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SCREENING FOR ANTAGONISTIC TROPICAL FUNGI AGAINST SELECTED MAIZE AND BEAN PATHOGENS

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A B S T R A C T

Phytopathogens are known to be the leading cause of important plant diseases which result in significant losses in agricultural crops. The need to maintain the level of yield both quantitatively and qualitatively is vital in order to curb the losses. So far there has been a positive advance recognized in research to the use of tropical fungi as biocontrol agents. The objective of this study was to screen for antagonistic tropical fungi against selected phytopathogens of maize (*Zea mays* L.) and beans (*Phaseolus vulgaris* L.) namely *Fusarium graminearum, Fusarium moniliforme, Pythium ultimum,* and *Colletotrichum lindemuthianum in vitro.* A total of 87 tropical fungi isolates were collected from Kakamega tropical rainforest, Kenya. Dual culture experiment was carried out to screen the tropical fungi against the selected phytopathogens. The bioassay was performed in a completely randomised design in triplicate and the inhibition zones recorded after every week for three weeks. Differential biocontrol ability among nine tropical fungi was noticed against *F. moniliforme* with the percentage inhibition increasing over time. *Fusarium solani* was the most active antagonist with an inhibition of 64% while *Phaeomarasmius* sp. had the lowest activity of 19.1% against *F. moniliforme*. *Epicoccum* sp. inhibited the mycelial growth of *P. ultimum* by 38% and also inhibited *C. lindemuthianum* by 58%. None of the fungal antagonists inhibited the mycelial growth of *F. graminearum*. The outcome of this study indicates that tropical fungi can be used as biocontrol agents and can be further explored and developed into effective fungicides for management of phytopathogens.

Keywords: antagonist, bioassay, biocontrol, fungal phytopathogens, tropical fungi.

INTRODUCTION

Plant diseases, in particular pathogens, play a direct role in the destruction of natural resources in agriculture causing important losses, fungi being the most aggressive (Abou-Zeid *et al*., 2008; Yu *et al*., 2010). Maize and beans are not only staple foods but also a major source of dietary in most homes in Kenya due to their nutritional composition. Therefore these two crops are an important key determinant of food security for small holder farming communities. Disease outbreaks are key constrain to maize and beans production. For instance, yield losses of up to 90% as a result of maize lethal necrosis led to grain loss of 126,000 metric tons valued at $52 million in Kenya in the year 2012 (Mahuku *et al*., 2015). There have been cases of crop losses like the recent aflatoxin production by *Fusarium* species in Kenya, 2004 (Nyikal *et al*., 2004; Azziz-Baumgartner, 2006). These emerging diseases are not only important at a global scale in terms of crop production but pose a high risk on a small scale especially small holder farmers.

Although, chemical compounds (fungicides) have shown promising results in controlling fungal diseases, phytotoxicity and fungicide residues are serious problems leading to environmental pollution and human health hazards (Patil *et al*., 2012). Their negative impact on the environment and their abuse in application which has favoured the development of pathogens resistant to fungicides has rendered them unattractive to farmers. In this context, the great task now facing scientists is to develop alternative, environmentally friendly disease management strategies such as biological control which

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has been proposed for several plant pathogens. Biological control of plant pathogens by use of natural antagonistic microorganisms has emerged as a promising alternative and realistic approach to reduce the use of chemical pesticides in agriculture.

Numerous microorganisms have been identified and play a considerable role in limiting the populations of phytopathogens. *Trichoderma* species have widely been studied and proven to be a potential antagonist against a variety of fungal phytopathogens (Schubert *et al*., 2008; Živković *et al*., 2010; Patil *et al*., 2012; Martínez-Medina *et al*., 2014). Tropical forest have a rich biodiversity of fungal communities (Rosa *et al*., 2011) and the magnitude of fungal diversity in tropical forests is unclear, and new species remain to be described (Hawksworth, 2004). Among the tropical fungi, endophytes have been studied and known to produce a wide range of secondary metabolites with biological activities (Huang *et al*., 2001; Chomcheon *et al*., 2006). Most of the tropical fungi belong to Ascomycota phylum and have been documented to have remarkable richness in antimicrobial activity (Arnold and Lutzoni 2007; Vaz *et al*., 2009; Vieira *et al*., 2011). The present study addressed the biocontrol efficacy and mechanisms of tropical fungi as biocontrol agents against selected phytopathogens of maize and beans.

MATERIALS AND METHODS

Collection and isolation of tropical fungi: A total of 87 tropical fungi were collected from Kakamega tropical rain forest located at 00^{o} 16' N, 34^{o} 53' E, where both the primary and secondary forests exist with indigenous plant species acceptable by the neighboring communities as medicinal. Random sampling of the tropical fungi was done in September 2014 based on ethno botanical information obtained from the local community. Isolation was done immediately upon collection. The inner part of the basidiomycetes sample was picked with a fine sterile forceps and stuck onto the inner top side of a petri dish containing Potato Dextrose Agar (PDA) media amended with streptomycin sulphate 250 mg/L by the help of silicone high vacuum grease, where the spores were left to drop on the media plate overnight. Thereafter, a sterile spatula was used to cut out the stamp of spores and placed on a media plate. For the ascomycetes, the perithecia were cut open with a sterile scalpel to release spores, which were picked with a fine needle, and plated in sterilized Yeast Malt Glucose (YMG) media containing streptomycin sulphate 250 mg/L and incubated at 25°C. The bacteria free isolates were then transferred to pure YMG media at pH of 6.3, after which they were transferred to YMG media plates amended with ivermectin 50 mg/L. The mite free isolates were finally plated on pure YMG media. For identification purposes the program BLAST (Basic Local Alignment Search Tool) was used to compare the consensus sequences of the isolates with published sequences of the "Genbank" (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Within this tool published sequences of a database are compared to the consensus sequences generated out of the raw data. The tropical fugi isolates examined were considered authentic if the best hits of the BLAST search (depending on query coverage and max identity) were nearly related to the strain presumed or at least belonged to the same family.

Isolation of Plant Pathogens: Four phytopathogens; *Fusarium graminearum*, *Fusarium moniliforme*, *Pythium ultimum*, and *Colletotrichum lindemuthianum*, which cause ear rots, root rots and bean anthracnose, respectively were isolated from infected plant materials collected from Nakuru, Narok and Bomet counties. The infected plant materials were washed under running tap water to remove any soil and blotted dry. Small sections were cut and surface sterilized for 10 seconds with 2% sodium hypochlorite containing 0.1% Tween 20. The plant tissues were rinsed three times each in two washes of sterile distilled water and blotted dry with sterile paper towels. Thereafter, they were plated on PDA and SDA plates amended with streptomycin sulphate 250 mg/L to inhibit any bacterial growth (Whiteside, 1986). The plates were then incubated at 25°C for 4-7 days and monitored for mycelial growth. The cultures obtained were sub-cultured to obtain pure and axenic cultures. Identification of the isolates on basis of symptomatological evidence was done at the Department of Biological Sciences of Egerton University. Identity of the *Colletotrichum lindemuthianum* was further confirmed through pathogenicity test on a bean plant both in green house and in the field.

Antimicrobial assays of the tropical fungi against the phytopathogens: All the tropical fungi were subjected to bioassay *in vitro* against each of the four phytopathogens using the dual culture method. Mycelia agar blocks were cut using a cork borer (7mm in diameter) from the actively growing tropical fungal cultures and inoculated opposite the phytopathogens approximately 4 cm apart on PDA media. The plates were incubated in a dark room with temperatures regulated at 25°C and monitored for growth inhibition.

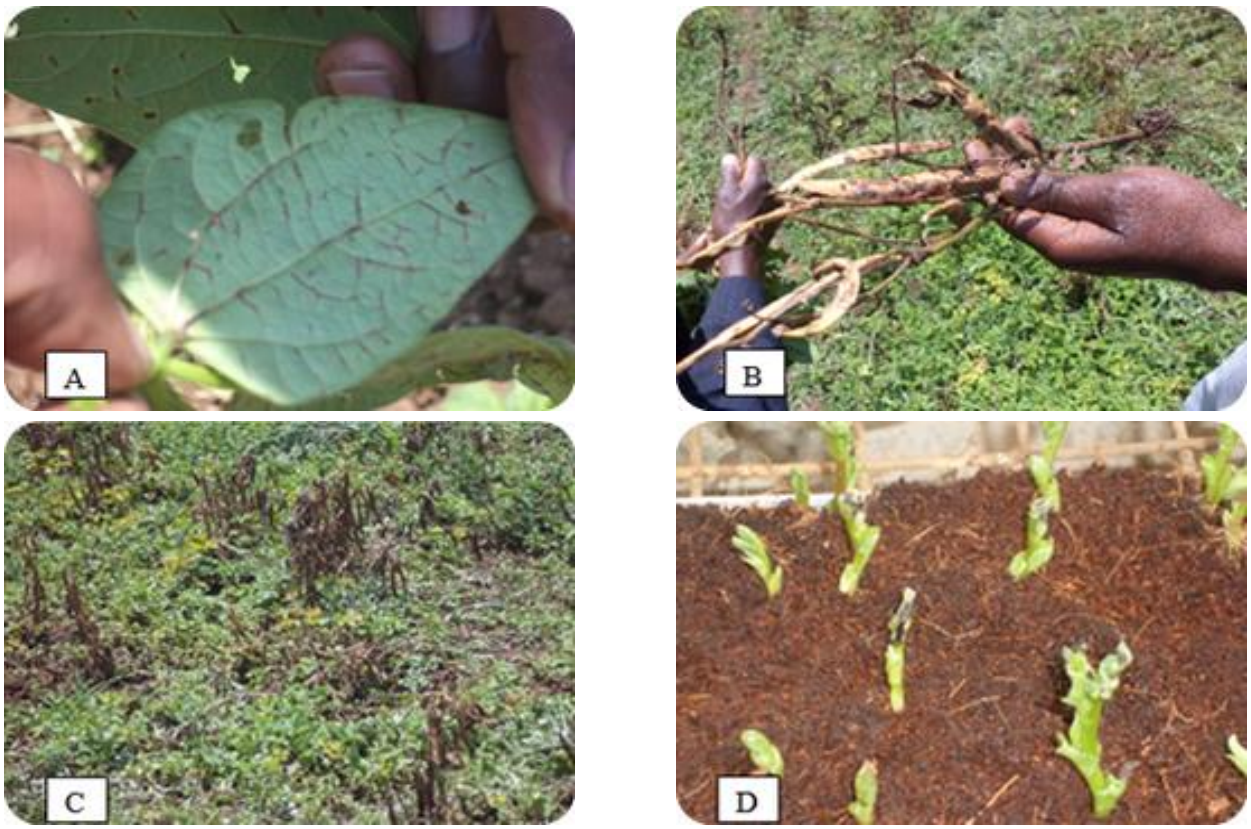

Figure 1. *Symptoms of bean anthracnose manifesting itself on leaves* (A)*, pods* (B and C) *and seedlings* (D).

RESULTS

Antimicrobial activity of tropical fungi against the four phytopathogens: Nine different isolates of tropical fungi inhibited the mycelial growth of *F. moniliforme* (Table 1) while one isolate (*Epicoccum* sp.) inhibited the growth of *P. ultimum* and *C. lindemuthianum*. However, none of the tropical fungal isolates inhibited *F. graminearum*. After 10 days of incubation the *Epicoccum* sp. inhibited radial growth of *P. ultimum* and *C. lindemuthianum* by14% and 63% respectively, and at 21 days the percentage inhibition for *P. ultimum* increased to 38% while for *C. lindemuthianum* it reduced to 58%. Results showed that among the nine bioactive tropical fungal isolates, *Phaeomarasmius* sp., and *Epicoccum* sp. proved to be the least potent bioagents against *F. moniliforme* while *Fusarium solani* and *F.oxysporum* were the most active. The percentage inhibition was generally seen to increase as the days progressed from 10 days to 21 days (Table 1). Six of the isolates inhibited more than 50% of the mycelial growth except for *Phaeomarasmius* sp., *Epicoccum* sp. and *Pestalotiopsis* sp. Differential biocontrol ability among the nine antagonists was noticed against *F. moniliforme*. Figures 2, 3 and 4 show different modes of action exhibited by the tropical fungi on the three phytopathogens; *F. moniliforme*, *P. ultimum* and *C. lindemuthianum*.

Table 1. Inhibition zones displayed by different tropical fungi against *Fusarium moniliforme* and their percentage inhibition.

Tropical fungi / Treatment	Inhibition zone (mm) (*Fusarium moniliforme)*	Percent Inhibition (%)	
		10 days	21 days
Fusarium solani	34.5±3.5^{ab}	63.3	64.0
Fusarium oxysporum	32±4.4^{bc}	54.2	61.9
Fusarium sp.	30.5±5^{bcd}	46.7	55.8
Fusarium sp.	28.2±2.7^{bcd}	57.5	57.1
Pezizomycetes sp.	23.8±2.5^{bcde}	46.7	59.2
Phomopsis sp.	20.5±2.5^{cdef}	37.5	53.1
Pestalotiopsis sp.	20.5±2.5^{cdef}	37.5	46.3
Epicoccum sp.	14.83±2.15^{ef}	25.83	39.46
Phaeomarasmius sp.	10±1.88^{f}	26.67	19.05
Negative control	44.50±2.01^{a}	-	-

Means with same letter within a column are not significantly different according to Turkey's test.

Inhibition zones between the test organisms and phytopathogens (C-T) were measured in a period of 7-21 days and the resulting percentage inhibition zones were determined as:

$$L = \frac{(C - T)}{C} \times 100$$

L= inhibition of radial mycelial/colony growth; C= radial growth measurement of pathogen in control; T= radial growth measurement of pathogen in the presence of antagonist (Hajieghrari *et al*., 2008).

The data collected on inhibition zones as a result of antagonistic tropical fungi were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS Institute, 2001) software. Treatment means were separated using Turkey's HSD test whenever ANOVA showed significant treatment effects.

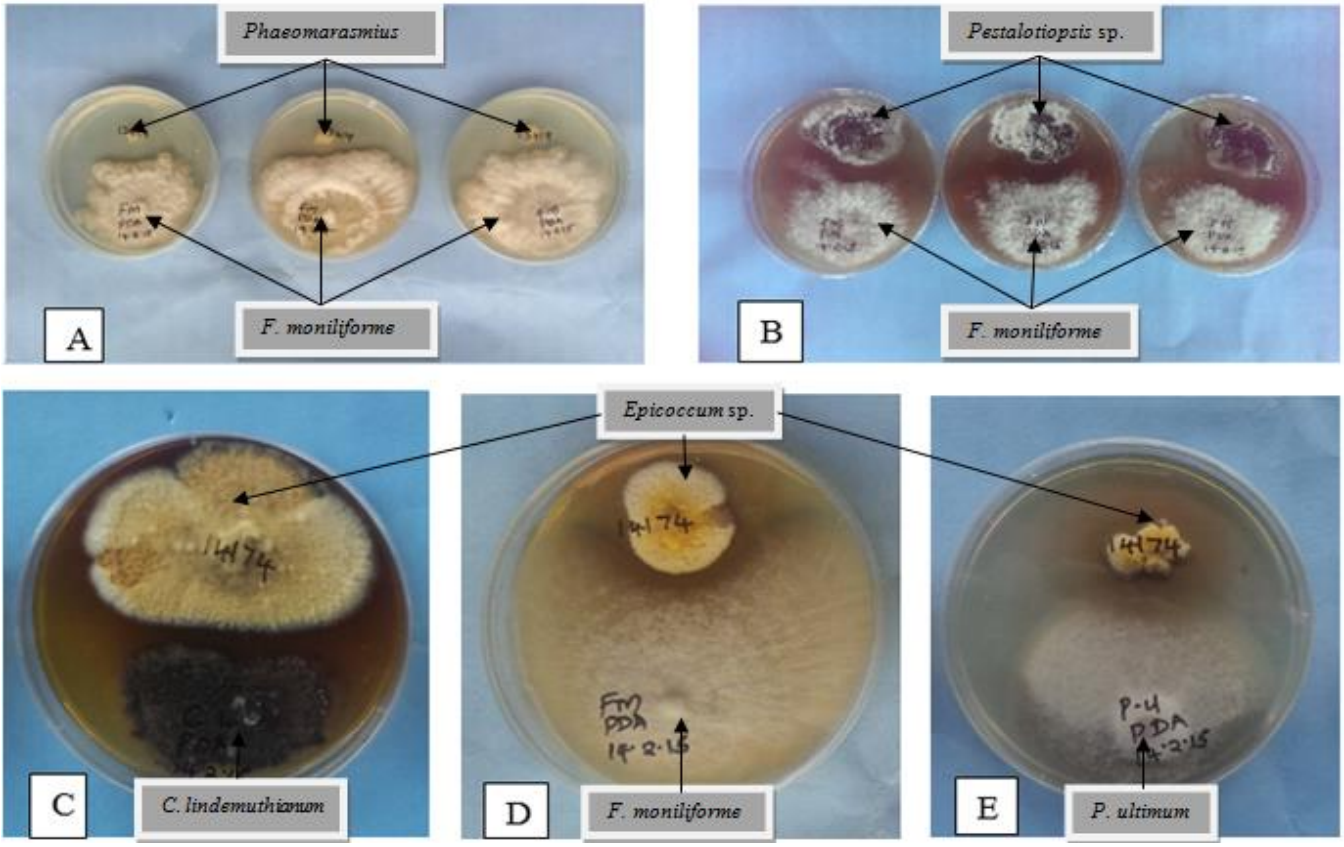

Figure 2. Different tropical fungi displaying antibiosis as a mechanism of inhibition against phytopathogens after 21 days. Activity of tropical fungal isolates *Phaeomarasmius* sp. (A), *Pestalotiopsis* sp. (B) and *Epicoccum* sp. (D) against *F. moniliforme*. Activity of *Epicoccum* sp. against *C. lindemuthianum* (C) and *P. ultimum* (E).

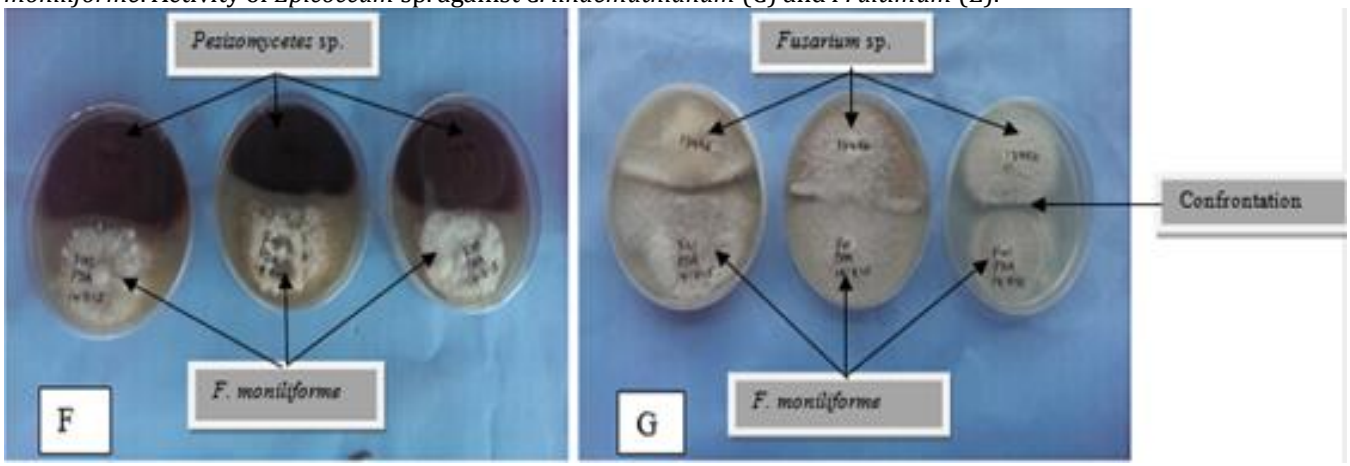

Figure 3(a). Dual antagonism demonstrated by different tropical fungi against *Fusarium moniliforme* after 21 days.

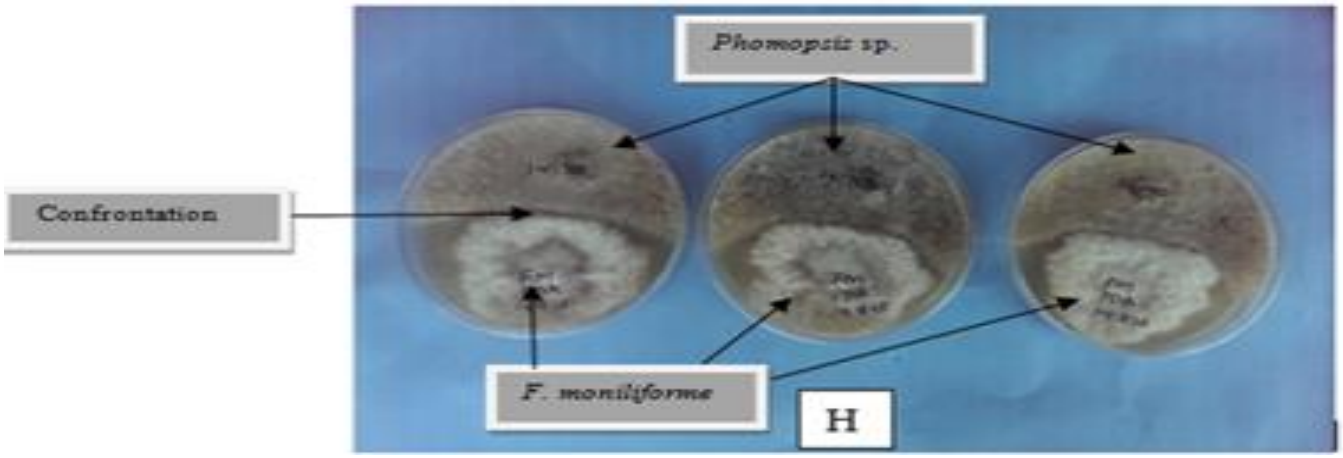

Figure 3. Dual antagonism demonstrated by different tropical fungi against *Fusarium moniliforme* after 21 days.

Both *F. moniliforme* and the tropical fungi isolate (F and G) were noted to be inhibiting each other while the tropical fungus seemed to be inhibited by the *F. moniliforme* (H). *Epicoccum* sp. inhibited both the growth and sporulation of *Pythium ultimum* (Figure 4). This was also evident with isolate 14327 (*Fusarium* sp.). The change of colour where both the tropical fungi isolate and pathogen almost meet could be attributed to lack of sporulation or production of chemical component responsible for inhibition. This was observed with *Epicoccum* sp. and *Fusarium* sp. as the antagonistic mechanism against *P. ultimum.*

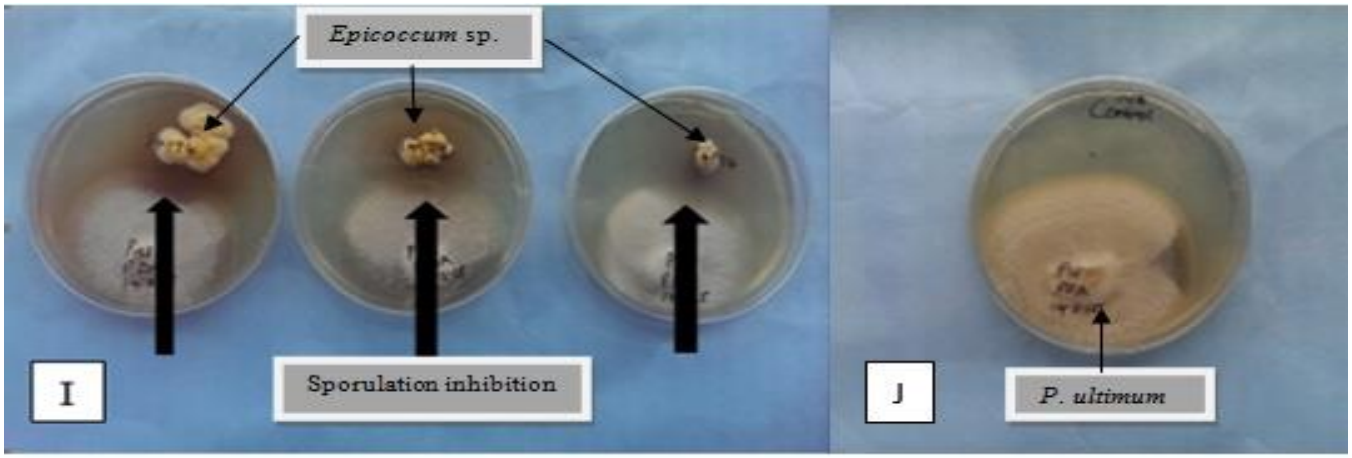

Figure 4. Tropical fungus 14174 (*Epicoccum* sp.) inhibiting sporulation of *Pythium ultimum* after 21 days.
Pythium ultimum (I) *inhibited by Epicoccum sp. Pythium ultimum* (J) *not inhibited by any tropical fungi (negative control).*

DISCUSSION

Antagonistic fungal isolates have been used as bio-agents to control plant pathogens. Rhizospheric, saprophytic and endophytic microorganisms are the most studied antagonists which have proven to be potent in controlling fungal plant pathogens (Schubert *et al*., 2008). Tropical fungi which include the above named types have been on the target by many researchers for the past decades due to their ability to control or suppress growth of phytopathogens especially soil-borne and post-harvest fungal plant pathogens (Verma *et al*., 2007; Fatima *et al*., 2009; Ara *et al*., 2012). Some of the active tropical fungal isolates identified in this study have been known to exist as endophytes. For instance, isolate 14174 which was identified as *Epicoccum* sp. was effective against *F. moniliforme*, *P. ultimum* and *C. lindemuthianum*. *Epicoccum* spp. especially *E. nigrum* has been known to be an endophyte which produces active metabolites (Wang *et al*., 2014). Use of epicoccolides as antibacterial and antifungal polyketides from *Epicoccum* sp. associated with *Theobroma cacao* has been shown to have antimicrobial activity against *P. ultimum* (Talontsi *et al*., 2013). *Epicoccum* sp., *Pestalotiopsis* sp. (isolate 14179) and *Phomopsis* sp. (isolate 14170) have also been associated with antimicrobial activity against target fungal organisms (Vieira *et al*., 2014). *Pestalotiopsis* sp. has recently been reported to produce a novel compound which is antibacterial and antiyeast (Subban *et al*., 2013).

Other ascomycetes like *Trichoderma*, *Xylaria* and *Fusarium* species form the most frequent antagonistic fungi encountered (Bacon *et al*., 2001; Joseph and Priya 2011; Vieira 2011). From this current study, the named active tropical fungi isolates 14167, 14164, 13427 and 13427 were *Fusarium* sp. Isolate 13419 identified as *Phaeomarasmius* sp. a basidiomycete, had the lowest antifungal activity against *F. moniliforme* among the active tropical fungi isolates and has also been known to inhibit plant pathogens (Thomas *et al*., 2008). Several studies involving basidiomycetes in production of bioactive compounds against microbes have been carried out and they have been proved to be effective against microorganisms (Stadler and Hoffmeister, 2015). Mushroom fruiting bodies (basidiomes) seem to be particularly talented in producing unique terpenoids, and the molecular background behind the biosynthesis of some of those compounds has only recently been elucidated (Quin *et al*., 2014).

Three antagonistic mechanisms were displayed in this study of tropical fungi against phytopathogens. Antifungal enzymes production could have been the main mode of inhibition demonstrated by the tropical fungi isolates 14167, 14164, 14327, 14179, 14174 and 13419; identified as *F. solani*, *F. oxysporum*, *Fusarium* sp., *Pestalotiopsis* sp., *Epicoccum* sp. and *Phaeomarasmius* sp. respectively. The mechanism of antifungal antagonists could be due to the secretion of hydrolytic enzymes such as chitinase-b-3 glucanase, chitosanase, and proteases (Moreno- Perez *et al*., 2014) which degrade the fungal cell wall or the secretion of antifungal compounds (Khamna *et al*., 2009; Elamvazhuthi and Subramanian, 2013). Dual antagonism exhibited by *Phomopsis* sp., *Fusarium* sp. And *Pezizomycetes* sp was another observed mechanism of inhibition. There was a clear confrontation between the antagonist and the phytopathogen with no distinct inhibition zone. Competition for space and nutrients perhaps was the mode of action between the antagonists (*Fusarium* sp., *Phomopsis* sp. and *Pezizomycete* sp.) and *F. moniliforme.* Generally, either the combination of extracellular hydrolytic enzymes and secondary antifungal metabolite(s) or the secondary antifungal metabolite(s) alone can be assumed to play a major role in the inhibition of fungal growth (Prapagdee *et al*., 2008).

Inhibition of sporulation was shown by *Epicoccum* sp. (isolate 14174) against *P. ultimum*. A change in the mycelia colour was noticed where the antagonist was in close proximity with *P. ultimum*. Sporulation is a key component for several purposes because fungal spores are frequently used as propagules to infect plants (Rodrigues *et al*., 2010). Several studies have reported different biocontrol agents inhibiting sporulation of fungal pathogens both *in vitro* and in field trials as a mechanism of control against phytopathogens. For instance, sporulation of *F. moniliforme* was completely inhibited by three species of *Trichoderma* and one *Penicllium* sp. *in vitro* (Begum *et al*., 2015). Various isolates of *Trichoderma* spp. have been screened against *F. oxysporum* f. sp. *Lycopersici* by dual culture technique and noted to inhibit its sporulation (Sundaramoorthy and Balabaskar, 2013). Field studies have also reported success in managing fungal pathogens by inhibiting sporulation. In a farm in Panama, treatment with *C. rosea* reduced the incidence of Cacao pods with sporulating leisons of *Monillophthora roreri* by 10% (Mejía *et al.,* 2008). These examples support the potential of tropical fungi as biocontrol agents against pathogens.

CONCLUSION

Nine of the collected tropical fungi; *Fusarium solani*, *F. oxysporum*, two unidentified *Fusarium* spp., *Pestalotiopsis* sp., *Epicoccum* sp., *Phaeomarasmius* sp., *Phomopsis* sp. and *Pezizomycetes* sp., had antimicrobial activity against three fungal phytopathogens; *Fusarium moniliforme*, *Colletotrichum lindemuthianum* and *Pythium ultimum* indicating that the tropical fungi are potential antifungal agents that can be used to manage plant diseases.

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