FUNGITOXIC POTENTIALS OF EXTRACTS OF PLANT ORIGIN AGAINST FUGAL ROOT ROT OF CASSAVA (*Manihot esculenta* Crantz) IN STORAGE

ABSTRACT

Investigations were carried out on the use of the water and ethanolic extracts of *Piper guineense, Ocimum graticimum, Casia alata,* and *Tagetes erecta* in the management of postharvest deterioration of cassava root caused by *Aspergillus flavus* and *Rhizopus stolonifer*. Water and ethanolic extracts of the plant materials had significant differences ($p \leq 0.5$) in their rates of fungitoxicity on the pathogenic organisms. Water and ethanol extracts of *C. alata* and *T. erecta* respectively at 50% concentration gave the same highest radial growth inhibition of 80.20% on *A. flavus in vitro* followed by ethanol extracts of *C. alata, O. graticimum,* and *P. guineense*. The ethanolic extract of *T. erecta* at 50% concentration gave the highest inhibitory effect of 53.50% on *R. stolonifer* followed by ethanol extracts of *C. alata, O. graticimum,* and *P. guineense* whereas the least growth inhibition of 0.17% was recorded by aqueous extract of *P. guineense* on *R. stolonifer*. *In vivo* test of the plant extracts applied before and after inoculation with spore suspension (1×10^5 spores/ml of distilled water) of test fungi showed significant reduction in root rot incidence and severity. The lowest incidence and severity of cassava root rot of 16.5% and 1.45 respectively were recorded with *T. erecta* ethanol extracts applied before inoculation of *A. flavus* indicating that the extracts of the plant materials could be better used as protectant than eradicant in the control of post harvest fungal deterioration of cassava root. *R. stolonifer* showed stronger resistance to the extracts of the plant materials than *A.flavus* during pathogenesis *in vivo*.

Key words: Cassava, root rot, postharvest, *Piper guineense, Ocimum graticimum, Casia alata,* and *Tagetes erecta*

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), is an important crop contributing to the survival of human beings and livestock by providing a ready and cheap source of carbohydrate for food and feed as well as raw material for industries (Nweke, 2015; Markson *et al*., 2012). It is the third largest source of carbohydrate after rice, sugar and maize in the world and a basic staple food and main source of energy for majority of the people in Africa and many other parts of the world (Amadioha, 2012; Echebiri and Edaba, 2008; Bua and Okello, 2011; Bukanga, 1999).

Cassava is a basic staple food to more than 70% of Nigerian population (Eke-okoro and Njoku, 2012) and a reliable and convenient source of food for tens of millions of rural and urban dwellers in Nigeria in its processed form (IITA, 2010; Nweke *et al*., 2002; Taiwo, 2006; Philip *et al.*, 2006). In addition to human consumption, cassava is used for the production of bioethanol, animal feed, and starch for industrial products (Plucknett *et al*., 2003).

Cassava root rot diseases are some of the major constraints to achieving the full potentials in cassava production in Nigeria and many areas in Africa (Chalwe *et al.*, 1999; Onyeka *et al.*, 2008; Onyeka, 2002; Bua and Okello, 2011). Apart from reducing cassava yield, root rot diseases caused by different organisms including fungi, bacteria and other pest organisms can also reduce the quality of cassava roots harvested and their products. Some of the fungi found to be pathogenic on cassava roots include *Sclerotium rolfsii, Fusarium oxysporum Schlecht, Botryodiplodia theobromae* Pat, *Aspergillus niger* Van Tieghem, *Aspergillus flavus* Link, *Rhizopus spp; Fusarium solani* (Mart) Sacc., and *Macrophomina phaseolina* (Tassi) Goidanich (Okigbo *et al*.,2009a; IITA, 1990). Different control measures have been suggested and used for the control of post-harvest cassava root rot diseases. In view of the problems associated with curing and use of synthetic chemicals in the control of storage rot of cassava root, natural plant extracts have been evaluated as pesticide alternatives in the management of postharvest root rot diseases of cassava incited by *A. flavus* and *R. stolonifer*..

MATERIALS AND METHODS

Source of plant Materials

The cassava root (TME 419 Variety) both infected and healthy (uninfected) were obtained from the National Root Crops Research Institute, Umudike, Abia State, Nigeria. The fresh leaves of *Ocimum gratissimum* and *Piper guineense* **w**ere obtained from open market stalls in Umuahia, Abia State while *Cassia alata* and *Tagetes erecta* were collected from Umudike, Abia State.

Culture Medium

Potato Dextrose Agar (PDA) (39g) was poured into one liter conical flask and made up to a liter with sterile distilled water, mixed thoroughly and melted in an electric water bath and then sterilized by autoclaving at 120^{o}C for 15 minutes. The sterile medium was allowed to cool (46^{o}C) and 15ml dispensed into sterile Petri-dishes and allowed to solidify. The sterile solidified medium was used for all the microbial cultures and other investigations

Isolation and Identification of Fungal Pathogen

The rotted cassava roots were washed with tap water, surface sterilized with 70% ethanol solution and rinsed in sterile distilled water. Pieces of the rotted tissue (3 mm diameter) were collected from the boundary of the infected and healthy root and placed on the culture medium in Petri dishes. The inoculated plates were transferred into the microhumidity chamber and incubated at 26^{0}C. The emerging fungal colonies were sub-cultured on fresh sterile culture medium of PDA to obtain pure cultures of the isolates. Peathogenicity was carried out on the isolates using fresh healthy, washed and sterilized cassava roots (Amadioha, 2001). On establishment of rot condition, re-isolation was carried out to obtain pure cultures of the inoculated isolates which were then compared with the original isolates. The isolates that caused root rot of the cassava were regarded as pathogens and identified (Sangoyomi, 2004; 2010).

Leaf Extracts.

Fresh leaves of *O. gratissimum, P. guineense, C. alata* and *T. erecta* were washed under running tap water and rinsed with sterile distilled water, air dried at room temperature (27^{o}C) and then dried in an oven at 60°C for 24 hours. The dried leaves of the test plant materials were ground into powder and separately weighed (10g, 20g, 30g, 40g and 50g) into a beaker before adding 100ml each of the extracting solvent (ethanol or sterile distilled water). Each solution was thoroughly mixed and left to stand for 24 hours and then filtered separately using a four –fold cheese cloth into a beaker. These filtrates constituted 10%, 20%, 30%, 40% and 50% concentrations of cold water or ethanol leaf extracts of the test plant materials. The purity of the extracts was confirmed using the method of Cheesbrough (2000).

Effect of Extracts on the radial growth of fungal pathogens *in vitro*

The method of Amadioha and Obi (1998) was used to evaluate the antifungal effect of extracts of the test plants against fungal growth *in vitro.* 2 ml each of the extract concentrations (10%, 20%, 30%, 40% and 50%) was separately transferred into a sterile Petri dish with the aid of a sterile pipette. Freshly prepared molten PDA (15ml) was aseptically poured into the plates. The plates were rotated gently for easy mixing of the PDA-extract media which were allowed to solidify. A 5mm diameter disc of each pathogen was then dropped separately at the centre of the solidified extract-PDA plates. The treatments were replicated three times. The control plates consisted of only PDA (15ml) + 2ml of distilled water (no extracts) inoculated with the test fungi. The inoculated Petri dishes were incubated at 27^{0}C and observed daily for fungal growth. The mycelial radial growth of each fungus was measured with a ruler along the two directions on the perpendicular lines drawn on the reverse side of the plates after the growth in the control experiment had reached the edge of the plate. The mean colony diameter of the three replicates was taken as the mean growth of each treatment. Fungitoxicity was calculated as percentage colony inhibited by the extracts (Amadioha, 2004).

$$\% \text{ Fungal Growth inhibition} = \frac{DC - DT}{DC} \times \frac{100}{1}$$

Where DC = Average diameter of colony in control experiment.

DT = Average diameter of fungal colony with extract treatment.

In vivo Screening of Plant Extracts against fungal pathogens

The 50% extract concentration of each of the water and ethanol plant extracts which gave the highest radial growth inhibition of the pathogens *in vitro*, was used in this experiment. Two sets of ten surface sterilized healthy cassava roots were each treated as a group with spore suspension (1×10^5 spores/ml of distilled water) of each of the test fungal pathogens (Amadioha and Markson, 2007a) as follows:

Group A - ten uninfected cassava roots each dipped into the extract concentration of test plants and allowed to air dried for 2 hrs before spray-inoculating with the spore suspension of the test fungal pathogens.

Group B - ten uninfected cassava roots each spray-inoculated with the spore suspension, air dried for 2 hours and then dipped into the extract concentration.

The control experiment constituted the spray-inoculated cassava roots that were not treated with the extract concentration. Each of the treated cassava roots including the control was enclosed separately in polyethylene bags with cotton wool soaked with distilled water (micro humidity chamber) and incubated at 28 ± 2^0C. The experiment was replicated two times. The samples were observed daily for rot development for 14 days. The disease incidence and severity were assessed.

$$\text{Disease incidence (\%)} = \frac{\text{No. of rotted cassava roots}}{\text{Total No. of cassava roots}} \times \frac{100}{1}$$

Disease Severity was assessed (Murugan and Luaina, 2013) on a 0-5 scale as follows:

0. - No infection

1 - Slight infection (≤ 10 - 20% of cassava root infected)

2 - Moderate infection (21 - 40% of root infected)

3 - High infection (41 - 60% of root infected)

4 - Extensive infection (61 - 80% of root infected)

5 - Complete rot (81 - 100% of root infected)

$$\text{Disease severity index} = \frac{\text{Sum of all scores}}{\text{Number of plants scored (N) x Highest score (5)}} \times \frac{100}{1}$$

Where; N is the total number of cassava root assessed; 5 - the maximum score of the scale used.

RESULTS

Effect of plant extracts on the radial growth of pathogens

The *in vitro* screening of the plant extracts against the radial growth of the test fungal pathogens, *A. flavus* and *R. stolonifer* showed that the plant extracts had significant (P≤ 0.05) inhibitory effects on the organisms tested. The inhibitory effect of the test plants increased with concentration and differed with extracting solvents across the test organisms. *Tagetes erecta* ethanol extract recorded the highest mean inhibitory effect on *A. niger* (66.9%) while the least was *P. guineense* aqueous extract with mean inhibition of 10.5% on *R. stolonifer*. Both ethanol and water extracts of the test plant materials at 50% concentrations had the highest inhibitory effects on the radial growth of all the pathogenic organisms whereas the lowest mean values were recorded with 10% concentration of test plant materials. Both the water and ethanol extracts of *C. alata* and *T. erecta* respectively at 50% concentration gave the highest radial growth inhibition (80.20%) of *A. flavus* in culture. All the concentrations tested recorded more inhibitory effects with ethanol as extracting solvent than water except *C. alata* that gave the same highest inhibitory effect with ethanol extract of *T. erecta* at 50% concentration (Table 1). *R. stolonifer* showed a stronger resistance across all the plant extracts tested.

Table 1: Percentage growth inhibition of aqueous and ethanol plant extracts on *R. stolonifer* and *A. flavus* in culture.

Plant Extract Concentration	Fungal Radial Growth Inhibition (%)			
	A. flavus		*R. stolonifer*	
	WE	EE	WE	EE
Piper guineense				
10	6.17	23.0	0.17	7.33
20	23.0	30.0	3.33	16.8
30	37.0	40.0	12.7	25.5
40	43.7	49.0	17.5	30.5
50	50.2	54.5	21.5	40.5
Control	0.00	0.00	0.00	0.00
Mean	30.8	38.5	10.5	23.6
Occimum graticimum				
10	13.3	25.5	0.50	10.5
20	32.8	39.3	11.8	25.2
30	40.5	53.2	19.7	30.2
40	56.5	63.2	24.5	41.8
50	62.8	67.5	26.2	48.2
Control	0.00	0.00	0.00	0.00
Mean	40.0	48.5	16.0	30.6
Cassia alata				
10	24.3	34.5	1.67	7.33
20	37.3	46.7	6.50	15.5
30	63.3	64.3	10.9	20.5
40	71.5	71.8	24.8	30.8
50	80.2	74.0	32.3	34.8
Control	0.00	0.00	0.00	0.00
Mean	53.9	57.1	14.7	21.3

Tagetes erecta				
10	28.5	45.0	6.17	16.0
20	46.2	64.7	21.8	24.5
30	61.5	72.3	29.8	33.5
40	73.2	76.8	35.0	40.2
50	79.3	82.0	49.2	53.5
Control	0.00	0.00	0.00	0.00
Mean	56.5	66.9	27.9	33.0
LSD (5%) Conc.	3.78		3.03	
LSD (5%) Extract	1.96		1.58	

WE = water extract, EE = ethanol extract
Values are means of three replicates in two separate experiments.

Effect of plant extracts on disease incidence and severity *in vivo*

The *in vivo* screening of plant extracts applied before and after spray-inoculating with the spore suspension ($1x10^5$ spores/ml of distilled water) of the test fungi indicated that incidence of cassava root rot was significantly reduced by the treatment both before and after inoculation when compared with the control experiment (Table 2). The same trend was observed in the severity of cassava root rot (Table 3). *P. guineense* water extract had the highest percentage disease incidence of 48.6% when it was applied after the inoculation with spore suspension of *R. stolonifer* followed by *O. graticimum*, *C. alata* and *T. erecta.* The lowest incidence and severity of cassava tuber rot of 16.5% and 1.45 respectively were recorded with *T. erecta* ethanol extracts applied before inoculation of *A. flavus*. Generally, the extracts of *T. erecta* had a stronger inhibitory effect on the pathogens than the other three plant extracts whereas *R. stolonifer* showed stronger resistance to the plant extracts than *A. flavus* both before and after spray-inoculating with the pathogenic organisms.

Table 2: Effect of aqueous and ethanol plant extracts applied before and after inoculation on the disease incidence by *R. stolonifer* and *A. flavus in vivo*

Treatment	Pathogens and Disease Incidence (%)			
Plant Extracts	***A. flavus***		***R. stolonifer***	
	A	**B**	**A**	**B**
Piper guineense				
Water extract	43.3	34.2	48.6	38.9
Ethanol extract	32.3	28.2	40.4	32.0
Ocimum graticimum				
Water extract	40.5	27.0	47.5	36.8
Ethanol extract	30.5	21.9	38.2	27.2
Cassia alata				
Water extract	40.3	25.4	43.3	33.8
Ethanol extract	30.3	20.3	31.9	22.3
Tagetes erecta				
Water extract	34.2	24.5	40.5	30.5
Ethanol extract	26.8	16.8	30.3	20.5
Control	50.50		82.83	
LSD (5%)	3.71		1.48	

Values are means of three replicates in two separate experiments.

Table 3: Effect of aqueous and ethanol plant extracts applied before and after inoculation on the disease severity of cassava root incited by *R. stolonifer* and *A. flavus.*

Treatment	Pathogens and Disease Severity Index			
Plant Extracts	***A. flavus***		***R. stolonifer***	
	A	**B**	**A**	**B**
Piper guineense				
Water extract	4.30	3.47	3.67	4.73
Ethanol extract	3.14	2.20	2.20	2.47
Ocimum graticimum				
Water extract	4.17	3.20	3.13	4.10
Ethanol extract	3.03	2.20	3.20	1.97
Cassia alata				
Water extract	3.43	3.50	3.33	3.30
Ethanol extract	3.17	1.97	2.17	2.10
Tagetes erecta				
Water extract	3.30	2.50	3.10	3.17
Ethanol extract	3.23	1.45	2.37	1.77
Control	4.37		5.47	
LSD (5%)	**0.42**		**0.38**	

Values are means of three replicates in two separate experiments.

DISCUSSION

The pathogens, *Aspergillus flavus* and *Rhizopus stolonifer* inciting cassava root rot in this study have been previously linked with postharvest rot of cassava, yam and cocoyam (Okigbo *et al*., 2015; 2014; 2009a; Umana *et al.*, 2016; Amadioha and Markson, 2007b). Okigbo *et al*., (2009b) implicated *A. niger* as the leading cause of postharvest fungal rot of cassava which is at variance with *A. flavus* in this study. The difference could be due to the variety and age of the cassava root used and location especially the prevailing environmental factors where the studies were conducted.

The result of the inhibitory potentials of the water and ethanolic extracts of test plant materials against the radial growth of the two fungal pathogens showed significant differences ($p \leq 0.5$) in their rates of fungitoxicity on *A. flavus* and *R. stolonifer*. The ethanolic extract of *T. erecta* at 50% concentration gave the highest inhibitory effect on *A. flavus* whereas the least radial growth inhibition was recorded by 10% water extract of *P. guineense* on *R. stolonifer.* This implies that the two fungal pathogens showed differences in their rates of resistance or susceptibility to the extracts of the test plants with *R. stolonifer* being less susceptible than *A. flavus* indicating that the fungus may have devised means of resisting the effect of the plant extracts (Umana *et al*., 2016). The different concentrations of the plant extracts also showed significant differences in their mean growth inhibitory effects with the higher concentrations (50%) having more inhibitory effects on the pathogens than the lower concentrations. This corroborates the work of Suleiman (2010) and Amadioha (2006) who recorded a significant difference in mycelial growth inhibition by various plant extract concentrations with higher concentrations giving remarkable fungitoxic effect. This shows that the antimicrobial potentials of the test plant materials can effectively be realized at higher concentrations of extracts (Nwinyi *et al*., 2009). Also, the higher concentrations of the test plant materials may have contained more active ingredients due to higher dilution in the extracting solvent than the lower concentration with low dilution due to some inhibitory factors (Umana *et al*., 2016). This finding also agrees with the reports of Amadioha, (2000) and Okigbo *et al*., (2009a) that the difference in the fungitoxicity of extracts may be due to the differences in the solubility of the active ingredients or compounds in extracting medium. The study on the fungitoxic potentials of the various extracts of the plant materials showed that *T. erecta* and *C. alata* were more fungitoxic and exhibited the highest percentage growth inhibition on the radial growth of the rot pathogens suggesting that the plant materials contain some active compounds/phytochemicals that affected the radial growth of the rot pathogens in culture. The ethanolic extracts recorded the highest radial growth inhibition in culture across all the concentrations and plant materials. This observation suggests that ethanol as extracting medium dissolved more active compounds present in the plant materials than water which disolved less active principles or compounds (Anukworji *et al.*, 2012). *P. guineense g*ave the lowest growth inhibitory effect whereas *T. erecta* recorded the highest inhibitory effects across the test fungi indicating that the pathogenic organisms reacted differently to the phytochemicals of test plant materials extracted by different extracting solvents.

The results of *in vivo* test of the plant extracts applied before and after inoculation with spore suspension of test fungi and their effect on the incidence and severity of cassava root rot indicated significant differences ($p \leq 0.05$) with the application of the extracts before inoculation recording a lower disease incidence and severity by the rot causing organisms than application of the extracts after inoculation. However, the water and ethanolic extracts of the test plant materials showed significant differences in their reduction of disease incidence and severity caused by the test organisms when compared with the control experiment. The least percentage tuber rot was recorded in tubers treated with *T. erecta* ethanol extract applied before inoculation of *A. flavus* while the highest incidence of cassava root rot was recorded with *P. guineense* water extract applied after inoculation of *R. stolonifer*. It was observed that *T. erecta* and *C. alata* reduced the growth and spread of the test fungi during pathogenesis than *P. guineense* and *O. graticimum in vivo* while *R. stolonifer* had more resistance to the plant extracts than *A. flavus.* Extracts of *T. erecta* and *C. alata* could therefore be exploited as biopesticide and alternative to synthetic chemicals by resource poor farmers in the control of storage rot of cassava caused *A. flavus* and *R. stolonifer.*

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