

Biological Control of Post Harvest Fungal Rot of Water Yam (*Dioscorea alata* L.) Tubers in Storage with *Cryptococcus nemorosus*, *Rhodotorula toruloides* and *Nakaseomyces glabratus*

ABSTRACT

Water yam tubers are staple food consumed in the South-Eastern Nigeria and are susceptible to microbial contamination in storage. This investigation focused on antagonistic potentials of saprophytic fungi, *Cryptococcus nemorosus*, *Rhodotorula toruloides* and *Nakaseomyces glabratus* against the spoilage fungi of water yam tubers in storage. The diseased tissues of the water yam samples were inoculated on PDA in the Petri dishes. The microbial pathogens obtained were; *Botryodiplodia theobromae*, *Aspergillus niger*, *Rhizopus stolonifer*, *Fusarium solani* and *Penicillium* spp. Analysis of Variance (ANOVA) was employed and the Duncan's New Multiple Range Test (DNMRT) was used to test the difference among treatments. The results of the *in vitro* assessment of fungal pathogens on water yam samples revealed that *Cryptococcus nemorosus* showed the highest zone of inhibition (13.50 ± 0.12 mm) on *Botryodiplodia theobromae* while the least zone of inhibition (9.14 ± 0.26 mm) was recorded for *Fusarium solani* after 48 hours of incubation. *Rhodotorula toruloides* recorded the highest zone of inhibition (22.10 ± 0.45 mm) on *Rhizopus stolonifer* whereas the least zone of inhibition (9.05 ± 0.36 mm) was observed on *Botryodiplodia theobromae* after 48 hours of incubation. *Nakaseomyces glabratus* showed the highest zone of inhibition (27.80 ± 0.13 mm) on *Botryodiplodia theobromae* while the least zone of inhibition (14.84 ± 0.20 mm) was recorded against *Fusarium solani* after 48 hours of incubation. The results of the *in vivo* mean mycelia radial growth of the fungal pathogens revealed that *Cryptococcus nemorosus* showed the highest growth (73.75 ± 0.11 mm) against *Aspergillus niger* while the least growth (64.80 ± 0.15 mm) was observed on *Rhizopus stolonifer* after 48 hours of incubation. *Rhodotorula toruloides* had the highest growth (68.45 ± 0.50 mm) on *Aspergillus niger* while the least growth (55.50 ± 0.09 mm) was recorded against *Rhizopus stolonifer* after 48 hours of incubation. *Nakaseomyces glabratus* showed the highest growth (67.00 ± 0.12 mm) on *Rhizopus stolonifer* whereas the least growth (51.90 ± 0.12 mm) was recorded against *Botryodiplodia theobromae* after 48 hours of incubation. The fungitoxic potentials of these biological antagonists on water yam rot can provide an alternative to synthetic fungicides since it is less expensive, environmentally friendly and easy to prepare.

Keywords: Biological control, Fungal rot, Postharvest, Water Yam, *Cryptococcus nemorosus*, *Rhodotorula toruloides*, *Nakaseomyces glabratus*

Introduction

Yam (*Dioscorea* spp) belongs to the genus *Dioscorea* (Family *Dioscoreaceae*) and is the second most important tropical root crop in West Africa after cassava (Adisa *et al.*, 2015) and form an

important food source in other tropical countries including East Africa, the Caribbean, South America, India and South East Asia (Coursey, 1967; Okigbo, 2002).

Okigbo (2002) estimated that the world production of yams is around 20 million tonnes per year. The greatest part of the world yam production (over 90%) is derived from West Africa (Coursey, 1967; Okigbo, 2002; Sahel, 2014) and Nigeria alone produces three-quarters of the world total output of yam (Modu *et al.*, 2015; Okigbo, 2002). Yams are t

he fifth most harvested crops in Nigeria, following after cassava, maize, guinea corn and beans/cowpeas. More so, after cassava, yams are the most commonly harvested tuber crops in the country (NBS, 2012). Yams do not only serve as the main source of earnings and food consumption, but also as a major employer of labour in Nigeria.

Despite the importance of yams to people, the attention to its production is still questionable (Verter and Becvarova, 2014). Studies by Zaknayiba and Tanko (2013) reveal that lack of access to inputs, finance, poor producer prices, inadequate of storage facilities, incidences of pests and diseases have negatively affected yam production. Similarly, (Maikasuwa and Ala, 2013) examined some determinants of yam production in particular regions in Nigeria. They found that the factors of production such as labour, finance and material inputs like fertilizer have influenced yam production in the region.

Among the wide species reported, only about ten species are estimated to have been domesticated across Africa, Asia and Latin America for food and income generation (Scarcelli *et al.*, 2017). Of the ten cultivated species of yam, the six most important in Nigeria are: *Dioscorea rotundata* Poir (white yam), *Dioscorea alata* L. (water yam), *Dioscorea cayenensis* Lam. (yellow yam), *Dioscorea dumetorum* (Kunth) Pax. (cluster or bitter yam), *Dioscorea bulbifera* L.

(aerial yam) and *Dioscorea esculenta* (Loir) Bark (Chinese yam) (Adeniji, 1970a; Okigbo, 2004).

Water yam (*Dioscorea alata* L.) is the most economically important yam species which serve as a staple food for millions of people in tropical and subtropical countries (Coursey, 1967; Hahn, 1995). *Dioscorea alata* is a crop with potential for increased consumer demand due to its low sugar content necessary for diabetic patients (Udensi *et al.*, 2010). According to Scott *et al.* (2000), water yam (*Dioscorea alata* L.) is the most widely distributed species of yam, though the total quantity produced is less than that of white yam. Water yam (*Dioscorea alata* L.) is grown widely in tropical and subtropical regions of the world. They are plants yielding tubers and contain starch between 70% and 80% of dry matter (Zhang and Oates, 1999; Shang *et al.*, 2007).

The tuber is the only economically important part of the crop and according to Sangoyomi (2004), it is consumed roasted, fried, boiled, pounded or as flour which can be reconstituted with hot water. Yam tubers are of a very high value, as in food, where it is a major source of carbohydrate, minerals of calcium, phosphorus, iron and vitamin B and C (Coursey, 1967; Okigbo and Ogbonnaya, 2006). It provides other nutritional benefits such as proteins, lipids, vitamins and minerals (Lasztity *et al.*, 1998).

Across different ethnic communities and geographic regions, diverse species of *Dioscorea* have been adapted within different habitation as a food source due to the high nutritional benefits and therapeutic values toward treatment and cure of certain health problems (Dutta, 2015; Trimanto and Hapsari, 2015). While yam is one of the most important staple root and tuber crops worldwide, it is still classified as an orphan crop because it is highly underutilized and receives little investment or research attention toward crop improvement. Yam plays a significant role in food security, medicine and economy in the developing countries. Its importance places it as the

fourth most essential and utilized root and tuber crop globally after potatoes (*Solanum* spp.), cassava (*Manihot esculenta*) and sweet potatoes (*Ipomoea* spp.) and the second in West Africa after cassava (Dansie *et al.*, 2013). This is evident in annual global production, especially in West Africa.

In addition, the significance of yam in the cultural, social and religious environment of West Africa cannot be overemphasized (Obidiegwu and Akpabio, 2017). Its symbolism as king of crops is manifested in its use in ceremonies such as those for fertility and marriages, as well as an annual festival held to celebrate its harvest. Importantly, the cultural and linguistic diversity that cuts across West Africa has no influence on the beliefs, social values and religious practices attached to the yam crop. In spite of the importance of yams as major staple food and its socio-cultural value in the lives of the people of the West and Central Africa sub-region, research and documentation on this important staple food crop is very limited (Sangoyomi, 2004). This present investigation focused on the indigenous knowledge of water yam in storage in the South-Eastern Nigeria.

Materials and Methods

Sources of Sample Materials

Water yams (*Dioscorea alata* L.) with symptoms of post harvest rot were obtained from 25 farmers' yam barns in major yam markets within the five States that made up the South East, Nigeria and was authenticated by the head of yam programme, National Root Crops Research Institute, Umudike, Abia State. The collected samples were taken to the Central Service Laboratory of the Institute for further studies. Ten fresh and healthy tomato, orange and lime fruits each were collected from Onitsha Main Market. The method of Adetunji (2014) was adopted in sampling diseased yam (*Dioscorea alata* L.) tubers, i.e. tubers that showed symptoms

of rot. The rot signs of the infected yam tubers were black patches, browning, discolouration, softening and foul or offensive odour/smell.

Preparation of Culture Media

Potato Dextrose Agar (PDA) was used as the medium for the growth and maintenance of the fungal isolates. The Potato Dextrose Agar (PDA) was prepared in accordance with the manufacturer's instruction. Thirty-nine (39g) of dehydrated PDA powder was weighed into one-liter glass conical flask. The weighed PDA powder was dispersed in about 800ml of distilled water and heated in an electric water bath until the agar melted. It was then made up to one-liter with distilled water and its pH was measured using a digital pH meter to ensure compliance with the standard (5.4 to 5.8). The prepared medium was transferred to a Pyrex media bottle and sterilized in the autoclave at 121⁰C, pressure of 15 (Psi) for 15 minutes (Cheesbrough, 2000; Jawetz *et al.*, 2004). After sterilization, the medium was allowed to cool to about 45°C and 1ml of 10% concentration of lactic acid solution was added to it to suppress bacterial growth and contaminations. It was mixed properly and then aseptically dispensed into 15ml aliquots to sterile glass Petri dishes. They were allowed to cool down and gel. After solidification, each agar plate was aseptically wrapped round (externally) with masking tape until needed for use. These plates were stored in the refrigerator.

Isolation of Fungal Pathogens from Rotten Water Yam Tubers

The collected rotten water yam tubers were rinsed in sterilized distilled water and surface sterilized with 70% ethanol. Each was cut open and three pieces of the infected tissues were removed from the point of advancement of rot using 3mm diameter cork borer and inoculated on the solidified Potatoes Dextrose Agar (PDA) in Petri dish. Three replicates of each were made. The inoculated plates were incubated at room temperature ($28 \pm 2^{\circ}\text{C}$) for 48 hours and

observations were made daily for fungi growth. Stock cultures was prepared using slants of PDA in McCartney bottle and stored in a refrigerator at 50°C for further use (Ezeibekwe and Ibe, 2010).

Sub-culturing/Purification and Identification of Fungal Isolates

All the sub-cultured and purified isolates were viewed under a compound microscope. Identification of fungal isolates was based on the morphological or structural features as seen on the culture plates as well as slides viewed under the compound microscope. Generally, morphological or structural features of colony, colour, and extent of growth, presence or absence of mycelia, spores, etc. and the nature of colony surface were observed. Microscopic examination involved slide mounts of test isolates and stained with Lactophenol Cotton Blue Stain (LCBS). Morphological or structural features as observed from each isolates were matched against those present in standard manual or identification guides (Barnett and Hunter, 2012; Dugan, 2006). Using morphological traits and matching the results to establish keys as given by Nwachukwu and Osuji (2008), fungi were so identified as being isolated.

Determination of Percentage Frequency Occurrence of the Fungal Isolates

This was done to determine the incidence of occurrence of the different fungal isolates associated with Yam (*Dioscorea alata* L.) tubers. The total number of each isolate in all samples was obtained against the total number of all the isolates in all the samples screened. Frequencies of occurrence of different fungal isolates were therefore determined using methods described by Giridher and Ready, 1997; Okigbo *et al.*, 2015). Number of times each fungus was encountered was recorded and the percentage frequency of occurrence was calculated using the formula described by Ebele (2011) thus:

$$\frac{\text{Number of times a fungus was encountered} \times 100}{\text{Total fungal isolations}}$$

Pathogenicity Test

Ten healthy yam tubers were washed under running tap water to removed soil (dirt). Surface sterilization was made by dipping each yam tuber in to 10% concentration of sodium hypochlorite for two minutes and rinsed twice in sterile distilled water (SDW). The tubers were placed on sterile paper towels in the Laminar Air Flow Cabinet (Environmental Air Control, Inc. USA) to dry for 20 minutes. Cylindrical holes were drilled at the proximal and distal ends of the yam tubers using a sterile 10 mm cork borer. Discs of five days old pathogen cultures were plug in the holes created in the yam tubers and the disc of the tuber in the cork borer was placed back. Then Vaseline was applied on the point of inoculation and incubated at room temperature. Three replications of each were made. After 72 hrs, the yams were cut transversely and the observation of the symptoms produced was recorded and the pathogens were re-isolated (Agrios, 2005).

Isolation, Purification and Identification of Saprophytic Fungi

Saprophytic yeasts were isolated from the surface of fresh apparently healthy tomato, orange and lime fruits collected from Main Market, Onitsha. Each was placed in a 500ml beaker containing 200ml sterilized distilled water (SDW) placed on a rotary shaker at 100 rpm for 12 hrs. 0.1ml of suspension was taken from the beaker, spread on a Potato Dextrose Agar (PDA) plates and incubated at ambient temperature ($28 \pm 20^{\circ}\text{C}$) for 24 hrs for yeast colonies to develop. Sub-cultures were made to obtain pure isolates. Pure isolates were kept on slants and put in refrigerator at 10.50°C for further use.

Harvesting of Saprophytic Fungi

Three plates (9cm in diameter) of fungal isolates, comprising both rot pathogens and biological antagonists were harvested. Sterile Distilled Water (SDW) was poured into the Petri dishes

containing fungal isolates of 14-days old and a sterile scalpel was used to scrape the cultures gently. The dispersed fungi in the liquid medium were then poured into a beaker of 300 litre SDW and covered with sterile cheese cloth. The cheese cloth traps the mycelia and allows the entrance of the spores/broken hyphae. Triton X was added in the liquid medium to aid in the evenly disperse of spores.

Counting of the Colonies

Using a hand tally counter, a counting tool that resembles a stopwatch, the number of colonies in each plate was counted after the plates had been incubated (Dhawale and La Master, 2003). The count's mean was calculated, and it was then multiplied by the proper dilution factor.

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 ($\mu\text{g/ml}$). 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 30 minutes. The plates were then incubated at 30°C for 48 hours. 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 Zone of Inhibition (*In Vitro*) of Fungi Isolates by Saprophytic Fungi 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 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 100 µg/ml) of the different anti-fungal agents were prepared. Then, 100 µl 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 30°C for 48 hours. The zones of inhibition were measured using a well calibrated transparent meter ruler.

***In Vivo* Effects of Biological Control Agents on Rot Pathogens of Water Yam Tubers**

The prospective antagonists, saprophytic fungi that showed the highest antagonism *in vitro* experiments were tested against fungal isolates on water yam tubers. The selected tubers were healthy and commonly 300 mm long with a girth of 55 mm (middle region). Water yam tubers were surface sterilized with 70% ethanol for 3 minutes and a 3 mm deep and 6 mm wide inoculation well was made in the middle region of the water yam tubers using a cork borer. The inoculum was 0.1 ml of a conidial suspension of 2×10^4 spores/ml prepared in potato dextrose (PDA) broth from a five-day-old PDA culture of a test fungus.

Following inoculation, the cylinder of water yam tissue originally removed to create the inoculation well was replaced and the edges of the well were sealed with sterile molten candle wax. Four replicate tubers were prepared for each of the treatments and these were incubated at room temperature for four weeks before the tubers were cut open through the inoculation sites, and along the main axis of the tuber, to assess the extent of rot. Rot was estimated, as the sum of the width (along the main axis of tuber) and depth (perpendicular to main axis) of the rot affected tissue. The extent of rot estimated for a fungus-antagonist pairing was compared with that of the appropriate control (fungus alone) to obtain a measure of the level of disease control and thus, of *in vivo* antagonism by the antagonist. The degree of rot was evaluated by comparing with the

controls and the percentage inhibition of rot by the antagonists over the pathogenic fungi was also evaluated according to the method described by (Whipps, 1987).

$$\text{Percentage inhibition} = \frac{R_1 - R_2}{R_1} \times 100\%$$

Where; R_1 is the furthest radial distance of pathogen in control tubers; R_2 is the furthest radial distance of pathogen in antagonist-incorporated tubers.

The inhibition percentage was determined as a guide in selecting the Minimum Inhibition Concentration (MIC) that will be effective in controlling the rot-causing fungi. Antagonists were also rated for their inhibitory effects using a scale by Sangoyomi (2004).

$\leq 0\%$ inhibition (not effective),

$> 0 - 20\%$ inhibition (slightly effective),

$> 20 - 50\%$ inhibition (moderately effective),

$> 50 - < 100\%$ inhibition (effective),

100% inhibition (highly effective).

Experimental Design/Statistical Analysis

The experimental design used was randomized complete block design (RCBD) with three replicates. Test of variance was calculated using Analysis of Variance (ANOVA) via statistical analysis system (SAS) of version 9.1 and means were separated with Duncan's Multiple Range Test (DNMRT) at $P < 0.05$.

Results

Fungi Count of Rotten Water Yam Tubers

The fungal pathogens that were constantly isolated from the rot-infested tissues of the water yam tubers were *Botryodiplodia theobromae* Pat., *Aspergillus niger* Van Tiegh, *Rhizopus stolonifer* Vuill, *Fusarium solani* Matt. and *Penicillium* spp. (Table 1). The results revealed that sample 2 gave the highest colony count (2.80×10^7) which was followed by sample 1 with colony count of (2.50×10^7). Whereas sample 5 had the lowest colony count of (1.90×10^7) (Table 1).

Table 1: Mean fungi count of rotten water yam tubers

Sample	Mean Total Fungi Count (cfu/g)
Sample 1	2.50×10^7
Sample 2	2.80×10^7
Sample 3	2.00×10^7
Sample 4	2.45×10^7
Sample 5	1.90×10^7

Percentage Frequency of Occurrence of Fungal Isolates on the Water Yam Tubers

The results of the percentage occurrence of fungal isolates from water yam samples revealed that *B. theobromae* recorded the highest percentage occurrence of 80.00%, followed by *A. niger* with

percentage occurrence of 22.00%. Whereas the lowest percentage occurrence (9.00%) was recorded against *F. solani* and *Penicillium* spp respectively (Table 2).

Table 2: Incidence occurrence of fungal isolates associated with water yam tubers

Fungal Isolates	% Occurrence
<i>B. theobromae</i>	80.00
<i>A. niger</i>	22.00
<i>R. stolonifer</i>	15.00
<i>F. solani</i>	9.00
<i>Penicillium</i> spp	9.00

Pathogenicity Test of Fungal Isolates from Water Yam Tubers

The results of the pathogenicity test showed that the test fungi (*B. theobromae*, *A. niger*) were pathogenic, hence causing the same disease and rot type noticed on the rot infected sample (Plate 1) after three (3) days of inoculation. The more virulent spoilage fungi was *B. theobromae*, causing 15% rot on the *D. alata*, followed by *A. niger* with 8% rot on *D. alata* (Table 3).

Table 3: Pathogenicity test of fungi isolated from water yam tubers

Fungal Isolates	Percentage Rot
<i>B. theobromae</i>	15

Zone of Inhibition (*In Vitro*) of Fungi Isolates with Biofungicides

The results of the *in vitro* assessment of fungal pathogens on water yam tubers revealed that *Cryptococcus nemorosus* showed the highest zone of inhibition (13.50 ± 0.12 mm) on *Botryodiplodia theobromae* while the least zone of inhibition (9.14 ± 0.26 mm) was recorded for *Fusarium solani* after 48 hours of incubation. *Cryptococcus nemorosus* recorded the highest zone of inhibition (22.10 ± 0.18 mm) against *Rhizopus stolonifer* while the least zone of inhibition (12.10 ± 0.36 mm) was observed on *Fusarium solani* after 96 hours of incubation. *Cryptococcus nemorosus* had the highest zone of inhibition (23.80 ± 0.10 mm) against *Rhizopus stolonifer* while the least zone of inhibition (16.80 ± 0.35 mm) was observed on *Fusarium solani* after 144 hours of incubation. *Cryptococcus nemorosus* showed the highest zone of inhibition (32.60 ± 2.10 mm) on *Aspergillus niger* whereas the least zone of inhibition (20.30 ± 0.70 mm) was recorded against *Fusarium solani* after 192 hours of incubation (Table 4).

The results revealed that *Rhodotorula toruloides* recorded the highest zone of inhibition (22.10 ± 0.45 mm) on *Rhizopus stolonifer* whereas the least zone of inhibition (9.05 ± 0.36 mm) was observed on *Botryodiplodia theobromae* after 48 hours of incubation. *Rhodotorula toruloides* recorded the highest zone of inhibition (25.10 ± 0.22 mm) on *Rhizopus stolonifer* while the least zone of inhibition (12.10 ± 0.11 mm) was noticed on *Fusarium solani* after 96 hours of incubation. *Rhodotorula toruloides* had the highest zone of inhibition (28.50 ± 0.37 mm) on *Rhizopus stolonifer* whereas the least zone of inhibition (15.70 ± 0.12 mm) was observed on

Fusarium solani after 144 hours of incubation. *Rhodotorula toruloides* had the highest zone of inhibition (39.75 ± 0.15 mm) on *Aspergillus niger* while the least zone of inhibition (18.66 ± 0.10 mm) was noticed on *Fusarium solani* after 192 hours of incubation (Table 4).

The results revealed that *Nakaseomyces glabratus* showed the highest zone of inhibition (27.80 ± 0.13 mm) on *Botryodiplodia theobromae* while the least zone of inhibition (14.84 ± 0.20 mm) was recorded against *Fusarium solani* after 48 hours of incubation. *Nakaseomyces glabratus* had the highest zone of inhibition (32.20 ± 0.12 mm) on *Rhizopus Stolonifer* while the least zone of inhibition (19.84 ± 1.00 mm) was observed on *Fusarium solani* after 96 hours of incubation. *Nakaseomyces glabratus* showed the highest zone of inhibition (38.00 ± 0.10 mm) on *Rhizopus stolonifer* whereas the least zone of inhibition (22.50 ± 0.25 mm) was noticed on *Fusarium solani* after 144 hours of incubation. *Nakaseomyces glabratus* showed the highest zone of inhibition (42.40 ± 0.10 mm) on *Rhizopus stolonifer* whereas the least zone of inhibition (26.17 ± 0.31 mm) was observed on *Fusarium solani* after 192 hours of incubation (Table 4).

Table 4: Mean zone of inhibition (*in vitro*) of fungal pathogens using *Cryptococcus nemorosus*, *Rhodotorula toruloides* and *Nakaseomyces glabratus* (mm)

Biofungicides/ Period	<i>Botryodiplodia theobromae</i>	<i>Aspergillus niger</i>	<i>Rhizopus stolonifer</i>	<i>Fusarium solani</i>	<i>Penicillium spp</i>
<i>Cryptococcus nemorosus</i>					
192 hrs	20.70±0.53 ^a	32.60±2.10 ^d	29.60±0.15 ^c	20.30±0.70 ^a	25.40 ^c ±0.12 ^b
144 hrs	17.10±0.20 ^a	19.00±2.17 ^b	23.80±0.10 ^c	16.80±0.35 ^a	19.30±0.05 ^b
96 hrs	16.18±0.08 ^c	13.33±0.10 ^a	22.10±0.18 ^d	12.10±0.36 ^a	15.00±0.90 ^b
48 hrs	13.50±0.12 ^c	10.75±0.12 ^a	12.10±0.18 ^b	9.14±0.26 ^a	11.03±0.60 ^b
<i>Rhodotorula toruloides</i>					
192 hrs	30.00±0.16 ^b	39.75±0.15 ^e	33.40±0.28 ^c	18.66±0.10 ^a	35.20±0.12 ^d
144 hrs	24.90±2.11 ^c	23.45±0.12 ^c	28.50±0.37 ^d	15.70±0.12 ^a	21.10±0.15 ^b
96 hrs	14.00±1.00 ^b	22.07±0.18 ^d	25.10±0.22 ^e	12.10±0.11 ^a	18.11±0.11 ^c
48 hrs	9.05±0.36 ^a	17.10±0.11 ^c	22.10±0.45 ^d	10.10±0.10 ^a	15.60±0.10 ^b
<i>Nakaseomyces</i>					

glabratus

192 hrs	39.90±0.15 ^b	39.10±0.10 ^b	42.40±0.10 ^d	26.17±0.31 ^a	40.50±0.38 ^c
144 hrs	33.70±1.00 ^c	30.16±0.03 ^b	38.00±0.10 ^d	22.50±0.25 ^a	36.70±0.17 ^e
96 hrs	30.80±0.11 ^c	25.60±0.02 ^b	32.20±0.12 ^d	19.84±1.00 ^a	30.20±3.10 ^c
48 hrs	27.80±0.13 ^e	18.00±0.10 ^b	26.20±2.00 ^d	14.84±0.20 ^a	21.70±0.15 ^c

Values are presented as means ± standard error; Figures bearing similar superscripts within columns are not significantly different at P<0.05 using Duncan New Multiple Range Test.

***In Vivo* Effects of Biological Control Agents on Rot Pathogens of Water Yam Tubers**

The results of the *in vivo* mean mycelia radial growth of the fungal pathogens revealed that *Cryptococcus nemorosus* showed the highest growth (73.75 ± 0.11 mm) against *Aspergillus niger* while the least growth (64.80 ± 0.15 mm) was observed on *Rhizopus stolonifer* after 48 hours of incubation. *Cryptococcus nemorosus* recorded the highest growth (78.33 ± 0.16 mm) against *Aspergillus niger* whereas the least growth (68.66 ± 0.07 mm) was noticed on *Rhizopus stolonifer* after 96 hours of incubation. *Cryptococcus nemorosus* caused the highest growth (82.60 ± 0.12 mm) against *Aspergillus niger* whereas the least growth (72.20 ± 1.20 mm) was recorded against *Fusarium solani* after 144 hours of incubation. *Cryptococcus nemorosus* showed the highest growth (88.10 ± 0.10 mm) against *Rhizopus stolonifer* while the least growth (76.80 ± 0.10 mm) was observed on *Fusarium solani* after 192 hours of incubation (Table 5).

The results revealed that *Rhodotorula toruloides* had the highest growth (68.45 ± 0.50 mm) against *Aspergillus niger* while the least growth (55.50 ± 0.09 mm) was recorded against *Rhizopus stolonifer* after 48 hours of incubation. *Rhodotorula toruloides* showed the highest growth (71.10 ± 0.11 mm) against *Fusarium solani* whereas the least growth (62.10 ± 0.12 mm) was noticed on *Rhizopus stolonifer* after 96 hours of incubation. *Rhodotorula toruloides* showed the highest growth (84.20 ± 0.30 mm) against *Penicillium* spp while the least growth (68.40 ± 0.13 mm) was recorded against *Rhizopus stolonifer* after 144 hours of incubation. *Rhodotorula toruloides* had the highest growth (90.60 ± 0.11 mm) against *Botryodiplodia theobromae* and the least growth (72.10 ± 0.10 mm) was observed on *Aspergillus niger* after 192 hours of incubation (Table 5).

The results revealed that *Nakaseomyces glabratus* showed the highest growth (67.00 ± 0.12 mm) against *Rhizopus stolonifer* whereas the least growth (51.90 ± 0.12 mm) was recorded against

Botryodiplodia theobromae after 48 hours of incubation. *Nakaseomyces glabratus* had the highest growth (70.90 ± 0.60 mm) against *Rhizopus stolonifer* while the least growth (63.70 ± 0.30 mm) was indicated against *Botryodiplodia theobromae* after 96 hours of incubation. *Nakaseomyces glabratus* showed the highest growth (79.60 ± 0.10 mm) against *Rhizopus stolonifer* whereas the least growth (72.50 ± 0.02 mm) was observed on *Fusarium solani* after 144 hours of incubation. *Nakaseomyces glabratus* caused the highest growth (80.40 ± 0.15 mm) against *Rhizopus stolonifer* while the least growth (75.00 ± 0.10 mm) was noticed on *Botryodiplodia theobromae* after 192 hours of incubation (Table 5).

The results of the control in the *in vivo* study of the mycelia growth of the fungal pathogens revealed that the control showed the highest growth (92.10 ± 1.04 mm) against *Penicillium* spp whereas the least growth (83.60 ± 1.00 mm) was recorded against *Rhizopus stolonifer* after 48 hours of incubation. The control had the highest growth (93.10 ± 0.10 mm) against *Penicillium* spp while the least growth (89.80 ± 0.05 mm) was observed on *Rhizopus stolonifer* after 96 hours of incubation. The control caused the highest growth (96.00 ± 0.13 mm) against *Rhizopus stolonifer* while the least growth (91.60 ± 0.12 mm) was noticed on *Fusarium solani* after 144 hours of incubation. The control had the highest growth (98.10 ± 1.00 mm) against *Penicillium* spp whereas the least growth (95.80 ± 0.11 mm) was observed on *Fusarium solani* after 192 hours of incubation (Table 5).

Table 5: Mean radial growth (*in vivo*) of fungal pathogens using *Cryptococcus nemorosus*, *Rhodotorula toruloides* and *Nakaseomyces glabratus* (mm)

Biofungicides/ Period	<i>Botryodiplodia theobromae</i>	<i>Aspergillus niger</i>	<i>Rhizopus stolonifer</i>	<i>Fusarium solani</i>	<i>Penicillium spp</i>
<i>Cryptococcus nemorosus</i>					
192 hrs	85.00±0.40 ^b	85.30±0.30 ^b	88.10±0.10 ^c	76.80±0.10 ^a	87.40±0.09 ^c
144 hrs	80.00±2.14 ^c	82.60±0.12 ^d	80.65±0.10 ^c	72.20±1.20 ^a	72.30±0.17 ^b
96 hrs	77.10±0.30 ^c	78.33±0.16 ^c	68.66±0.07 ^a	69.10±1.12 ^a	72.30±0.14 ^b
48 hrs	68.70±0.51 ^b	73.75±0.11 ^c	64.80±0.15 ^a	65.14±1.10 ^a	68.20±0.10 ^b
<i>Rhodotorula toruloides</i>					
192 hrs	90.60±0.11 ^d	72.10±0.10 ^a	87.10±0.10 ^c	77.10±0.12 ^b	88.20±0.50 ^c
144 hrs	78.50±0.10 ^d	70.07±0.16 ^b	68.40±0.13 ^a	74.66±0.10 ^c	84.20±0.30 ^c
96 hrs	71.00±0.17 ^d	69.75±0.18 ^c	62.10±0.12 ^a	71.10±0.11 ^d	64.60±0.10 ^b
48 hrs	66.30±0.15 ^c	68.45±0.50 ^d	55.50±0.09 ^a	56.70±0.09 ^a	62.10±0.40 ^b
<i>Nakaseomyces glabratus</i>					

192 hrs	75.00±0.10 ^a	76.10±0.18 ^a	80.40±0.15 ^c	78.84±0.80 ^b	75.70±0.16 ^a
144 hrs	69.90±1.00 ^a	73.16±0.12 ^b	79.60±0.10 ^c	72.50±0.02 ^b	73.70±0.22 ^b
96 hrs	63.70±0.30 ^a	68.10±0.11 ^c	70.90±0.60 ^d	65.84±0.01 ^b	67.20±0.10 ^c
48 hrs	51.90±0.12 ^a	60.70±0.15 ^b	67.00±0.12 ^c	59.17±0.13 ^b	66.00±0.40 ^c
Control					
192 hrs	96.40±0.25 ^a	96.10± 0.11 ^a	97.60± 0.12 ^a	95.80± 0.11 ^a	98.10±1.00 ^a
144 hrs	94.60± 0.33 ^b	94.20± 3.10 ^b	96.00± 0.13 ^c	91.60± 0.12 ^a	94.10± 1.33 ^b
96 hrs	92.10± 0.70 ^c	90.10± 0.03 ^b	89.80± 0.05 ^a	90.30± 0.15 ^b	93.10±1.10 ^c
48 hrs	90.10± 1.10 ^d	86.10±0.17 ^b	83.60± 1.00 ^a	88.00± 0.17 ^c	92.10± 1.04 ^c

Values are presented as means ± standard error; Figures bearing similar superscripts within columns are not significantly different at P<0.05 using Duncan New Multiple Range Test.

Discussion

The fungi associated with post-harvest rot of water yam obtained from different markets in the South-Eastern Nigeria were *B. theobromae*, *A. niger*, *R. stolonifer*, *F. solani* and *Penicillium* spp. This study agrees with the findings by (Okigbo, 2002, 2005; Okigbo and Ogbonnaya, 2006). The results of the percentage frequency of occurrence revealed that *B. theobromae* recorded the highest percentage occurrence of 80.00% while the lowest percentage occurrence of 9.00% was recorded against *F. solani* and *Penicillium* spp respectively. The results of the pathogenicity test

revealed that the test fungi (*Botryodiplodia theobromae*, *Aspergillus niger*, *Rhizopus stolonifer*, *Fusarium solani* and *Penicillium* spp) were pathogenic, hence causing the same disease and rot type noticed on the rot infected samples. This was due to the ability of the pathogen to utilize the nutrient of water yam as a substrate for growth and development. This result is in line with the finding of Bali *et al.* (2018) who implicated *Aspergillus niger* for post-harvest deterioration in sweet orange and acid lime at the field. *Aspergillus niger*, *Alternaria* spp, *Botryodiplodia theobromae*, and *Colletotrichum gloeosporioides* were isolated from the damaged cocoyam, according to Okereke *et al.* (2021). The prevalence of these fungi which developed black mould on some fruits and crops generates strong mycotoxins called ochratoxins that can be dangerous to humans and animals. This outcome is consistent with many reports on other root and tuber crops (Okigbo *et al.*, 2018). The more virulent spoilage fungi was *B. theobromae*, causing 15% rot on the *D. alata*, followed by *A. niger* with 8% rot on *D. alata*. This study is similar to the report on fungi associated with Nigerian yams by (Okigbo and Ikediugwu, 2000).

In this study, biological antagonists, *Cryptococcus nemorosus*, *Rhodotorula toruloides* and *Nakaseomyces glabratus* were used to control the pathogens of *D. alata* and these produced a significant inhibition on the growth of the pathogenic fungi on postharvest rot of water yam tubers. The results of the *in vitro* assessment of fungi pathogens on water yam tubers revealed that *C. nemorosus* had the highest zone of inhibition (13.50 ± 0.12 mm) on *Botryodiplodia theobromae* while the least zone of inhibition (9.14 ± 0.26 mm) was recorded for *Fusarium solani* after 48 hours of incubation. *C. nemorosus* recorded the highest zone of inhibition (22.10 ± 0.18 mm) against *Rhizopus stolonifer* while the least zone of inhibition (12.10 ± 0.36 mm) was observed on *Fusarium solani* after 96 hours of incubation. *C. nemorosus* had the highest zone of inhibition (23.80 ± 0.10 mm) against *Rhizopus stolonifer* while the least zone of inhibition

(16.80±0.35 mm) was observed on *Fusarium solani* after 144 hours of incubation. *C. nemorosus* showed the highest zone of inhibition (32.60±2.10 mm) on *Aspergillus niger* whereas the least zone of inhibition (20.30±0.70 mm) was recorded against *Fusarium solani* after 192 hours of incubation.

The results revealed that *R. toruloides* recorded the highest zone of inhibition (22.10±0.45 mm) on *Rhizopus stolonifer* whereas the least zone of inhibition (9.05±0.36 mm) was observed on *Botryodiplodia theobromae* after 48 hours of incubation. *R. toruloides* recorded the highest zone of inhibition (25.10±0.22 mm) on *Rhizopus stolonifer* while the least zone of inhibition (12.10±0.11 mm) was noticed on *Fusarium solani* after 96 hours of incubation. *R. toruloides* had the highest zone of inhibition (28.50±0.37 mm) on *Rhizopus stolonifer* whereas the least zone of inhibition (15.70±0.12 mm) was observed on *Fusarium solani* after 144 hours of incubation. *R. toruloides* had the highest zone of inhibition (39.75±0.15 mm) on *Aspergillus niger* while the least zone of inhibition (18.66±0.10 mm) was noticed on *Fusarium solani* after 192 hours of incubation.

The results revealed that *N. glabratus* showed the highest zone of inhibition (27.80±0.13 mm) on *Botryodiplodia theobromae* while the least zone of inhibition (14.84±0.20 mm) was recorded against *Fusarium solani* after 48 hours of incubation. *N. glabratus* had the highest zone of inhibition (32.20±0.12 mm) on *Rhizopus Stolonifer* while the least zone of inhibition (19.84±1.00 mm) was observed on *Fusarium solani* after 96 hours of incubation. *N. glabratus* showed the highest zone of inhibition (38.00±0.10 mm) on *Rhizopus stolonifer* whereas the least zone of inhibition (22.50±0.25 mm) was noticed on *Fusarium solani* after 144 hours of incubation. *N. glabratus* showed the highest zone of inhibition (42.40±0.10 mm) on *Rhizopus*

stolonifer whereas the least zone of inhibition (26.17 ± 0.31 mm) was observed on *Fusarium solani* after 192 hours of incubation.

The results of the *in vivo* mean mycelia radial growth of the fungal pathogens revealed that *C. nemorosus* showed the highest growth (73.75 ± 0.11 mm) against *Aspergillus niger* while the least growth (64.80 ± 0.15 mm) was observed on *Rhizopus stolonifer* after 48 hours of incubation. *C. nemorosus* recorded the highest growth (78.33 ± 0.16 mm) against *Aspergillus niger* whereas the least growth (68.66 ± 0.07 mm) was noticed on *Rhizopus stolonifer* after 96 hours of incubation. *C. nemorosus* caused the highest growth (82.60 ± 0.12 mm) against *Aspergillus niger* whereas the least growth (72.20 ± 1.20 mm) was recorded against *Fusarium solani* after 144 hours of incubation. *C. nemorosus* showed the highest growth (88.10 ± 0.10 mm) against *Rhizopus stolonifer* while the least growth (76.80 ± 0.10 mm) was observed on *Fusarium solani* after 192 hours of incubation.

The results revealed that *R. toruloides* had the highest growth (68.45 ± 0.50 mm) against *Aspergillus niger* while the least growth (55.50 ± 0.09 mm) was recorded against *Rhizopus stolonifer* after 48 hours of incubation. *R. toruloides* showed the highest growth (71.10 ± 0.11 mm) against *Fusarium solani* whereas the least growth (62.10 ± 0.12 mm) was noticed on *Rhizopus stolonifer* after 96 hours of incubation. *R. toruloides* showed the highest growth (84.20 ± 0.30 mm) against *Penicillium* spp while the least growth (68.40 ± 0.13 mm) was recorded against *Rhizopus stolonifer* after 144 hours of incubation. *R. toruloides* had the highest growth (90.60 ± 0.11 mm) against *Botryodiplodia theobromae* and the least growth (72.10 ± 0.10 mm) was observed on *Aspergillus niger* after 192 hours of incubation.

The results revealed that *N. glabratus* showed the highest growth (67.00 ± 0.12 mm) against *Rhizopus stolonifer* whereas the least growth (51.90 ± 0.12 mm) was recorded against

Botryodiplodia theobromae after 48 hours of incubation. *N. glabratus* had the highest growth (70.90 ± 0.60 mm) against *Rhizopus stolonifer* while the least growth (63.70 ± 0.30 mm) was indicated against *Botryodiplodia theobromae* after 96 hours of incubation. *N. glabratus* showed the highest growth (79.60 ± 0.10 mm) against *Rhizopus stolonifer* whereas the least growth (72.50 ± 0.02 mm) was observed on *Fusarium solani* after 144 hours of incubation. *N. glabratus* caused the highest growth (80.40 ± 0.15 mm) against *Rhizopus stolonifer* while the least growth (75.00 ± 0.10 mm) was noticed on *Botryodiplodia theobromae* after 192 hours of incubation.

The results of the control in the *in vivo* study of the mycelia growth of the fungal pathogens revealed that the control showed the highest growth (92.10 ± 1.04 mm) against *Penicillium* spp whereas the least growth (83.60 ± 1.00 mm) was recorded against *Rhizopus stolonifer* after 48 hours of incubation. The control had the highest growth (93.10 ± 0.10 mm) against *Penicillium* spp while the least growth (89.80 ± 0.05 mm) was observed on *Rhizopus stolonifer* after 96 hours of incubation. The control caused the highest growth (96.00 ± 0.13 mm) against *Rhizopus stolonifer* while the least growth (91.60 ± 0.12 mm) was noticed on *Fusarium solani* after 144 hours of incubation. The control had the highest growth (98.10 ± 1.00 mm) against *Penicillium* spp whereas the least growth (95.80 ± 0.11 mm) was observed on *Fusarium solani* after 192 hours of incubation.

The biological antagonists, *C. nemorosus*, *R. toruloides* and *N. glabratus* may have acted by the production of antibiotic substances that inhibited the growth of these pathogenic fungi. This is in line with the findings of (Siameto *et al.*, 2011; Mokhtar and Aid, 2013; Singh and Sharma, 2014) who reported the production of both non-volatile antibiotics by species of *Trichoderma*. These substances produced by *C. nemorosus*, *R. toruloides* and *N. glabratus* may be responsible in the biological control of storage rot of water yam tubers. This is in accordance with the works of

(Ubalua and Oti, 2007; Okigbo and Emeka, 2010; Markson *et al.*, 2012; Nikolajeva *et al.*, 2012) where species of *Trichoderma* have been exploited in the control of rot fungi of tubers, fruits and vegetable diseases. The antagonistic potential of *C. nemorosus*, *R. toruloides* and *N. glabratus* to inhibit the growth of the isolated fungi in storage is similar to the result of Okigbo and Emeka (2010) who studied the biological control of rot-inducing fungi of water yam (*Dioscorea alata*) with *Trichoderma harzianum*, *Pseudomonas syringae* and *Pseudomonas chlororaphis* and found that the three antagonists significantly inhibited the growth of *Botryodiplodia theobromae* and *Fusarium solani* on yam tubers in storage. Ubalua and Oti (2007) reported inhibition of *B. theobromae*, *A. flavus*, *F. solani* and *Rhizopus* sp. during storage of cassava roots inoculated with *T. viride* and recorded percentage rot of between 0% and 3% in the paired treatments. The use of *C. nemorosus*, *R. toruloides* and *N. glabratus* in controlling postharvest fungal pathogens of water yam tubers in storage is similar to the work earlier conducted by Okigbo and Ikediugwu (2000) who used a single application of *T. harzianum* and protected yam tubers in storage for up to six months.

Bacterial organisms have also been widely used to control fungal rot of tuber crops. Okigbo (2005) in his study used *Bacillus subtilis* to control post harvest fungal rot of yams in storage. In other studies, the saprophytic strain of the bacterium *Pseudomonas syringe* (L-59-66) also satisfactorily controlled the difficult grape rots (*B. cinerea*) and blue mould of *Citrus* (*P. citrinum*) (Arya, 2010). This saprophyte has been developed into a commercial brand (Ecosuinx). In this study, *C. nemorosus*, *R. toruloides* and *N. glabratus* were used to control pathogenic fungi that cause rot in water yam tubers. The antagonists were able to displace the fungi organisms and inhibit their growth significantly.

The study showed that there was an inhibition of the pathogenic fungi organisms when paired with the biological antagonists; *C. nemorosus*, *R. toruloides* and *N. glabratus* which may probably be attributed to the displacement of the pathogenic fungi on the water yam tubers by causing reduction in the percentage rot observed. These antagonists were effective in controlling rot caused by fungal organisms in yam tubers. In addition, *C. nemorosus*, *R. toruloides* and *N. glabratus* may also have produced antifungal substances which function by breaking down the polysaccharides, chitin and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity and limiting the growth of these pathogens (Anita *et al.*, 2012). The result agreed with the findings of Ubalua and Oti (2007) who recorded 0% infection in cassava tubers when *T. viride* was inoculated on the tubers and stored for three weeks. The control tubers that were not treated with the antagonists did not produce any rot symptoms on the water yam tubers throughout the period of incubation. The finding has revealed that *C. nemorosus*, *R. toruloides* and *N. glabratus* are good biological antagonists used to control rot causing pathogens in post harvest diseases of water yam tubers. This can complement or provide better alternative ways of reducing rot in yam tubers than in the use of chemical fungicides which are often very expensive and environmentally hazardous.

Conclusion

The study has demonstrated that *C. nemorosus*, *R. toruloides* and *N. glabratus* are good biological antagonists in the control of postharvest rot fungi pathogens of yam tubers in storage. The biological control agent could therefore, be considered as a substitute to synthetic fungicides in managing postharvest tuber rots of yams since it is eco-friendly, cheap and biodegradable.

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