fjrs.futa.edu.ng

FUTA Journal of Research in Sciences

ISSN: 2315-8239

FUTA Journal of Research in Sciences, 2016 (1): 156-166

BIOASSAY OF PLANT EXTRACTS ON TWO FUNGAL PATHOGENS OF CASSAVA TUBER ROT IN KOGI STATE, NIGERIA.

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ABSTRACT

Antifungal effects of *Jatropha curcus* (L.), *Psidium guajava* (L.) leaf extracts and mancozeb solution on *Rhizopus stolonifer* (Link) and *Penicillium expansum* (Link) isolated from cassava tuber rot whose pathogenicities were proven were assessed. Mancozeb solution was used as a standard fungicide to compare its effects on the fungi with those of the leaf extracts. Antifungal properties by the extracts and mancozeb solution were separately assessed on inhibition of fungal growth on Potato Dextrose Agar (PDA). The experiment was a completely randomized design with three replications per treatment. The inhibitory action of the extracts and mancozeb on mycelial growth increased with increasing concentrations. Both *J. curcus* and *P. gujava* leaf extracts reduced the radial growth of the pathogens; *J. curcus* leaf extracts reduced the growth of *Rhizopus stolonifer* by 84.44% and reduced that of *P. expansum* by 88.89%. The percentage reduction of the growth of *Rhizopus stolonifer* in *P. guajava* leaf extract is 92.67% and reduced *P. expansum* by 93.11% ($P \leq 0.05$). Mancozeb solution reduced the radical growth of *Rhizopus stolonifer* and *P. expansum* by 90.22% and 92.22% respectively.

The extracts and fungicide were however, more inhibitory to *Penicillium expansum* than to *Rhizopus stolonifer*.

Keyword: Concentration, fungi, inhibition, mycelial growth, plant extract

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a dicotyledonous plant belonging to the botanical family euphorbiaceae (Salami and Osonubi, 2002). It is a major food crop in the tropical and sub tropical world, which Nigeria is currently one of the largest world producers (Nyerhovwo, 2004). The genus *Manihot*, comprises a large number of species, of the family Euphorbiaceae (IITA, 2009). Early literature on cassava describes the genus with two edible species, *Manihot utissing and Manihot aipi* delineating species which have high and low cyanogenic glycoside concentration respectively. More recently, cassava was classified as being the same species *Manihot esculenta* applying taximetric method and is classified into 75 species. Cassava is grown for its starchy tuberous root which can be used for the production of ethanol, glucose, starch gum and pastes for industrial uses (Adewolu, 1999). The stems are variable in height and branching, and the light to dark green leaves is spirally arranged with numerous lobes of variable shape.

The mode of cassava utilization varies from one place to another. Studies revealed that cassava is one of the most important crops in Nigeria. More than two third of total production of cassava is used as food for humans, with lesser amounts being used for animals feeds and industrial purposes (Nwokoro *et al*., 2002). Nigeria alone currently produces over 14 million tones annually, representing about 25% of sub-saharan Africa's output, and playing an important role in the rural economy in southern agro ecological zone and is increasingly gain importance in other parts of Nigeria (Ayodeji, 2005). Among the starchy staples, cassava gives a carbohydrate like maize, rice and so on with the result that cassava is the cheapest source of calories for both human nutrition and animal feeding (Nyerhovwo 2004). Among the common product of cassava are 'gari', 'fufu', starch, cassava flour and so on and so forth. Also, leaves and tender shoot are consumed as vegetable in some countries (FAO, 2000).

The main biological constrains in cassava production in Kogi state, and by extension, North Central Nigeria, is diseases and sometimes pest. The extent of losses may be as high as 80%. The spoilage of cassava tuber arises from combination of physiological and pathological factors (Salami and Osonubi, 2002). Studies have shown that primary deterioration is essentially a wound response with increase in enzyme and phenol production (Nwokoro *et al*., 2002). Two distinct types of deterioration have been detected in cassava tubers; they are soft and dry rots. Biochemical analysis of infection process showed that the microbial pathogen must produce a set of enzyme capable of attacking the carbohydrate polymers and protein composition of the infected plant's cell wall (Odebode *et al.,* 2001). The fungi also play important role in producing amylase which is capable in degradation of starch tissue of

cassava. Cassava root and tuber rot disease is a fungal disease of cassava occurring throughout West Africa (IITA, 2009).

Amienyo and Ataga (2007), reported the isolation of different fungi from rotted cassava tubers. Some of the fungi found to be pathogenic on cassava tubers and root after re-inoculation in storage included *Fusarium solani, Rhizopus stolonifer, Phythophthora drechslera, Aspergillus niger and Botryodiplodia theobromae.* Others are *Fusarium oxysporum, Aspergillus flavus.* These fungi cause discolouration in the surrounding tissue of infected cassava tubers, resulting in change in appearance, deterioration of texture and flavour of taste of cassava product. Rot fungi result in post-harvest losses and reduction in market value of tubers (Amienyo and Ataga 2007).

The necessity to develop non-toxic, safe and effective biodegradable alternatives to synthetic pesticides has in recent years, led to global efforts at screening various plants for bioactivity against plant pathogenic organisms. It is estimated that about 10% of the over 250,000 different plant species in the world today have been examined chemically for antimicrobial activity (Wokocha and Okereke, 2005). A large reservoir of potential sources of botanical fungicides still exists especially in tropical forests awaiting exploitation (Wokocha and Okereke, 2005). The continued use of natural plant products for plant disease protection is particularly important in countries like Nigeria where synthetic fungicide are indiscriminately used. Different control measures so far suggested for post-harvest cassava tuber rot disease which include reduce temperature; resistances and use of chemical have been reported by Adewolu (1999). The use of synthetic fungicide apart from their potential danger to both farmers and environment are unaffordable by most of the cassava farmers. Recent studies on the use of plant extract have opened a new opportunity for the control of plant disease. In Nigeria, plant extracts have been used to control fungal disease of plants such as cowpea (Okigbo and Edmoghene, 2004 and Suleiman, 2011), yam, sweet potato and maize, but have been sparsely used in the control of cassava disease (Okigbo and Nmeka 2005).

Of the systemic fungicides, mancozeb (80% active ingredient), has been preferred because of its widest spectrum of fungitoxic activity compared with other systemic, and is neither phytotoxic nor toxic to mammals at the concentration used (Suleiman, 2011). The primitive and subsistence system of farming practiced in Kogi State where farmers often lack inputs such as improved seeds, irrigation, fertilizer, treatment and storage facilities has not favour large scale cassava cultivation. Also cassava production in the state is not left out of parasitic infection. Since cassava cultivation is important in Kogi State, it seemed desirable to study the fungi associated with it on field and in storage by

determining the damage caused and the possibility of minimizing such damages.

MATERIALS AND METHODS

Collection of Samples

Rotten cassava tubers were randomly collected from the farms and storage at Aiyegunle-Gbedde, Kabba, Okene, Isanlu, Anyigba, Dekina and Ugwalawo. These were packaged in polythene bags, labeled properly to avoid mix-up and taken to the laboratory in the Department of Biological Sciences, Kogi State University, Anyigba, for further investigations. The plant *Jatropha curcus and Psidium guajava* were collected from the staff quarters, Kogi State University, Anyigba, washed and air dried in the laboratory.

Pathogen isolation and identification: Isolations were made from diseased cassava tubers collected from seven towns in Kogi State, Nigeria. Tissue segment (2 mm diameter) were cut from the periphery of the infected tubers and sterilized for one minute in 0.1% mercuric chloride. The sterilized tissues were rinsed thrice in sterile distilled water and plated on water agar (WA) plates. The resulting mycelia after 5 days incubation on laboratory bench (27± 2^{o}C) were transferred to potato dextrose agar (PDA) plates and incubated for 7days (Chiejina, 2005). Several sub culturing were carried out on the isolated fungi, to obtain pure culture. Isolated fungi were identified based on the mycelia growth patterns of the cultures in PDA and examination under the microscope. The fungi were identified according to Barnett and Hunter (1992) and also Dutta (2005).

Pathogenicity tests

Ten fresh apparently healthy tubers were used for the pathogenicity test; out of which two served as control. They were washed with sterile distilled water and later surface sterilized with 70% alcohol. Cylindrical discs (5mm diameter) were removed from the tubers with a sterile cork borer. Mycelia discs (4mm diameter) were made from 7 day old PDA cultures of the isolates and used for the inoculation bore-holes of the tubers. The cassava tubers were replaced and inoculation sites smeared with petroleum jelly to prevent contamination by other microorganisms. The inoculated tubers and the controls (uninoculated) were placed separately in their groups of 5 in sterile polythene bags containing cotton wool soaked in sterile distilled water to provide a humid environment and to prevent entry of other pathogens (Suleiman, 2010). The bags were properly labeled and incubated at a temperature of 27 ± 2^{0}C for daily observation. Disease symptoms produced by artificial inoculating after the incubation period were compared with those observed on the naturally infected tubers collected from the farms. Re-isolation and re-examination from the inoculated tubers were cultured in PDA plates. The morphology of each pathogenic fungus was compared with that of the original culture according

to Koch's postulate (Agrios, 2005). The experiment was repeated twice.

Preparation of the plant extracts.

The fresh fully expanded leaves of *Jatropha curcus* and *Psidium guajava* were used. The collected leaves were washed with tap water to remove the trace of sand and dirty, rinsed severally with sterile distilled water and air dried. The extracts were prepared by the modified method of (Suleiman 2011) which involved pulverizing the dried leaves in a mortar with pestle. Twenty five grams each of the powder was soaked in 250 ml absolute ethanol for 24 hrs and the solution filtered through sterile cheese cloth into a beaker. The ethanol was allowed to evaporate after extraction of the active ingredient, and solution dried to powder by heating at low temperature in an oven. The powder was dissolved in sterile distilled water to give 25% concentrate of the leaf extract and kept in a fridge wrapped properly with cotton wool and aluminum foil to prevent contamination according to the method of Sangoyoni, (2004). From the stock solution (25%), subsequent concentrations (5%, 10%, 15%, and 20%) were prepared by serial dilution.

On the other hand, hot organic solvent extraction was carried out by weighing the same quantity of samples (25g), washed and soaked in 25mls of 75% ethanol in a 1000ml conical flask. They were then placed in pots of water and heated on the electric cooker at 100^{o}C. The filtrates was concentrated using the vacuum evaporator so as to regenerate the ethanol. From the crystal appropriate samples were weighed separately and dissolved in 25ml distilled water to give the final concentrations of 5%, 10%, 15%, and 20%., a modified method of Ojo and Olufolaji (2005). Accordingly, the weight of the fungicide (mancozeb) was calculated to give definite concentrations in parts per million (ppm) of its active ingredient (80%). Stock solution or suspensions were prepared by adding the desired grammes 1.25 g aseptically to the appropriate 1000ml of sterile distilled water in conical flask according to Suleiman (2011). Twenty five percent was prepared from the stock solution (1litre) to form the 25% serial dilution stock, subsequent solutions were then prepared. The concentrations used were 5%, 10%, 15%, 20% and 25%, and these were used for *in vitro* test as amendments on potato dextrose agar (PDA).

***In vitro* assay of leaf extracts and mancozeb solution on radial growth of the pathogens.**

One milliliter portions of the extracts (5, 10, 15, 20 and 20%) were separately infused into 5 Petri dishes per concentration per fungus. To the same number of Petri dishes, 1ml portions of 5, 10, 15, 20, and 25% of mancozeb solution made in sterile distilled water were infused. A control using sterile distilled water in place of leaf extracts or mancozeb solution was set up according to the method of Sangoyoni, (2004) and a modified method of Suleiman (2011). Melted PDA was poured into all these plates and left to solidity. The plates were inoculated with the

mycelial discs of the pathogens cut with a 5 mm diameter cork borer from 7 day old cultures. They were incubated at room temperature for 7 days and radial diameter measurements from two axes taken daily starting from the 2nd day of incubation. The experiment was repeated twice.

Statistical Analysis

Descriptive statistics of variables measured are presented as mean values in bar charts. In order to test the level of significant differences between the plant extracts and fungicide used and the control group, the smallest difference between means (least significant difference [LSD]) of mycelia growth inhibition was computed by *post hoc* tests to obtain multiple comparisons between treatments. Data obtained were analyzed by Analysis of Variance (ANOVA) at 5% level of significant.

RESULTS

Isolation and Identification of fungal pathogen

Two fungal pathogens were isolated and identified as *Rhizopus stolonifer and Penicillium expansum.* The two fungi were found to be associated with cassava tuber rot in the study area. *R. stolonifer* has coenocytic (aseptate) hyphae with whitish vegetative mycelium at early stage that was richly branched, slender and cylindrical. The mycelium gave rise to terminal or intercalary sporangia and is produced in sporangia. The thallus is richly filled with granular protoplasm and multinucleated. *P. expansum* is composed hyaline slender, branched and septate hyphae and are multinucleated. The hyphae spread on the surface of the substratum penetrating deep into it.

The result, as shown in (table 1), revealed that the isolated fungi were associated with cassava tuber rots in storage with varying frequencies. The fungi were isolated from different locations with *Rhizopus stolonifer* slightly showing highest percentage fungal infection rate of 50.9% while *Penicillium expansum* showed 49.1% total infection rate. Anyigba recorded the highest frequency occurrence with 22.16%, while Kabba had the lowest rate of occurrence with 6.53 %(Table 1).

Table 1: Frequency of isolated fungi obtained from different locations

Accession name	*Rhizopus stolonifer*	*Penicillium expansum*	Total no. fungi isolated	% frequency
Aiyetoro	20	15	35	9.94
Kabba	18	05	23	6.53
Okene	25	35	60	17.04
Isanlu	30	18	48	13.63
Anyigba	43	35	78	22.16
Dekina	23	34	57	16.19
Ugwalawo	20	31	51	14.49
Total no. of fungi	179	173	352	
% frequency	50.9	49.1		

Pathogenicity test on the isolated fungi

The fungi were found to be pathogenic as the symptom produced in the artificially inoculated cassava tubers were identical with those observed in the naturally infected tubers. *Rhizopus stolonifer* produced whitish-grayish mars of hyphae at inoculation sites on the tubers three days after inoculation. The hyphae rapidly covered substantial parts of the tubers and produced dark coloured sporangia. These fungi caused discolouration in the surrounding tissue of inoculated cassava tubers, resulting in change in appearance, deterioration of texture and flavor of taste of cassava product. Water oozed out freely due to disintegration of the tissues, giving off an offensive odour.

Penicillium expansum composed of hyaline, brightly coloured in mass (i-celled) and septate. Uprightly conidiophores and arising from the mycelium, singly or branched and form a brightly green mould on the tubers three days after inoculation. Progress of rot development was noticed on the inoculated tubers, but slower than *Rhizopus stolonifer*. These symptoms displayed by the fungal inoculations were similar to those on the naturally infected tubers, using Koch`s postulate.

Effect of leave extract and mancozeb solution on radial growth of the pathogens.

The leaf extracts of *Jatropha curcus*, *P. guajava* and mancozeb generally reduced the growth of the pathogen in PDA. The results showed that the two extracts and the fungicide had fungicidal properties, with *P. guajava* leaf extract generally more effective in retarding vegetative growth than *J. curcus* leaf extract and mancozeb (Fig. 1a). Both *J. curcus* and *P. gujava* leaf extracts reduced the radial growth of the pathogens; *J. curcus* leaf extracts reduced the growth of *Rhizopus stolonifer* by 84.44% and reduced that of *P. expansum* by 88.89%. The percentage reduction of the growth of *Rhizopus stolonifer* in *P. guajava* leaf extract is 92.67% and reduced *P. expansum* by 93.11% (fig 1a and 1b).

Mancozeb solution reduced the radical growth of *Rhizopus stolonifer* and *P. expansum* by 90.22% and 92.22% respectively (fig 1a and 1b).

Psidium. guajava leaf extract significantly inhibited the growth of the fungi at its highest concentration of 25%. At the end of the incubation period, both the extracts and the fungicide exhibited a drastic reduction of the mycelial growth of the isolation; with a significant (0.05) difference compared with untreated plates (control). Percentage inhibition generally increased with increase in concentration of leaf extracts and mancozeb (Fig. 1b). Percentage inhibition of mancozeb was however more on *P. expansum* than on *Rhizopus stolonifer* at 25ppm of 92.22% and 90.22% respectively at the end of the incubation period.

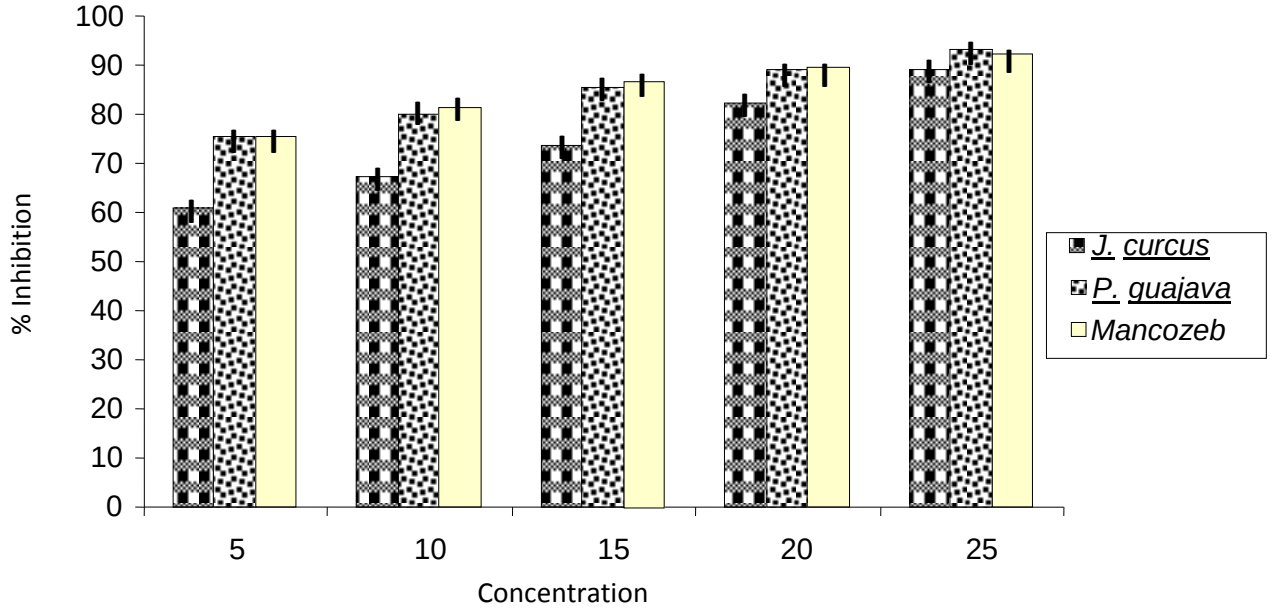

Fig. 1a: Mean Percentage Inhibition effects of extracts and fungicide on mycelial growth of *Penicillium expansum*

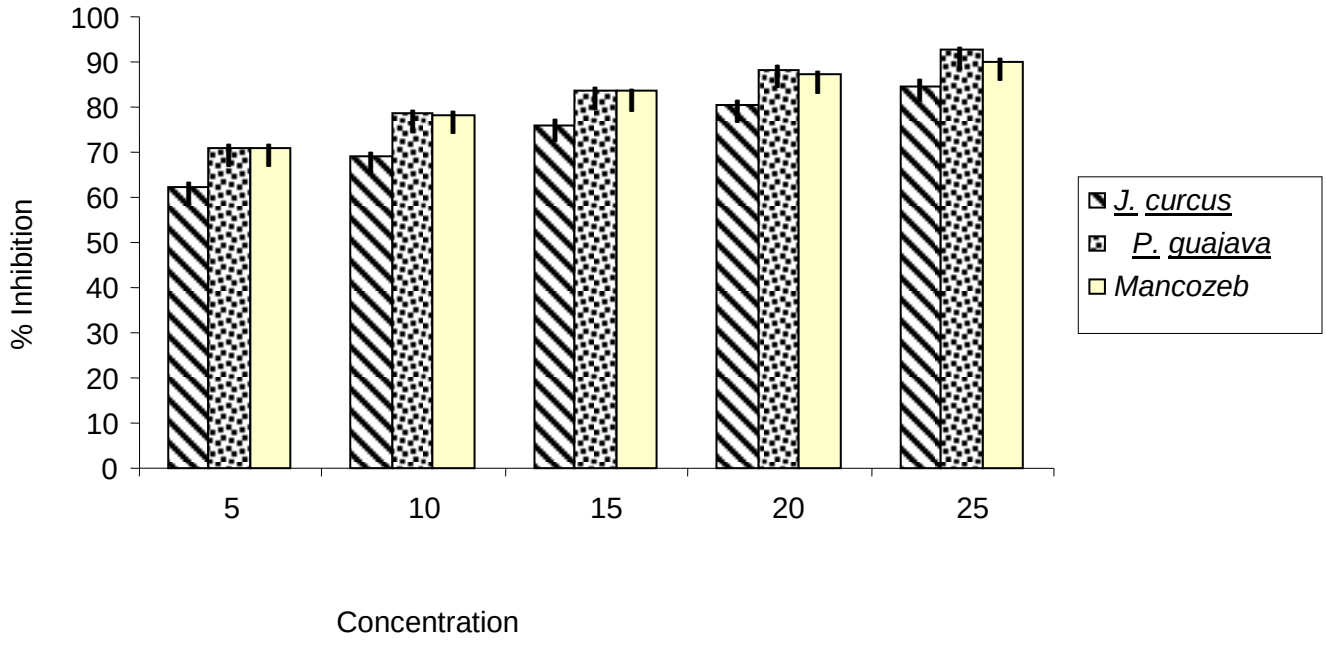

Fig. 1b: *Mean* Percentage Inhibition effects of extracts and fungicide on mycelial growth of *Rhizopus stolonifer.*

DISCUSSION.

Post harvest losses, one of the set back in cassava production has been shown to be due to infection by pathogenic fungi of cassava tuber rots like *P. expansum* and *Rhizopus stolonifer* which were isolated and identified as causal organisms of the cassava tubers rot in Kogi State . This is in agreement with Sangoyoni (2004) who reported isolation of *Penicillium* sp from Post harvest cassava tubers. *Penicillium* , *Aspergillus* and *Rhizopus* are regarded as saprophytic and parasitic fungi, their spores are cosmopolitan, found everywhere in the air and are often source of contamination and toxin production (Dutta, 2005). In most of the study areas, these fungi were found to gain entrance into cassava tubers through natural opening and wounds created during harvesting; transporting, handling and marketing similar to Amienyo and Ataga (2007).

The production of typical symptoms in tubers inoculated separately with the two fungi isolated from naturally infected cassava tubers and the subsequent re-isolation of identical fungi fulfils Koch's postulates and establishes the pathogenicity of the isolates.

Synthetic pesticides have long being used in controlling fungal diseases which has gulped millions of dollars in tropical and sub-tropical countries in importing them. There is equally a reported complex of health and ecological problem due to non-biodegradable of these pesticides (Ojo and Olufolaji). This has prompted the search for an alternative plant disease control. The two leaves extracts used for the present study recorded drastic retardation of vegetative growth of the fungi when compared with the control. This may be as a result of the presence of active water solution antifungal principles associated with each of the plant leaves.

If in the crude form the leaf extract could virtually stop the growth of the pathogens, their potentials as an effective control agent for these two fungi cannot be over looked. This study has revealed the potential of botanicals in the control of cassava tuber rotting fungi. This has gone a long way in providing better alternative to the over dependency on synthetic fungicides. Their effects would be enhanced if purifies and probably concentrated like mancozeb fungicide. The observed similar inhibitory effects in the leaf extracts and mancozeb indicate that the potency of the active ingredients in the extracts and mancozeb is similar when subjected to *in vitro* tests. The use of plant products in integrated pest management could reduce over reliance on one source of agricultural chemical to the farmer, as well as cut down cost production. It would therefore, be necessary to purify the extracts and determine the active ingredients which can be extracted and produced as a fungicide. Therefore, the facts that *J. curcus* and *P. guajava* are readily available, with easy method of extraction; they can be exploited in the control of cassava tubers rotting disease.

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