Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.8, No.17, 2018

Stability Studies of Some Antifungal Agents Activities Against Selected Phytopathogenic Fungi (Aspergillus niger) Spores

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Abstract
Fungi infections have been implicated as one source of yam tuber (genus *Dioscorea*) spoilage. The effects of temperature, pH and storage duration on antifungal activities of selected combined antifungal agents were investigated using the agar-well diffusion and agar dilution method against *Aspergillus niger* spores. The test combine antifungal agents are: Fluconazole/Sodium propionate (1/1µg/ml), Terbinafine Hcl/ Sodium propionate (1/10µg/ml), ketoconazole / Sodium propionate (1/2.5µg/ml), Fluconazole / Griseofulvin (1/1µg/ml), ketoconazole / Griseofulvin(1/2.5µg/ml) and Terbinafine Hcl/ Griseofulvin (1/10µg/ml). The combined antifungal agents activities at 25 - 70 ºC varies while at neutral and alkaline pH (pH 9) the antifungal activities of the chemical agents remained relatively unaffected. There was no significant changes in the antifungal activities of the test combined agents against *Aspergillus niger* spores at $p>0.05$ throughout the period of the six months of investigation. The significant of these finding with relation to stability of the chemical for preserving yam tubers is discussed in this work.
Keywords: Biological control, fungi, Antifungal Agents, Nigeria

Introduction

Yam tuber is one of the oldest foods cultivated since 50,000 BC in Africa and Asia. In addition to these continents, yams also currently grow in the tropical and subtropical regions of North and South America (IITA, 2009). It's one of the most popular and widely consumed foods globally. They have been taken as major diets in many countries, notably those in South America, Africa, the Pacific Islands, and the West Indies.

Worldwide yam production in 2007 amounted to 52 million tons, of which Africa produced 96%. Most of the world's production comes from West Africa representing 94%, with Nigeria alone producing 71%, equaling more than 37 million tons. African countries exported 15,500 tons of yam tubers, of which Nigeria contributed 12% (IITA, 2009).The Food and Agriculture Organization (1998) estimated that the world production of yam is around 30.2 million tons per year. Over 70% of world yam production has been reportedly produced in Nigeria, which is three quarter of the world production of yam (FAO; 1998).

Yam is a good source of energy, because of high carbohydrate content (low in fat and protein) (Amusa *et al*., 2003). Yams also contain modest amounts of Vitamin B_1 (thiamin) and Vitamin C. Yams also provide bulk fiber, which are needed to make the intestines or bowels work properly. It has been reported to provide a fair amount of iron and niacin (Coursey, 1983).

Medically, Undie and Akubue, (1986) discovered that yam could be used to treat disease like diabetes mellitus, to increase coronary flows and prevent hyper-costerolemia therefore controls blood pressure because of the potassium in it. Yam has been reported to be used in dermatology and gastroenterology infection and also sources of progesterone and cortisone (Coursey; 1983). Its medicinal use as a heart stimulant is attributed to its chemical composition, which consists of alkaloids of saponin and sapogenin (Amusa *et al.*, 2003).

Yam has been reported to be major source of incomes for farmers and traders in Nigeria and has also been reported to be useful as pharmaceutical excipients. The yam by-products have been reported useful in animal hunting and as insecticides (Degras; 1993). In spite of the great economic importance of this food, 20–30% (about 6.4 million tonnes) are lost during storage (FAO, 1998).

A report by Ala'a 2008 highlighted the ability of a hypersensitive chemical compound in an enclosed system to retain its physical, chemical, microbiological and toxic qualities is referred to as the stability of the compound. Some of the factors that can make the antimicrobial activities of chemical compounds unstable are: environmental factors like air (oxygen & carbon dioxide), other chemicals, light, heat, water (hydrolysis) and duration of material storage and before usage. All these factors have reported to lead to the instability of the chemicals agents (Oyi *et al.,* 2007).

Changes in wholesomeness during storage include wound repair, diseases (rot cause by Phytopathogens) and pests of stored tubers; hence yam tubers are lost after 4-5 months of storage. Traditionally, processed yam products are made in most yam-growing areas, usually as a way of preserving the tubers that cannot withstand long storage before microbial spoilage sets in. It is significant to note that rotting in storage probably started in the soil and progressed in storage. This may happen when infected tubers do not show perceptible external symptoms (Jones, 1985). Each type of rot is characteristic of its causal organism. The incidence of rotting varies with the species and with the varieties of the species of yam (Nnodu and Nwankiti, 1986). They also noted that it would probably vary from place to place. It has been observed that in the case of white yam, rotting appeared first at the end of yams

and then proceeds towards the head regions. Rot vary due to variations in the distribution of microorganism. It does not relate to the soil mineral status, because the differences in the mineral status are not known to be correlated with type of organism isolated nor total percentage of rot. It's been reported that over 50% of the yam tubers produced and harvested in Nigeria are lost in storage (Onayemi, 1983). These loses has been attributed to sprouting, transpiration, respiration and rot due to fungi & bacteriosis, insect, nematodes and mammals (Osunde, 2008). The disease causing agents reduce the quantity of yam produced and also reduce the quality by making them unappealing to the consumer. Yam is prone to infection right from the seedling stage through harvesting and even after harvesting in storage (Amusa *et al*., 2003). Yams are subjected to several diseases. There are different genera of fungi that have been reported in association with storage deterioration in yam tubers (Okigbo and Ikediugwu, 2000). The major microorganism causing diseases in yams are: - *Aspergillus flavus* Lark Ex Fr, *Aspergillus niger* Van Tiegh, *Botryodiplodia theobromae* pat, *Fusarium oxysporum* schlecht ex Fr, *Fusarium solani* (Mart). Sacc, *Penicillium chrysogenum* Thom Rhizoctinia sp, *Penicillium oxalicum* Curries and Thom, *Rhizopus stolonifer* (Enrend. ex Fr) Lind, *Rhizopus nodosus* N'amyslowski and *Trichoderma viride*. Per. ex S. F. Gray among others (Okigbo and Ikediugwu 2000, 2001, 2002; Okigbo 2004).

Decomposition in hydrolysis usually occurs in chemicals, if not in conducive temperature, pH and also in the presence of water, while oxidative reaction are strongly influenced by environmental factors such as light and metal ions to trigger off reaction. The rate of the hydrolysis depends on the quantity of water present, degree of temperature and the pH.

Aulton (2000) has also reported that deterioration of some thermo liable agents can occur as a result of the increase rate of chemical reaction since they can only withstand a particular range of temperature.

Since all chemical agents can be affected by above mentioned factors, there is the need to investigate the effect of these factors on the activities of combined antifungal agents on yam.

Materials and Methods

Test Organism

The micro organism (*Aspergillus niger*) used in this study was isolated from yam in Department of Pharmaceutics. & Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria.

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.

Effect of varying pH values on the antifungal activities of the formulated fungicide combinations:

Three different pH values 4, 7, and 9 were used, which was obtained and maintained using pH buffers. The buffer powder was added to the Formulated product which is in 10mls volume with sterile distilled water. The content was allowed to stand for 30minutes.

The standardized cultures of the test fungi spores was used to flood the SDA plates which was allowed to dry at 37 ºC in a sterile incubator. Using the cup plate method, a sterile cork borer (6mm diameter) was used to make a hole in each of the agar plates. The bottom sealed with two drops of the melted SDA at 45 ºC.

Precisely 0.1ml of the fixed concentration of the formulated product and sterile distilled water (which served as control) was then dispensed into the holes using micropipette. This was allowed diffuse into the agar at room temperature for one hour. This was then incubated at 30 ºC for 48 hours. The zones of inhibition were then measured to the nearest millimeters.

This procedure was carried out in triplicates and the same method was used for the other pH values.

Effect of varying temperature on the activities of the formulated fungicide combinations:

The formulated combined fungicides were dissolved in sterile distilled water, giving a known concentration of the test agent (1/20 of the concentrates). The set up was maintained at different temperatures (37 ºC, 45 ºC, 70 ºC, and 100 ºC) in the water bath for 30minutes.Exactly 0.1 ml of the product solution was aseptically transferred into the already made holes in the standardized test fungal spores suspension flooded SDA plates and incubated at 30ºC for 48hours. Positive and negative controls were also set up. The zones of inhibition of the test organism were then measured using a well calibrated meter rule.

Effect of duration of storage on the antifungal activities of the formulated fungicide combinations:
A solution of the formulated products was prepared in 10 ml volume with sterile distilled water at regular intervals during the six months. At interval of 1 month, solution of the formulation prepared was aseptically assessed for antifungal activity using the agar well diffusion method. An Inoculum of 0.1ml of the product solution was aseptically dispensed into the bored hole in the SDA containing the test organism. The plates were then allowed to stand for 1hour for diffusion. Positive and negative controls were set up and the plates were incubated at 30 ºC for 48 hours and the results of the zone of inhibition taken appropriately.

Result
The tested antifungal agents viz: Terbinafine Hcl, Ketoconazole, Fluconazole, Sodium propionate and Griseofulvin (Table 1) were effective against the test phytopathogenic fungi spores of *Aspergillus niger* isolated from yams in Zaria, Kaduna Nigeria. The activities of agents can be affected by some environmental factors.
The formulated antifungal agents were found to show the highest antimicrobial activity at pH of 7 and above while at acidic pH there was a decrease in antifungal effect (Table 2). Temperature was found to have a varying degree of effect on the antifungal activity of the test agents. The test agents were observed to be effective at temperature ranges of 37ºC and 45ºC. The observed antifungal activity decreases as the temperature increases above 45 ºC (Tables 3-6). There were no significant changes in the antifungal activity of the test agents within six months of storage at ambient temperature against *Aspergillus niger* at p>0.5 (Table 7-8).

Table 1 Minimum Inhibitory Concentration (MIC) of test antifungal agents against*, Aspergillus niger,*

Test antifungal Agent	*Aspergillus niger*
Terbinafine(µg/ml)	10.0
Ketoconazole(µg/ml)	20.0
Fluconazole(µg/m)	500.0
Sodium propionate (µg/ml)	1000.0
Griseofulvin (µg/ml)	>2000.0

Table 2: Effect of varying pH values on the antifungal activities of the formulated fungicidal combinations.

	Zone of inhibition (mm)			
Antifungal agent	pH 4	pH 7	pH 9	Distilled water (pH 7.05)
Fluconazole / Sodium. Propionate	19.0± 0.00	39.0±0.50	31.0± 0.00	41.0±0.00
Ketoconazole/ Sodium. Propionate	31.0± 0.00	35.0 ±0.0	Nil	37.0±0.50
Terbinafine / Sodium. Propionate	60.0±0.00	70.0±0.00	75.0±0.00	65.0±0.50
Fluconazole / Griseofulvin	25.0±0.00	28.0± 0.00	35.0±0.00	25.0±0.00
Ketoconazole / Griseovulvin	31.5±0.00	42.0±0.50	38.0±0.50	32.0±0.00
Terbinafine/ Griseofulvin	48.0±0.00	53.0±0.00	61.0±0.50	55.0±0.00

The result is expressed as mean ± standard deviation.
Nil = No zone of inhibition

Table 3: Effect of temperature on the antifungal activity of Fluconazole /Sodium propionate (5000µg/5000µg /ml)

Tempt (ºC)	Distilled Water	Tap water	Well water	River water	Pond water
Room	40.5±0.70	43.0±0.00	39.0±0.70	40.0±0.00	38.5±0.70
37 ºC	43.0±0.00	46.0±2.80	40.0±1.40	40.0±1.40	43.0±1.40
45 ºC	37.0± 0.70	44.0±1.40	38.5±0.70	42.5±1.40	45.0±1.40
70 ºC	33.5±1.40	34.0±1.40	32.5±2.80	34.0±0.70	33.5±1.40
100 ºC	35.0±0.00	32.5±0.70	35.5±1.40	37.0±1.40	38.0±0.70

The result is expressed as mean ± standard deviation.

Table 4: Effect of temperature on the antifungal activity of Terbinafine/Sodium propionate (500µg/5000µg/ml)

Tempt (ºC)	Distilled Water	Tap water	Well water	River water	Pond water
Room	64.0±0.00	52.0±0.70	59.0±0.70	60.0±0.00	58.5±0.70
37 ºC	63.5±1.40	50.0± 1.40	62.5±0.70	62.0±1.40	57.0±1.40
45 ºC	57.5±0.70	55.5±0.70	56.5±1.40	49.0±1.40	56.5±1.40
70 ºC	46.5±1.40	52.0±0.70	45.5±1.40	55.0±1.40	52 5±0.00
100 ºC	55.5±0.70	47.0 ±0.70	56.0±0.70	55.0±0.70	62.0±1.40

The result is expressed as mean ± standard deviation.

Table 5: Effect of temperature on the antifungal activity of Fluconazole /Griseofulvin (5000µg/5000µg/ml)

Tempt (ºC)	Distilled Water	Tap water	Well water	River water	Pond water
Room	40.5±0.70	43.0±0.00	39.0±0.70	40.0±0.00	38.5±0.70
37 ºC	31.5±0.70	31.5±0.70	32.5±2.80	29.5±2.80	28.0±0.70
45 ºC	42.5±1.40	44.0±0.70	47.5±1.40	41.0 ±1.40	33.5±0.70
70 ºC	30.5±0.70	36.0±0.00	34.0±0.00	31.5±0.70	38.0±1.40
100 ºC	31.0±1.40	34.5±1.40	32.0±0.70	32.5±0.70	31.5±0.70

The result is expressed as mean ± standard deviation.

Table 6: Effect of temperature on the antifungal activity of Terbinafine Hcl/ Griseofulvin (500µg/5000µg/ml)

Tempt (ºC)	Distilled Water	Tap water	Well water	River water	Pond water
Room	40.5±0.70	43.0±0.00	39.0±0.70	40.0±0.00	38.5±0.70
37 ºC	64.0±0.00	63.5±0.70	71.0±0.70	60.5±0.70	59.0±0.70
45 ºC	74.5±0.70	74.0±0.70	74.5±0.70	71.0±0.70	68.5±1.40
70 ºC	70.5±1.40	60.5±1.40	65.0±1.40	65.0±2.80	65.0±0.45
100 ºC	51.5±0.70	62.0±0.00	52.0±1.4 0	60.0±1.40	55.0± 1.70

The result is expressed as mean ± standard deviation.

Table 7: Effect of storage duration on the antifungal activities of the formulated fungicide combinations using *Aspergillus niger*

	Zone of inhibition (mm)		
Duration	Fluco/Sod prop	Keto/Sod prop	Terb. Hcl/Sod prop
Day 1	64.0±0.00	57.0±0.00	69.5±0.50
4weeks	63.0±0.00	56.5±0.00	69.0±0.00
2months	62.0±0.50	57.0±0.00	68.0±0.50
3months	61.0±0.50	54.5±0.50	68.0±0.00
4months	61.0±0.00	57.0±0.50	68.0±0.00
5months	60.0±0.00	58.0±0.50	68.0±0.00
6months	60.5±0.00	56.0±0.00	67.0±0.50

The result is expressed as mean ± standard deviation.

Key Fluco/Sod prop = Fluconazole / Sod propionate 5000/5000µg/ml
Keto/Sod prop = Ketoconazole / Sod propionate 2000/5000µg/ml
Terb. HCl/Sod prop = Terbinafine / Sod propionate 500/5000µg/ml

Table 8: Effect of storage duration on the antifungal activities of the formulated fungicide combinations using *Aspergillus niger* .

	Zone of inhibition (mm)		
Duration	Fluco/Griseo	Keto/Griseo	Terb Hcl/Griseo
Day 1	35.5±0.00	38.0±1.00	63.5±0.00
4weeks	35.5±0.50	37.5±0.00	62.5±0.00
2months	32.0±0.50	37.0±0.50	62.0±0.50
3months	31.0±1.00	38.0±0.00	62.0±0.00
4months	31.5±0.00	37.0±1.00	61.0±1.00
5months	31.0±0.00	38.0±0.00	60.5±1.00
6months	30.0±0.00	36.0±0.00	60.0±0.00

The result is expressed as mean ± standard deviation.

Key

Fluco/Griseo = Fluconazole / Griseofulvin 5000/5000µg/ml
Keto/Sod prop = Ketoconazole / Griseofulvin 2000/5000µg/ml
Terb HCl/Griseo= Terbinafine / Griseofulvin 500/5000µg/ml

Discussion

The antifungal activities of test antifungal agents: Fluconazole/Sodium propionate), Terbinafine Hcl/ Sodium propionate, Fluconazole /Griseofulvin and Terbinafine Hcl/ Griseofulvin on phytopathogenic *Aspergillus niger* spores in Zaria, Nigeria was investigated, and it was discovered that these test agents all have antifungal effects on the isolate spores . The MIC of these test agents individually was Terbinafine Hcl 10 µg/ml, Fluconazole 500 µg/ml, Sodium propionate 1000 µg/ml and Griseofulvin > 2000 µg/ml.

The study showed that at varying temperature, pH ranges and storage period investigated; there were no significant changes in the observed antifungal activities. The observation showed that at acidic pH there is a general decrease in antifungal activity; while at neutral to alkaline pH, there is an increase in activity.

These chemical agents antifungal activities were found to deceased with increase in temperature (45ºC to 100ºC). This could be due to denatured structure of the compounds at very high temperature which in turn affect the antifungal activity of the chemical agents. Aulton (2007) reported that the rate of reaction is markedly influenced by temperature. Furthermore it has been reported that using high-performance liquid chromatography the concentration of fluconazole was virtually changed when stored in the dark at above 45 C and sampled after 1, 2, 3, and 15 days (Yamreudeewong et al 1993).

Little is known about the use of these commonly known antifungal agents to be used for the purpose of preservation. Observation in this study support other reports (Skiba, et al 2000) that ketoconazole was unstable in acidic pH, hence the reduced activity.

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