*, *Fusarium equiseti*, *Fusarium chlamydosporum* and *Fusarium incarnatum* in the infected marama plant could primarily have contributed to the necrosis and leaf wilting observed in the growing seedlings. *Alternaria solani* is the causal agent of early blight disease in tomato and is responsible for significant economic losses sustained by tomato producers each year (Spletzer and Enyedi, 1999). It is also the causal agent of early blight disease of not only commercially-produced tomatoes but also potatoes and eggplants. Early blight may affect the foliage, stems and fruits of infected plants (Jones et al., 1991). Therefore, the presence of *Alternaria solani* among the fungal species isolated from the infected marama leaves is thus not surprising as foliage was affected at the seedling stage similar to what happens in tomato, potato and eggplant seedlings. *Penicillium olsonii* is among the

Table 1. Concerted identities of the various fungi isolated from the infected marama leaf tissues. As is determined by the BLAST search and CLUSTAL X tools, a total of 8 distinctive fungal isolates were successfully matched and positively identified.

Test isolate	Plant part	Accession no.	Closest species[a]	Similarity (%, x/y)	Reference
1	Leaf	AM948959.1	*Penicillium brevicompactum*	94 % (506/540)	Kasana and Salwan (2008, unpublished)
2	Leaf	KC106698.1	*Epicoccum sorghi*	99% (531/536)	Goncalves et al. (2012, unpublished)
3	Leaf	JQ606829.1	*Rhizopus stolonifer*	100% (129/129)	Wicklow (2012, unpublished)
4	Leaf	JF491193.1	*Alternaria solani*	96% (504/526)	Chowdappa et al. (2011, unpublished)
5	Leaf	JQ690085.1	*Fusarium equiseti*	98% (543/553)	Zhao (2012, unpublished)
6	Leaf	AY373925.1	*Penicillium olsonii*	98% (573/583)	Haugland et al. (2004)
7	Leaf	EU520242.1	*Fusarium chlamydosporum*	98% (543/552)	Yu (2008, unpublished)
8	Leaf	FN597588.1	*Fusarium incarnatum*	99% (519/522)	Santori et al. (2009, unpublished)

[a]Closest species was determined by comparing the ITS gene sequence of the test isolate with sequences in the NCBI database and then establishing the exact level of similarity. (x/y) = (isolate sequence size/NCBI sequence size).

rarest clearly distinct species of the genus *Penicillium* which are often associated with post-harvest diseases or are pathogenic to intact growing plants (Wagner et al., 2000) while *Rhizopus stolonifer* is found as a pathogen to vegetables, fruits and ornamentals (Kwon et al., 2001). Thus, these two can also possibly be potential pathogens to the vulnerable marama plant.

Overall, there were 2 *Penicillium* species and 3 *Fusarium* species identified in this study, as well as a single isolate each of the genera *Epicoccum*, *Alternari* and *Rhizopus*. These fungi are commonly known to be involved in plant diseases of some common legumes and other agronomically important crop plants. For instance, the genera *Penicillium*, *Aspergillus* and *Phoma* are the most common fungal species frequently isolated from the black and coloured classes of the bean (*Phaseolus vulgaris* L.), followed by the *Rhyzopus*, *Alternaria*, *Helminthosporium*, *Cladosporium*, *Botrytis*, *Fusarium*, *Trichoderma*, *Curvularia* and *Dreschelera* species (Freitas-Costa and Scussel, 2002). These two particular classes of the bean (black and coloured) are the ones commonly cultivated and consumed in the different regions of the State of Santa Catarina, south region of Brazil (Freitas-Costa and Scussel, 2002). In addition and in a study that involved the analysis of mycoflora and mycotoxins in dry beans, Tseng et al. (1995) identified several fungal species, which among them, included *Phaseolus*, *Fusarium, Rhizoctonia, Penicillium, Rhizopus, Sclerotinia, Gliocladium* and *Mucor*. Apparently, the most frequent species to be identified in that study was the *Alternaria* (Tseng et al., 1995). Conceivably, the presence and involvement of these fungal species in marama leaf decay should be noted with great concern and interest as marama bean has since been identified and earmarked as a potential leguminous crop for possible domestication. The fungal species identified here can probably be potential pathogens that could adversely affect yields in marama bean when it is adopted and utilized for both domestication and farming efforts.

Conclusion

A relatively wide community of the fungal organisms associated with the leaf decay symptoms of young marama bean seedlings was isolated and identified using molecular techniques whereby the ITS DNA region of the isolated fungi were amplified and sequenced followed by a comparison of the obtained sequences with the GenBank. From this attempt, the presence of a complex of various fungal strains with 8 commonly known species was revealed: *P. brevicompactum, E. sorghi, R. stolonifer, A. solani, F. equiseti, P. olsonii, F. chlamydosporum* and *F. incarnatum*. These fungi could have had some endophytic and/or pathogenic roles in the infection process of the affected plant of which whatever role(s) each species may or might be playing in this process still needs to be properly established through further investigations.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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