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Distribution and occurrence of indigenous strains of atoxigenic and toxigenic *Aspergillus* section *Flavi* in groundnut producing areas of Southern Tanzania

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Abstract

The objective of this study was to isolate and identify atoxigenic and toxigenic strains of *Aspergillus* section *Flavi* in southern Tanzania, and investigate possible application of atoxigenic strains in control of aflatoxin levels in groundnuts. Fungal communities in soils from groundnut fields were examined to see the distributions of aflatoxin-producing *Aspergillus* species and to spot endemic atoxigenic strains. Forty-five isolates belonging to *Aspergillus* section Flavi were collected randomly from soils of groundnut fields in three districts and characterized using morphological and physiological examination. *Aspergillus* section *Flavi* was detected in 40/45 (88.89%) of the soil samples collected in Mtwara, Tanzania. Members of *Aspergillus* section *Flavi* L-strain was the most common (79.5%), followed by S-strains (18.4%) and finally *Aspergillus tamarii* (1.8%). The mean colony forming unit (CFU) of the *Aspergillus* colonies per gram of soil was highly variable ($p < 0.05$) among the districts, ranging from 8.5×10^2 to 8.2×10^3. The mean pH across the gathering sites additionally varied (pH 5.5-6.8) which is within the optimal pH requirement for the members of *Aspergillus* section *Flavi*. Non-significant ($p > 0.05$) variation in temperature across the sampling sites was observed. The results also showed that *Aspergillus* flavus was detected in all the three districts. Atoxigenic strains have a potential value to be employed as biological control agents to mitigate aflatoxin in groundnuts.

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Introduction

The soil serves as a reservoir for many microbial communities of plants and herbs which can be producing, carbon dioxide and nitrogen cycle (Fontaine *et al.*, 2003). The microorganisms play major role in soil ecosystem. Soil is associate in nursing oligotrophic medium for the expansion of fungi as a result of the plant life growth (Parkinson *et al.*, 1989). *Aspergillus* and its teleomorphs have been investigated with polyphasic methods to examine variability among species. Currently, according to the polyphasic taxonomy, (Samson *et al.,* 2014); (Yilmaz *et al.*, 2014) proposed that the genus *Aspergillus* is classified into four subgenera (*Aspergillus*, *Circumdati*, *Fumigati* and *Nidulantes*) and 20 sections and each includes a number of related species.

According to (Sugui *et al.*, 2014) *Aspergillus* section *Fumigati* is one of the most species-rich sections in the genus *Aspergillus* and includes species with overall significance for medicine, pharmacology, biotechnology, food and soil mycology. At present, the section consists of 51 taxa: 21 strictly anamorphic *Aspergillus* species and 30 *Neosartorya* species (Samson *et al.*, 2007). The most known economically important species are *Aspergillus flavus* and *Aspergillus parasiticus*, which are saprophytic (living on dead or decaying material) during most of their life-cycle (Klich, 1993). They are also plant pathogens and are found on a wide variety of crops produced in Africa including cereals, legumes, oilseeds, roots and tubers, spices, and tree nuts (Logrieco *et al.*, 2003). Furthermore, *Aspergillus flavus* and *Aspergillus parasiticus* are very useful in biological control of aflatoxin in legumes and cereals (Reddy *et al.*, 2009).

In United Republic of Tanzania, groundnut is among the foremost necessary crop for rural household's farmers, providing each food and financial gain for households (Katundu *et al.*, 2014). Groundnut is extremely alimentary with variety of useful ingredients as fats, protein, carbohydrates, vitamins and minerals all of which are important in human and livestock feed (Sibuga *et al.*, 1992).

Due to the multiple uses of groundnut crop, this makes it an important food and cash crop for domestic consumption and export in many developing and developed countries (Kassie *et al.*, 2011). The consumption of groundnut and its products has received special attention in recent years due to food safety and food quality issues (Bourn & Prescott, 2002). The potential hazards associated with food include naturally occurring fungi which periodically cause severe contamination in invaded crops (Pitt, 2000). Aflatoxins are toxic metabolites produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*.

Aflatoxin B1 (AFB1) is a potent carcinogen, teratogen and mutagen (Ahsan *et al.*, 2010). Aflatoxin contamination reduces utilization options for the affected produce through complete rejection or reduced market value (Kumar *et al.*, 2017). According to (Dorner, 2009) biological control of aflatoxin production in crops in the United states of America (USA) has been approved by the Environmental Protection Agency (EPA) and two commercial products based on atoxigenic *Aspergillus flavus* strains are being used (Alfa-guard® and AF36®), for the prevention of aflatoxin in groundnuts, corn and cotton seed.

In Africa, atoxigenic strains of *Aspergillus flavus* have been identified to competitively exclude toxigenic fungi in the maize and groundnut fields. (Atehnkeng *et al.*, 2008) reported that atoxigenic strains reduce aflatoxin concentrations in both laboratory and field trials by 70 to 99% in Nigeria; a mixture of four atoxigenic strains of *Aspergillus flavus* of Nigerian origin has gained provisional registration as AflaSafe® to determine efficacy in on-farm tests. However, none known strains have been marketed in Tanzania and even if they will, there will a cost to be incurred by farmers higher than if the strains could be identified and produced in Tanzania. Therefore, objective of this research study was to identify natural occurrence of different toxigenic and atoxigenic *Aspergillus* strains in Mtwara, Masasi and Nanyumbu districts which will be useful in biological control approach.

Materials and methods

Field survey of study locations

Farmer's household fields' survey was conducted in 3 prominent groundnut producing districts of Masasi, Nanyumbu and Mtwara in Mtwara region, Southern zone of Tanzania. Three villages of Mnanje, Mpeta and Naliendele were purposively selected due to their potential in groundnut production in Mtwara region (Fig. 1). To determine fungal populations, soil samples were collected randomly from 45 groundnuts from 3 villages selected.

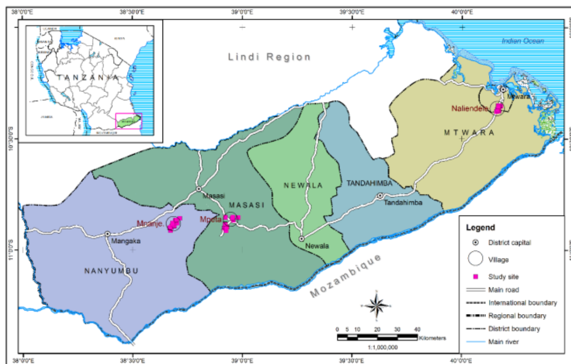

Fig. 1. A map of Mtwara region showing *Aspergillus* section *Flavi* study sites in Mtwara, Tanzania.

Sample collection and preparation

Forty-five soil samples were collected and processed as described by (Dorner, 2009). Three to five scoops of soil samples were randomly taken from each farmer's household field, thoroughly mixed to form a composite sample. Spoons used to scoop the soil at 4-10cm depth were surface sterilized using 70% ethanol to avoid cross contamination. The same procedure was repeated for all the randomly selected soil sample points in the same famer's household field which were distant at least four meters apart.

A one-kilogram sub-sample was drawn from the composite soil sample and labelled with the name of the farmer, village, Global Positioning Systems (GPS) co-ordinates, and the date of collection. The labeled samples were put in zip lock bags and placed in a cool box transported to laboratory further analysis.

Isolation and enumeration of fungal species

The soil samples were air dried (48 - 50°C for 48 hours) and then hammered to break it into a powder. It was then passed through a 2mm aperture laboratory test sieve (Endecott's Ltd, London, UK) to get a fine powder. Isolation and quantification of *Aspergillus* section *Flavi* were done using the dilution plate technique on Modified Rose Bengal Agar (MRBA). Soil sample weighing 1g each were put into a 15mL graduated dilution tube. Nine milliliters of 2% water agar was added to make a 10mL stock solution.

The stock solution was serial diluted by transferring 1 mL of the stock to 9mL of the diluent until a 10-3 dilution was attained. The diluted samples were placed in a rack in a water bath at 40°C and plated in a semi-selective medium. Plates were incubated within the dark for three days at 31°C. Colonies of *Aspergillus* section *Flavi* were then identified by colony morphology. About five - ten isolates per soil sample were transferred to 5/2 agar (5% V-8 juice and a couple of agar, pH 5.2) and grown for 5 more days, unilluminated at 31°C. Isolates were then classified on the premise of colony characteristics and conidial morphology at X400 magnification. *Aspergillus* section *Flavi* colonies were known by their characteristic growth pattern, retention of MRBA within mycelia, and production of characteristics conidiophores after 3 days on MRBA.

Isolates with abundant small sclerotia (average diameter < 400mm) were preliminary classified as strains S while isolates with smooth conidia and large sclerotia (average diameter > 400mm) were classified as L strains of *Aspergillus flavus*. *Aspergillus tamarii* were initially identified by colony and spore morphology. All preliminary identification was confirmed by color reaction on *Aspergillus flavus* and *Aspergillus parasiticus* (AFPA) agar. Numbers of *Aspergillus* section *Flavi* in soils were calculated as colony forming units (CFU) per gram of soil. The following equation was used to calculate the number of *Aspergillus* section *Flavi* per mL of diluted soil sample

$$\text{Number of } \textit{Aspergillus} \text{ section } \textit{Flavi} \text{ per mL} = \frac{\text{Number of colony forming unit (CFU)}}{\text{Volume plated (mL) x Total dilution used}}$$

Number of 3mm plugs of sporulating culture were transferred to 4-dram vials containing 10 mL of sterile distilled water. These conidial suspensions were maintained at 4°C for additional analysis.

Results

Morphological identification of *Aspergillus* section *Flavi* was done as described by (Afzal *et al.*, 2013). The fungal species were identified on the basis of morphology which comprises both macroscopic and microscopic characteristics. Plates were observed for colony colour using colour chart. All colonies that showed yellowish orange or pale yellow colour on the reverse side of colonies or black heads on the top of colonies were counted.

The colour of the colonies (Fig. 2A) was used for enumeration and identification of the sections. *Aspergillus* section *Flavi* had the highest frequency of (40/45) which is 88.8% from soils of the total *Aspergillus* species. Three species were identified namely *Aspergillus flavus* L-strains, *Aspergillus flavus* S-strains and *Aspergillus tamarii* with their respective colonies observed (Fig 2B, $2C_1$ & & $2C_2$) respectively.

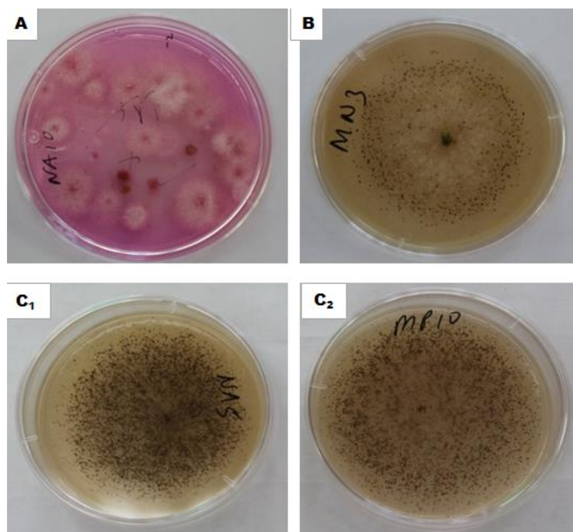

Fig. 2A. Colonies of soilborne fungi growing on Modified Dichloran Rose Bengal medium (MDRB) after dilution and ready for counting.

Fig. 2B. *Aspergillus flavus* colonies on selective media.

Fig. $2C_1$ & C_2. *Aspergillus tamarii* colonies on selective media from Naliendele and Mpeta villages respectively.

Aspergillus flavus (MN3)

Colony observed after 7 days of incubation at 31°C; the colonies were yellow green with white mycelia at the edges; formed sporulation rings; the conidia were rough; did not produce exudates and soluble pigments; Reverse colour was cinnamon brown as shown in Fig. 2B.

Aspergillus tamarii (N5 & MP10)

Colony observed after 7 days of incubation at 31°C. On organizer the colonies were cinnamon in color with white mycelia and rough conidia. They produced exudates but no soluble pigments. Reverse was cream yellow with deep yellow colour at the edges as shown in Figs. 2C1 & C2.

The results of this study indicated a high incidence of *Aspergillus* species from the soils of groundnut farmer's household fields in Southern Mtwara. *Aspergillus* section *Flavi* population average ranged between 8.479 x 10^2 in colony forming unit (CFU)/g and 8.2136 x 10^3CFU/g in all the three study villages summarized in Table 1. In this study, I documented the population densities of *Aspergillus flavus* across three villages in Southern Tanzania. Population densities varied among study villages.

Table 1. Average quantity of *Aspergillus* section *Flavi* population in soil from 45 groundnut fields after harvest season.

District[a]	Village name[b]	CFU/g[c]
Masasi	Mpeta	8.479 x 10^2
Mtwara	Naliendele	4.1158 x 10^3
Nanyumbu	Mnanje	8.2136 x 103

[a]Adminstrative districts; [b]Experiment localization; [c]Colony forming unit (CFU) of *Aspergillus* section *Flavi* in dry soil after harvest.

Aspergillus section *Flavi* was detected in 36 soil samples from 45 fields situated within the studied sites. A total of 402 section *Flavi* colonies were successfully transferred from MRBA to 5/2 agar and subsequently identified by macroscopic, microscopic and growth characteristics in AFPA medium. Distribution of *Aspergillus* section *Flavi* (Table 2) indicated *Aspergillus* species was the most predominant fungal genera identified.

Among *Aspergillus*, *Aspergillus flavus* was the most predominant where L-strains constituted 79.5% of the species identified, followed by S-strains constituted 18.4% while the frequency of the *Aspergillus tamarii* constituted 2%.

Table 2. Average percentage of *Aspergillus* section Flavi strains identified across study villages.

Village name	Average L strains (%)	Average S strains (%)	Average *A. tamarii* (%)
Mnanje	68.0	32.0	0
Mpeta	85.3	13.3	1.3
Naliendele	85.3	10.0	4.7
Mean	79.5	18.4	2.0

Results from Table 2 shown average high incidence of L-strains observed (85.3%) at Mpeta and Naliendele villages respectively; which followed by Mnanje village (68%); while average low incidence of S-strains were observed at Naliendele village (10%), followed by Mpeta village (13.3%), and finally Mnanje village (32%). The presence of both L and S-strains indicated the possibility using them to develop sustainable bio control method by using atoxigenic ones.

Discussion

Morphological characterization of Aspergillus section Flavi from soil isolates

Aspergillus section *Flavi* were detected in 40 soil samples out of total 45 (88.8%) soil samples collected from different farmer's household fields of groundnut producing areas in Mtwara, Tanzania. This current study complies to previous similar research study findings in Benin (Cardwell & Cotty, 2002), Nigeria (Donner *et al.*, 2009) and Kenya (Muluvi *et al.*, 2015). Members of the *Aspergillus* section *Flavi i*dentified in this study includes *Aspergillus* section *Flavi* strains of L-morphotypes, S-morphotypes, and *Aspergillus tamarii* which showed to be major aflatoxin-producing contaminants in the soils of groundnut producing areas. This study supports a high incidence of *Aspergillus* section *Flavi* with *Aspergillus flavus* (L-morphotypes) being the most predominant (79.5%) as indicated in Table 2.

Distribution of Aspergillus section Flavi in Southern Tanzania

The incidence of atoxigenic strains of *Aspergillus flavus* L-strains was higher in all the villages except in the Mnanje village; where frequency of atoxigenic strains were significantly (p< 0.05) higher than that of toxigenic S-strains. According to (Gonçalves *et al.*, 2012) *Aspergillus flavus* exists in two morphotypes the large (L) and small (S) sclerotia producing strains; the S-strains have consistent high aflatoxin-producing ability while the L-strains vary greatly in toxin production with atoxigenic strains commonly found in this group. In this study, I documented the population densities of *Aspergillus* section *Flavi* across three studied sites in southern Tanzania.

Population densities of *Aspergillus flavus* varied among sites. Mnanje and Naliendele villages which fall in warm areas had high populations of *A. flavus* as compared to Mpeta village, located in cooler weather. Recently study in Zambia (Njoroge *et al.*, 2016) documented the population densities of *A. flavus* across two agroecologies in eastern Zambia. Population densities of *A. flavus* varied among districts. The mean population density of *A. flavus* was 2.6, 1.8, 2.0, and 2.4log CFU/g of dry soil in Chipata, Mambwe, Nyimba, and Petauke districts, respectively.

Presence of *Aspergillus* section *Flavi* L-strains in higher percentages in all study villages of Mnanje, Mpeta, and Naliendele (68%, 85.3% and 85.3%) respectively; led to the identification of atoxigenic strains (MN03) which was employed to formulate groundnut seed based inoculant to manage aflatoxin-producing fungi in groundnut. Similar study conducted by (Probst *et al.*, 2014) in Kenya, reported the majority (75%) of isolates belonged to the L-strain morphotypes of *Aspergillus flavus*; minor percentages were *Aspergillus tamarii* (6%), *Aspergillus parasiticus* (1%), and isolates with S-strain morphotypes (3%)

Aflatoxin-producing fungi vary most widely in their characteristics which includes, virulence for crops and aflatoxin-producing ability (Cotty *et al.*, 2008). In many reported cases groundnut and maize crops are very vulnerable for aflatoxin infestation. *Aspergillus flavus* identified has both L and S strains which are commonly implicated as causal agents of aflatoxin contamination (Probst *et al.*, 2014).

Currently, biological control to mitigate aflatoxin is managed by use of atoxigenic *Aspergillus flavus* L-strain isolates which are very useful to competitively exclude aflatoxin producers during crop infection and thereby limit contamination in USA (Cardwell & Cotty, 2002). In Africa particularly Nigeria these strains have been reported to reduce aflatoxin in both laboratory and field trials by 70 to 99% (Atehnkeng *et al.,* 2008). Fungi used for competitive exclusion involved both atoxigenic and competitive strains. According to (Dorner, 2010), for competitive exclusion to be effective, the atoxigenic strains must be present at highly competitive levels when conditions make the crop susceptible to infection. This was supported by results in this study where atoxigenic strains were 79.5% in average population while toxigenic strains were 21.4%. Similar study conducted by (Kachapulula *et al.*, 2017) indicated the potential of atoxigenic members of the *Aspergillus flavus* L-morphotypes for management of aflatoxin in Zambia.

Conclusion

Evidence in distribution and occurrence of both toxigenic and atoxigenic species of *Aspergillus* section *Flavi*, in Mtwara region confirmed the possibility of using these fungal species as biological agents to mitigate aflatoxin contamination risks in both soils and groundnuts produced and traded in southern Tanzania.

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