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ISOLATION AND ANTI FUNGAL SUSCEPTIBILITIES OF PHYTOPATHOGENIC FUNGI FROM INFECTED YAM LEAVES IN ZARIA, NIGERIA

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ABSTRACT
In order to study the disease causing organisms that reduces the quantity and quality of yam produced, which makes them unappealing to consumers, isolation of phytopathogenic fungi from infected yam leaves in the field at Zaria was carried out. The phytopathogenic infected yam leaves were surface sterilized with 70% alcohol, swabbed aseptically with sterile cotton swab stick and then inoculated on sterile Sabourand dextrose agar (SDA) suplimented with 0.1% of penicillin and streptomycin. The inoculated plates were incubated at 30^0C for 5 days. The growth were isolated in pure culture, and then characterized fully. The fourteen fungal species identified namely Aspergillus niger, Cladosporium sp, Aspergillus ostianus, Scopolarium sp, Aspergillus flavus, Rhizopus sp, Penicillium citrinum, Aspergillus wentii, Penicillium oxalicum, Aspergillus cervinus, Penicillium sp, Aspergillus fumigatus, Aspergillus oryzae and Rhizopus stolonifer were subjected to different concentrations of the following antifungal agents: Terbinafine HCl; Fluconazole; Flucytosine; Benomyl and Sodium propionate. The susceptibilities (MICs and MFCs), the percentage of occurrence of the 14 identified isolates and their distribution in 12 farms and three towns studied were also determine.

Keywords: Disease, phytopathogenic fungi, yam, Zaria

INTRODUCTION

Yams (*Dioscorea* spp) belong to the family Dioscoreceae, order liliales, subclass lilidae, class liliopsida (monocotyledons), division magnoliophyta (seed plants), sub-kingdom spermatophyte (flowering plants), sub kingdom tracheobionta (vascular plants) and finally kingdom plantae (plants) (Coursey, 1967; Okigbo and Ikendiugwu, 2000; Okigbo and Nmeka, 2005 and USDA, 2008). The cultivated species in Nigeria are the *D. rotundata* (white yam), *D. cayenesis* (yellow yam), and *D. alata*, (water yam). There are also species of wild yam growing in Nigeria whose tubers are collected for eating in times of food shortage such as *D. dumtorum* (Cluster, or bitter yam), *D. esculenta* (Loir) bark Chinese yam) and *D. bulbifera* L. (aeria yam) (Adeniji, 1970; Okigbo, 2004).

In spite of its importance, post-harvest deterioration has been a major problem facing farmers and traders (Osagie, 1992; Knoth, 1993; Amusa *et al.,* 2003). It has been estimated that an average of over 25% of the yield is lost annually to diseases and pest (Arene, 1987, Ezeh 1998; FAO, 1998). These losses are attributed by many workers mostly to rot caused by bacteria, fungi and nematodes. The disease causing organisms not only reduce the quantity of Yam produced, but also reduce the quality by making them unappealing to the consumers. Reducing sugars reduce copper in some obscure chemical reaction. In tuber, reducing sugars are predominantly the 6-carbon sugars glucose and fructose, which result from the breakdown of starch from tuber reserves or the breakdown of sucrose transported from the plant leaves to the tubers. Yam is exposed to infections right from the seedling stage through to harvesting and even after harvesting, in storage (Amusa *et al*., 2003). Yams are susceptible to a variety of pest and diseases during growth as well as postharvest. Attack by microorganisms is devastating. The major postharvest disease of yam tuber rots was reported mostly caused by fungi (Opera, 1999). Most of the pathogens of yam tuber are soil-born, but manifestations of the tuber disease are observed mostly during storage (Jones, 1985). While these fungal infections originate in the fields, they often are not fully evident at the time of harvest and may carry on and spread during storage after harvest (Demand Media, 2010). These rots may also affect the growing plant when the setts consist of cut pieces of tuber (Adeniji, 1970; Enyi, 1970; Ekundayo and Naqvi, 1972; Ferguson, and Gumbs, 1977; coursey and Ferber, 1979). Treatment of yam tubers with fungicides such as benlate and captan has been reported effective in reducing fungal yam rot (Ogundana, 1971, 1981). This notwithstanding, there is a major problem of resistance emergence of the target organisms, chemicals potential accumulation in the ecosystem, chronic toxicity to humans and animals (Okigbo and Ikediugwu, 2000; Okigbo, 2004).

MATERIALS AND METHODS

Isolation and Identification of phytopathogenic fungi from infected yam leaves

Infected yam cultivars, was first identified through careful physical examination of the entire plant for symptoms of infection such as necrotic lesions on the leaves, petioles and stems.

The phytopathogenic fungi infected samples of yam leaves [about 3mm diameter] were surface sterilized with 70% ethanol and cut from advancing edge of a rot infected tissue. These samples were swabbed aseptically with sterile cotton swabbed stick and plated out on sabouraud dextrose agar (SDA) supplemented with penicillin (0.1%) and streptomycin (0.1%). The plates were incubated at 30^0C for 5 days. The fungal growths observed were identified using biochemical, morphological and cultural characteristics. The frequency of fungi isolates occurrence were also documented (Okigbo and Ikediugwu, 2000; Okigbo and Nmeka 2005). The isolated and identified fungi were culture on SDA slants in triplicates and kept until require for further investigations.

Stock culture preparation

Stock cultures of fungi spores were prepared using SDA slants incubated at 30^0C for five days and stored in refrigerator at 4^0C (Okigbo and Nmeka 2005; Olurinola *et al.,* 1992) until require for use or further studies.

Spores preparation

Fungal spores' suspensions were prepared from SDA slant culture. The spores were harvested using sterile glass beads and normal saline containing 0.05% Tween 80 to obtain homogenous spores suspension. The harvested fungal spores' suspensions were

standardized to approximately 10^8 spores per ml (Olurinola *et al.,* 1992).

Media preparation

Sabourand Dextrose Agar (SDA) and Sabourand Dextrose liquid medium (SDLM) were prepared according to the manufacturer's specification and sterilized at 121^0C for 15 minutes.

Preparation of inactivation agent

Sodium chloride (0.90g) was weighed and dissolves in 95ml of distilled water plus 5ml Tween 80 to make up 0.9%w/v and 5% v/v Tween 80.

Preparation of Harvesting medium

Sodium chloride (0.90g) was weighed and dissolves in 99.95ml of distilled water plus 0.05ml Tween 80 to make up 0.9%w/v and 0.05% v/v Tween 80 and heat to enhanced solubility

Determination of Minimum Inhibitory Concentration (MIC) of test antifungal agents

The graded concentrations of each test-antifungal agents Viz: Benomyl (0.50-2000µG/ml), Flucytosine (0.20-800µg/ml), fluconazole (0.5-2000µg/ml), Terbinafine (0.5-2000µg/ml), and Sodium propionate (2.0-10,000µg/ml) in 5ml volume were prepared aseptically. These were mixed with melted double strength (5ml) SDA at 45^0C and poured aseptically in to sterile petri dishes and allowed to set (Shettima *et al.,* 2005).

Three pairs of sterilized filter discs were equidistantly place on the dried SDA-plates aseptically. An aliquot of 20µl of each spectrophotometrically standardized test fungal spores suspension (10^8 cfu or10^8 spores / ml) was inoculated on each pair of filter paper discs on the test compound in SDA plates. The plates were allowed to stand for one hour (1hr) and then incubated at 30^0C for 72 hours (Shettima *et al.,* 2005).Control was also set up i.e. SDA plate without test chemical agent but inoculate with the test fungi spores (Olurinola *et al.,* 1992).

The lowest concentration of each test antifungal agent and in admixture that inhibited the growth of the test fungal spores was regarded as MIC (Olurinola *et al.,* 1992; Shettima *et al.,* 2000; Ehinmidu *et al.,* 2003; Godwin *et al.,* 2003; Shettima *et al.,* 2005).

Determination of Minimum Fungicidal Concentration (MFC) of test antifungal agents

The MFC of each test compounds was determined by inoculating the discs that showed no visible growth during MIC determinations. These were sub-cultured aseptically into 5ml Sabouraud Dextrose Liquid Medium, supplemented with 5% $^v/_v$ of Tween 80 as inactivator (Olurinola *et al.,* 1991; Shettima *et al.,* 2000; Shettima *et al.,* 2005).This medium with filter paper discs was incubated at 30^0C for 5 days (Olurinola *et al.,* 1991; Shettima *et al.,* 2000; Shettima *et al.,* 2005). The lowest concentration that showed no growth was regarded as MFC of the test Antifungal agent against test phytopathogenic fungi spore.

RESULTS AND DISCUSSION

Many different fungal species were isolated from tweelve randomly selected different farms of Dioscoria species in Zaria, between new Jos road and Kaduna road, (KwauyenAliDakaci, Zaria L.G; Wanka, Soba L.G. and Rafinyashi, Giwa L.G „Latitude 10057"E and 11012"N, Longititude 7045"E and 806"N), and these species were confirmed to be *D. Rotundata* in the department of botany, Ahmadu Bello University, Zaria. Fourteen of these fungi were identified with their percentage of occurrence of the 14 identified isolates and their distribution in 12 farms and three towns studied in various farms as shown in the table 1, 2 and 3.

Table 1: Percentage of occurance of phytopathogenic fungi isolates from leaves of yams in 12 farms in three towns in Kaduna state, Nigeria.

S/N	Organisms	% of occurance
1	*Penicillium citrinum* (PC)	7.813
2	*Penicillium oxalicum* (Po)	4.688
3	*Penicillium sp* (Pen sp)	4.688
4	*Aspergillus cervinus* (Ac)	4.688
5	*Aspergillus fumigatus* (Afm)	4.688
6	*Aspergillus niger* (An)	15.625
7	*Aspergillus wentii* (Aw)	6.250
8	*Aspergillus ostianus* (Ast)	10.938
9	*Aspergillus oryzae* (Aory)	3.125
10	*Aspergillus flavus* (Afv)	7.813
11	*Scopolarium* sp (Sco)	7.813
12	*Cladosporium* sp (Clod)	10.938
13	*Rhizopus* sp sp (R. sp)	7.813
14	*Rhizopus stolonifer* (Rs)	3.125

Table 2: Distribution of fungi isolates from leaves of *D. rotundata* in 12 farms in three towns in Kaduna state, Nigeria.

Farms Studied	Number of species	Type of Organisms Isolated
A	7	Pc, Pen. sp, Sco, Clo, Afv, Rs, A. ost
B	8	Pc, Ac, Pen. sp, An, Clo, Afm, Afv, R. sp,
C	5	Po, An, Sco, R. sp, A. ost
D	8	Aw, Po, Pen. sp, An, Afm, Afv, A.ost, R.sto,
E	4	An, Sco, Clo, R. Sp
F	5	Pc, An, Sco, Clo, A. ory
G	9	Pc, Aw, Po, Ac, An, Clo, Afv, A.ost, A. ory
H	7	Pw, Ac, An, Clo, Afv, A.ost, R. sto,
I	3	An, Afv, R. Sp
J	5	Pen. sp, An, Sco, Clo, A. ost
K	2	Aw, A. ost
L	2	An, Pc

Key:

1. *Penicillium citrinum* (PC)
2. *Aspergillus wentii* (Aw)
3. *Penicillium oxalicum* (Po)
4. *Aspergillus cervinus* (Ac)
5. *Penicillium sp* (pen sp)
6. *Aspergillus niger* (An)
7. *Scopolarium* sp (Sco)
8. *Cladosporium* sp (Clod)
9.*Aspergillus fumigates* (Afm)
10. *Aspergillus flavus* (Afv)
11. *Rhizopus* sp (2)Rs
12.*Aspergillus ostianus* (Ast)
13. *Aspergillus oryzae* (Aory)
14. *Rhizopus stolonifer* (1)Rs

Table 3: Distribution of fungi isolates from leaves of *D. rotundata* in 12 farms in three towns in Kaduna state, Nigeria.

	Isolates number				
Town studied	As	Rs	Ps	Scs	Cls
Zaria	12	4	7	2	2
Soba	13	2	4	2	4
Giwa	7	1	2	1	1
Total isolates	32	7	13	5	7

Key: Ps=*Penicillium* sp, As=*Aspergillus* sp, Cls=*Cladosporium* sp, Scs=*Scoppolarium*sp, Rs=*Rhizopus* sp

Minimum Inhibitory Concentration (Mic) Of Test Antifungal Compounds Against Phytopathogenic Yam Fungal Isolate Spores

The result of minimum inhibitory concentration of Terbinafine, Sodium propionate, Fluconazole, Flucytosine and Benomyl against the isolated phytopathogenic yam fungi shows that: benomyl has a higher antifungal inhibitory activity with a lower range of 0.98-15.25 µg/ml; followed by terbinafine with a range of 0.98-31.25 µg/ml; flucytosine with range of 0.78-200 µg/ml; fluconazole with a range of 1.95-500 µg/ml while sodium propionate has the lowest inhibitory activity with a higher range value of 19.53-10,000 µg/ml (Table 4 and 5).

Table 4:The Range of Minimum Inhibitory Concentration (MIC) values of test antifungal compounds against phytopathogenic yam fungal isolate spores at 30^0C after 48hours.

	MIC(µg/ml)				
Test Organisms	**Terbinafine**	**Fluconazole**	**Sodium Propionate**	**Flucytosine**	**Benomyl**
Ps	7.81-31.25	62.50-125.00	1250.00-2500.00	6.25-100.00	0.49-0.98
As	0.98-15.25	1.95-500.00	78.25-10000.00	0.78-200.00	0.98-15.25
Scs	1.85-3.91	31.25-62.50	625.00-1250.00	3.125-6.25	0.98-1.95
Cls	0.49-0.98	1.95-3.91	62.25-312.50	50.00-100.00	7.81-15.25
Rs	1.95-250.00	3.91-1000.00	19.53-2500.00	0.78-200.00	0.98-500.00

Key:
Ps *Penicillium* sp (11)
As *Aspergillus* sp (34)
Cls *Cladosporium* sp (5)
Scs *Scoppolarium* sp (7)
Rs *Rhizopus* sp (7)

Table 5: The Minimum Inhibitory Concentration (MIC) values of test antifungal compounds against 10^8 cfu/ml phytopathogenic yam fungal isolate spores suspension at 30^0c after 48hours (µg/ml)

MIC					
	TERBINAFN	FLUCONAZOLE	NaPROPIONET	FLUCYTOSINE	BENOMYL
Sco	3.91	62.50	1250	6.25	1.95
Clad	0.98	3.91	312	100	15.25
Afm	1.95	1.95	1250	200	7.81
Aflv	1.95	1.95	1250	0.78	0.98
Rs	1.95	3.91	19.53	0.78	0.98
Ast	1.95	3.91	78.25	0.78	0.98

Key: *Scoppolarium* sp (Sco), *Cladosporium* Sp (Clad), *Aspergillus fumigatus* (Afm), *Aspergillus flavus* (Afv), *Rhizopus* Sp (2)Rs, *Aspergillus ostianus* (Ast)

Minimum Fungicidal Concentration (Mfc) Values Of Test Antifungal Compounds Against Phytopathogenic Yam Fungal Isolates

The result of minimum fungicidal concentration of terbinafine, Sodium propionate, fluconazole, flucytosine and Benomyl against the isolated phytopathogenic yam fungi spores (Table 6 and 7) showed the following order of antifungal activity Viz: terbinafine (1.95-2000µg/ml); fluconazole (3.91-4000µg/ml); benomyl (15.25-4000µg/ml); flucytosine (200-1600µg/ml) and sodium propionate (156.25-20,000µg/ml). The sporicidal activities of the test antifungal agents depend on the test phytopathogenic fungal spores involved. Generally, observations from this research have shown that, the order of test fungal spores susceptivbility to test antifungal agents was *Cladosporium* sp > *Scoppolarium* sp > *Penicillium* sp > *Rhizopus* sp > *Aspergillus* sp (Table 7).

Table 6: The Range of Minimum Fungicidal Concentration (MFC) values of test antifungal compounds against phytopathogenic yam fungal isolate spores at 30^0C after 48hours

	MFC(µglml)				
Test Organisms	**Terbinafine**	**Fluconazole**	**Sodium Propionate**	**Flucytosine**	**Benomyl**
Ps	15.63-62.50	125.00-1000.00	2500.00-5000.00	400.00-800.00	62.50-1000.00
As	1.95-7.81	3.91-4000.00	156.00-20000.00	200.00-800.00	15.25-2000.00
Scs	3.91-7.81	62.50-125.00	1250.00-2500.00	400.00-800.00	500.00-1000.00
Cls	1000.00-2000.00	3.91-7.81	10000.00->10000.00	200.00-400.00	31.25-62.50
Rs	3.91-500.00	7.81-2000.00	312.00-5000.00	400.00-1600.00	250.00-2000.00

Key:

Ps *Penicillium* sp (11)
As *Aspergillus* sp (34)
Cls *Cladosporium* sp (5)
Scs *Scoppolarium* sp (7)
Rs *Rhizopus* sp (7)

Table 7: The Minimum Fungicidal Concentration (MFC) values of test antifungal compounds against 10^8 cfu/ml phytopathogenic yam fungal isolate spores suspension at 30^0C after 48hours (µg/ml)

MFC					
	TERBINAFN	FLUCONAZOLE	NaPROPIONET	FLUCYTOSINE	BENOMYL
Sco	7.81	125	2500	800	1000
Clad	2000	7.81	>10000	400	62.50
Afm	3.91	3.91	>10000	400	>2000
Aflv	3.91	3.91	>10000	800	250
Rs	3.91	7.81	312.50	400	250
Ast	3.91	7.81	156.25	800	500

Fungal infection of yam leaves is a major factor leading to reduction in yam tuber growth and spoilage in storage (Aboagye-Nuamah *et al.,* 2005). Many of these fungi attack the yam leaves right in the field. The post-havest infections of yam tuber which are later transported to store have been implicated in most yam tuber spoilage. Rot of yam tubers may be caused by a wide variety of fungi, including those isolated in this study from yam leaves in the field and have also been found associated with post harvest rot and store yam rot (Adeniji, 1970; Ogundana *et al.,* 1970; Okigbo and Ikediugwu, 2001).

The yam phytopathogenic fungi isolated and identified in the field from white yam infected leaves in this work include: *Aspergillus niger; Aspergillus fumigatus; Aspergillus flavus; Aspergillus wentii; Aspergillus ostianus; Aspergillus oryzae; Aspergillus cervinus; Rhizopus stolonifer; Rhizopus* sp; *Penicillium citrinum; Penicilium oxalicum*; *Penicillium* sp*; Clodosporium* sp and

Scoppolarius sp.This coresponds with the works of several researchers in the area of yam tuber rot and in the field (Ogundana *et al.,* 1970; Adeniji, 1970; Okigbo and Ikediugwu, 2000; Okigbo, 2002; Okigbo, 2004; Okigbo and Nmeka, 2005; Aboagye-Nuamah et al., 2005; Okigbo,2005).

The fungal infection of yam leaves in the farms has been reported to drastically affect yam tuber production (Amusa *et al.,* 1996; Amusa, 1999). Hence, the observed myriads of infective fungi from the leaves of yams in the twelve farms studied potent a serious danger for high yam tuber production and subsequent low income to farmers and the subsequent food insecurity to the nation that depend on this food stuff to feed their populace. This low yield of the yam tuber could result in instability in the society due to lack of food and loss of livelyhood in need of food.

The need to reduce this high level of fungal infection of yam leaves in farms with effective fungicides cannot be over-emphasized.

Susceptibility Test

The susceptibility of spores of fungi isolated from the yam leaves which were also implicated in yam postharvest rot to test chemical compounds, such as Benomyl, Terbinafine, Fluconazole, Flucytosine and sodium propionate showed that Benomyl had the best inhibitory activity with MICs range of 0.98-15.25µg/ml, This result is consistent with values previously obtained with wild-type *A. nidulans* strains (Oakley, 1981; Van Tuyl,1977; Yulia *et al.,* 1999); Terbinafine had a range of 0.98-31.25µg/ml; Flucytsine had a range of 0.78-200µg/ml; Fluconazole with a range of 1.95-500µg/ml while Sodium propionate had the lowest inhibitory activity with a higher range value of 19.53-10,000µg/ml. From this result the order of fungistatic activity of the test compounds was Benomyl > Terbinafine > Fluconazole > Flucytosine > Sodium propionate. The sensitivity of the test isolated fungi spore varied.

A similar pattern of the antifungal activity was observed in Minimum Fungicidal Concentration of the same test agents determined in this study. These test compounds show different degree of fungicidal activities against test fungal isolates spores. Terbinafine HCL (1.95-2000µg/ml); fluconazole (3.91-4000µg/ml); benomyl (15.25-4000µg/ml); flucytosine (200-1600µg/ml) and sodium propionate (156.25-20,000µg/ml) and the order of this lethal activity was, Terbinafine HCL > fluconazole > benomyl > flucytosine > sodium propionate.

Generally, Terbinafine HCl was the most active in terms of inhibitory and lethal activities (i.e MIC and MFC) in this study, while sodium propionate was the least active followed by fluconazole. Other workers have reported high MICs values of azole against *Aspergillus* species; *Rhizopus stolonifer* and *Penicillium citrinum* as observed in this study. (Moore *et al.,* 2000; Walsh *et al.*, 2004; Qiao *et al.,* 2008; Donald *et al.*, 2009; Susan *et al.,* 2009).

CONCLUSION

This work shows that Terbinafine could be used to reduce fungal growth. It has been reported that indiscriminate use of any of these antifungal agents singly may affect their effectiveness due to resistance development. Thus a combination study of terbinafine HCl with other fungicides could be a possible way out of the challenges of resistance development. Therefore the effectiveness of terbinafine as an antifungal agent could be maximized with two antifungal compounds with one potentiating the other with lesser toxicity and cost effectiveness with possible delay in resistance development.

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