Antifungal activity of selected chemical agents against phytopathogenic fungi spores.

Abstract

Postharvest deterioration has been a major problem associated with yam storage for both famers and traders and it is caused mostly by micro-organisms especially fungi. During the storage of yam, many organisms such as *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer* are often reported to cause rotting of the stored yams. The aim of this research is to find out the antifungal effect of some commonly used anti-dermatophytic agents,(Fluconazole, Terbinafine Hcl, Ketoconazole, Sodium propionate and Griseofulvin) on the above named fungi spores. This was carried out using zone of inhibition, MICs, MFCs, FICs and FFCs to measure the antifungal activities of the test antifungal agents against the isolated phytopathogenic fungi spores. These agents were found to have fungitoxic effects on the test organisms in the order of: Terbinafine Hcl > Fluconazole > Ketoconazole > Sodium propionate > Griseofulvin. This work indicated that the test antifungal agents were able to inhibit the fungi spores that are widely reported to be associated with yams rot when stored. The observation in this study showed that a good and efficient fungicide against the test organisms that are known to cause yam rot during storage can be effectively arrested with combination of these fungicides.

Key words: Antifungal agents; Fungitoxic; Phytopathogenic fungi spore; Postharvest deterioration; Yam

Introduction

Yams are good source of carbohydrates (Adelusi & Lawanson, 1987). Protein, fats, calcium, phosphorous, iron, sodium and potassium has been widely reported to be found in yam, which are basic nutrients that the body needs. Some other nutritional component that are reported in yams are fibers (helps in bowel cleansing), Vitamins such as Thiamine, Riboflavin (growth promoting factor in human), Niacin (essential for metabolism) and Ascorbic acid (antioxidant) are also found in yam (Osagie, 1992).

Out of the global yam production of about 47million metric tons (MT) with 96% of this coming from Africa, Nigeria alone produce about 70% of world production (Okigbo, 2004).

Despite all the importance of yam, its production and preservation have being a worldwide problem. The yam storage challenge has been attributed to be by postharvest rot (Cornelius at el, 1999). Bonire (1985) estimated this loss to be 40% while Okigbo & Ikeiugwu (2000) indicated between 20 and 39.5% of stored yam may be lost to decay. Okigbo (2008) also reported that over50% of the yam tubers produced and harvested in Nigeria are lost during storage. Many fungi are responsible for this rot among which are *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer*.

Many reported works has been done towards the reduction of post harvest microbial losses of yam during storage. The use of wood ash and palm oil (Oduro et al, 1991) and use of lime and

local gin (Ogali et al 1991) have all been researched. Some chemicals have also been used to reduce storage losses of yam e. g. sodium orthophenylphenate, borax, captan, thiobenazole and benomyl (Okigbo & Ikeiugwu, 1999), Sodium hypochlorite (Nnodu & Nwankiti, 1986) and organotins (Olurinola at el 1992). Plant extract have also been proven to be effective in the control of yam rot e. g. *Xylopia aethiopica* and *Zingiber officinale* by Okigbo & Nmeka , (2005), also Onifade (2002) used *Azadirachta indica*. Biological method have also been employed, Bacillus subtilis (Okigbo, 2002) and *Trichoderma viride* (Osuinde et al, 2002 and Okigbo & Ikeiugwu, 2000) controls rot of yam during storage.

It is therefore important to find an effective method that can control this yam rot during storage. Hence the use of the already known anti-dermatophytic agents, (Fluconazole, Terbinafine Hcl, Ketoconazole, Sodium propionate and Griseofulvin) in very minute quantity against *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer* which are sometimes responsible for rotting of yam tubers during storage.

MATERIALS AND METHOD

Test Organism

The micro organisms *(Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer)* were obtained from Department of Pharmaceutics & Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria.

Determination of Zone of Inhibition using the Cup plate method

The single strength SDA (20ml) prepared were melted and poured into sterile plates aseptically. They were then allowed to solidify. Standardized spore suspension of the fungal at 10^6 cfu/ ml was used to flood the agar surface. The number 4 (6mm) sterile cork borer was flamed red hot, allowed to cool and used to bore holes in the agar. Secondly, various concentrations (2000, 1500, 1000, 500, 250 and 100µg/ml) of the different anti-fungal agents were prepared. Then, 100µl of the varying concentrations were dispensed into each of the holes on the SDA.The plates were allowed to stand for an hour and later incubated at 30ºC for 48hours. The zones of inhibition were measured using a well calibrated transparent meter ruler.
The same procedure was repeated using similar concentrations for Fluconazole, Terbinafine, Ketoconazole, Sodium propionate and Griseofulvin.

Determination of Minimum Inhibitory Concentration (MIC) using Agar Dilution method.

Ten milliliters (10mls) volume of double strength SDA was melted and mixed aseptically with 10mls volume of varying concentration of the test anti-fungal agents such as Fluconazole viz 2, 5, 10, 20, 40, 60, 100, 200, 500, 1000, 2000 and 4000 (µg/ml). Each admixture was aseptically poured into sterile plates and allowed to set. The standardized spores of test fungi (10^6 cfu spores/ml) were aseptically inoculated (10.0 µl) in duplicates on sterile filter paper disc plated at equidistance on the SDA test antifungal plates.

The inoculated organisms were allowed to diffuse for a period of 30minutes. The plates were then incubated at 30ºC for 48hours. The first lowest concentration that showed no growth of inoculated test fungi spores was considered as the MIC of the test anti-fungal agent.

Determination of Minimum Fungicidal Concentration (MFC)

In determining the MFC of the different anti- fungal agents, the filter paper disc that showed no growth were aseptically transferred into the already prepared Saboraud Dextrose Liquid medium supplement 5% Tween 80as in activator. These were then incubated at 30ºC for 72hours in an incubator. Visual observations for any visible growth were made. The lowest concentration of each of the anti-fungal agents that showed no visible growth was taken as the MFC of the test anti-fungal agent.

Determination of Fractional Inhibitory Concentration (FIC) of Admixture using Agar Dilution method.

Each varying concentrations of the test anti-fungal sub-inhibitory level (e.g. Sodium propionate 50, 100, 200, 300, 500µg/ml) in 5mls volume each were mixed with fixed sub-inhibitory concentration of another test anti-fungal agents (e. g. Fluconazole 500µg/ml) in same 5mls. Each of these admixtures in 10ml volume was mixed with melted 10ml volume of sterilized double strength of SDA aseptically in a Petri-dish. This was allowed to set. 10µml of standardized fungi spores (10^6 cfu/ ml) were inoculated on a sterilized duplicate filter paper discs aseptically placed at equidistance on the test anti-fungal agents contained in the SDA.
The inoculates were allowed to diffuse into the SDA for 30minutes. These were then incubated at 30ºC for 48hours and the lowest mixed concentration of test-antifungal agents that showed no growth was taken as combined anti-fungal agents MIC.
This same procedure was carried out for other anti-fungal agents combination such as Terbinafine/Sodium propionate.

Determination of Fractional Fungicidal Concentration (FFC) of Admixtures

In determining the combined FFC of admixture of test anti-fungal agents, the filter paper disc that showed no visible growths during combined MIC of test antifungal agents were aseptically transferd into 5ml volume of the sterilized Saboraud Dextrose Liquid medium supplemented with 5% tween 80and 3%w/v yeast extract determinations. These were then incubated at 30ºC for 72hours, the lowest concentration of combined anti-fungal agents that showed no growth was taken as the combined test antifungal agents FFC.

Result

Table 1 Zone of inhibition of Fluconazole against *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer*

Test antifungal Concentration (µg/hole)	*Aspergillus flavus* (mm)	*Aspergillus niger* (mm)	*Penicillium citrinum* (mm)	*Rhizopus stolonifer* (mm)
200	41.5±0.70	33.5±0.70	34.5±0.70	20.5±0.70
150	41.0±1.40	32.5±0.70	31.5±0.70	20.0±0.00
100	38.5±0.70	31.5±0.70	30.0±0.00	15.5±0.70
50	38.0±0.00	30.5±0.70	29.5±0.70	15.0±0.00
25	35.5±0.70	29.5±0.70	29.0±1.40	14.5±0.70
10	34.0±0.70	26.0±0.00	24.5±0.70	13.0±0.00

Table 2 Zone of inhibition of Terbinafine against *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer*

Test antifungal Concentration (µg/hole)	*Aspergillus flavus* (mm)	*Aspergillus niger* (mm)	*Penicillium citrinum* (mm)	*Rhizopus stolonifer* (mm)
200	60.0±0.00	58.5±0.70	69.5±0.70	19.5±0.70
150	59.5±0.70	58.0±1.40	64.0±0.00	15.5±0.00
100	56.0±1.40	56.5±0.70	62.5±0.70	14.0±0.00
50	45.5±0.70	54.5±0.70	61.5±0.70	13.5±0.70
25	45.0±0.00	53.5±0.70	61.0±0.00	12.5±0.70
10	36.5±0.70	52.5±0.70	57.0±1.40	Nil

Table 3 Zone of inhibition of Ketoconazole against *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer*

Test antifungal Concentration (µg/hole)	*Aspergillus flavus* (mm)	*Aspergillus niger* (mm)	*Penicillium citrinum* (mm)	*Rhizopus stolonifer* (mm)
200	46.5±0.70	23.5±0.70	25.0±0.00	12.5±0.70
150	42.5±0.70	18.5±0.70	24.5±0.70	Nil
100	35.5±0.70	12.0±0.00	23.5±0.70	Nil
50	34.5±0.70	Nil	23.0±0.00	Nil
25	21.0±1.40	Nil	15.0±0.00	Nil
10	20.0±0.00	Nil	Nil	Nil

Table 4 Zone of inhibition of Sodium propionate against *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer*

Test antifungal Concentration (µg/hole)	*Aspergillus flavus* (mm)	*Aspergillus niger* (mm)	*Penicillium citrinum* (mm)	*Rhizopus stolonifer* (mm)
200	20.5±0.70	Nil	Nil	Nil
150	20.0±1.40	Nil	Nil	Nil
100	17.5±0.70	Nil	Nil	Nil
50	16.5±0.70	Nil	Nil	Nil
25	14.0±0.00	Nil	Nil	Nil
10	Nil	Nil	Nil	Nil

Table 5 Zone of inhibition of Griseofulvin against *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer*

Test antifungal Concentration (µg/hole)	*Aspergillus flavus* (mm)	*Aspergillus niger* (mm)	*Penicillium citrinum* (mm)	*Rhizopus stolonifer* (mm)
200	Nil	Nil	Nil	16.5±0.70
150	Nil	Nil	Nil	14.5±0.70
100	Nil	Nil	Nil	12.0±0.00
50	Nil	Nil	Nil	Nil
25	Nil	Nil	Nil	Nil
10	Nil	Nil	Nil	Nil

Table 6 Minimum Inhibitory Concentration (MIC) of test antifungal agents against *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer*

Test antifungal Agent	*Aspergillus flavus*	*Aspergillus niger*	*Penicillium citrinum*	*Rhizopus stolonifer*
Fluconazole(µg/m)	100.0	500.0	1.0	1000.0
Terbinafine(µg/ml)	1.0	10.0	1.0	50.0
Ketoconazole(µg/ml)	10.0	20.0	10.0	50.0
Sodium propionate (µg/ml)	100.0	1000.0	100.0	2000.0
Griseofulvin (µg/ml)	200.0	>2000.0	100.0	>2000.0

Table 7 Minimum Fungicidal Concentration (MFC) of test antifungal agents against *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer*

Test antifungal Agent	*Aspergillus flavus*	*Aspergillus niger*	*Penicillium citrinum*	*Rhizopus stolonifer*
Fluconazole(µg/ml)	500.0	1000.0	5.0	2000.0
Terbinafine(µg/ml)	250.0	50.0	50.0	250.0
Ketoconazole(µg/ml)	50.0	250.0	100.0	1000.0
Sodium propionate(µg/ml)	500.0	>2000.0	>2000.0	>2000.0
Griseofulvin(µg/ml)	>200.0	–	>2000.0	–

Table 8 Fractional Inhibitory Concentration (FIC) of combined Test fungicides against Phytopathogenic fungi spores (10^6cfu/ml).

Fungicide Combination	*Aspergillus flavus*	*Aspergillus niger*	*Penicillium citrinum*	*Rhizopus stolonifer*
Fluconazole/ Sodium Propionate	0.50	0.19	0.83	0.09
Terbinafine/ Sodium Propionate	0.83	0.44	0.63	0.38
Ketoconazole/ Sodium Propionate	0.59	0.35	0.59	0.43
Fluconazole/ Griseofulvin	0.39	0.15	0.83	0.09
Terbinafine/ Griseofulvin	0.71	0.39	0.83	0.38
Ketoconazole/ Griseofulvin	0.49	0.28	0.59	0.43

Key: FIC>4=Antagonistic
FIC=1-4=Indifference
FIC<1=Synergistic

Table 9 Fractional Fungicidal Concentration (FFC) of combined Test fungicides against Phytopathogenic fungi spores (10^6cfu/ml).

Fungicide Combination	*Aspergillus flavus*	*Aspergillus niger*	*Penicillium citrinum*	*Rhizopus stolonifer*
Fluconazole/ Sodium Propionate	0.19	0.11	0.29	0.07
Terbinafine/ Sodium Propionate	0.29	0.38	0.29	0.33
Ketoconazole/ Sodium Propionate	0.43	0.35	0.26	0.14
Fluconazole/ Griseofulvin	0.13	_	0.29	_
Terbinafine/ Griseofulvin	0.22	_	0.29	_
Ketoconazole/ Griseofulvin	0.31	_	0.26	_

Key: FFC>4=Antagonistic
FFC=1-4=Indifference
FFC<1=Synergistic

Discussion /Conclusion

Food preservation usually involves preventing the growth of bacteria, fungi (such as yeasts), and other microorganisms (although some methods work by introducing benign bacteria, or fungi to the food), as well as retarding the oxidation of fats to the food (Wikipedia). This work is novel, in which few workers have researched into. Many other methods have been employed in the post harvest control of yam rot, like the use of chemical Sodium orthphenylphenate, borax (Sodium borate), captan (ethanethiol or ethyl mercaptan), thiobendazole, benomly (Acephate) and sodium hypochlorite have been found to significantly reduce storage rot in yam

Otoo et al, (2001) reported that a combination of wood ash and broad spectrum antifungal Benlate or Thiabendazole has been used for protection of yam minisetts against rot. Wood ash and palm oil was also discovered by Oduro et al, (1991) to delay or prevent rot caused by *Aspergillus niger, Penicillin specie* and *Rhizopus stolonifer* when applied to the cut surface of yam tubers. Though treatment with wood ash alone gave good result but in combination with the antifungal was much better.

However Ogundana & Dennis (1981) also used worked on fungicide fornthe preservation of storage rot of yam tubers.This investigation shows that all the test antifungal agents displayed inhibitory effect on the different isolates of the test phytopathogenic fungi spores. Fluconazole, Ketoconazole, Terbinafine Hcl, Sodium propionate and Griseofulvin all showed marked antifungal activities. In combination better antifungal activities were observed with lower concentration because of the synergistic effect of the Fluconazole/Sodium propionate, Ketoconazole/Sodium propionate and Terbinafine Hcl/Sodium propionate.

In conclusion, having proven that Fluconazole, Ketoconazole, Terbinafine Hcl, Sodium propionate and Griseofulvin could be used to inhibit *Aspergillus flavus, Aspergillus niger, Penicillium citrinum and Rhizopus stolonifer* isolated from rotted yams, there use should be encouraged to reduce the loss of yam year in and year out.

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