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BOTANICAL CONTROL OF PATHOGENIC FUNGI ASSOCIATED WITH CASSAVA CHIPS IN UMUAHIA MARKETS, SOUTH EAST, NIGERIA

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Abstract
Laboratory experiment was carried out in the College of Crop and Soil sciences, Michael Opkara University of Agriculture, Umudike to determine the level of contamination of dry Cassava chips from local markets in Umuahia metropolis. Potato Dextrose Agar was used to isolate the fungi involved in the contamination of chips. Through serial dilution, plating the frequency of occurrence of the major fungi was obtained. Fungi which include: *Aspergillus niger*, *Fusarium solani, Aspergillus flavus, Aspergillus fumigatum, Rhizopus stolonifer* and *Penicillium digitatum* were observed and 80% frequency of occurrence recorded. Three fungi: *Aspergillus niger*, *Rhizopus stolonifer* and *Penicillium digitatum* were the most consistently recorded in the three markets sampled. The least recorded was *Penicillium digitatum* at 49.36%. The average total fungal counts were 26.2 x 10^5 (*Aspergillus niger*); 12.0 x 10^5 (*Rhizopus stolonifer*) and 19.2 x10^5cfu/g (*Penicillium digitatum*). Aqueous extracts of *Newbouldia laevis* gave the highest inhibitory effect (91.067%), on *Aspregillus niger*. The inhibitory effect of *Newbouldia laevis* was significantly ($p<0.05$) higher than that of *Vitex doniana*. *Newbouldia laevis* demonstrated antifungal potential on cassava chips spoilage causing fungi and can be recommended for use as natural fungicide on cassava chips in storage.

Keywords: Cassava chips, moulds, identification, mycotoxins, and plant extracts

Introduction

Cassava (*Manihot esculenta* Crantz) is a dicotyledonous plant, belonging to the family *Euphorbiaceae* (Alves, 2002) and is cultivated mainly in south-east region over a wide range of environmental and soil conditions. According to Alexandratos (1995) cassava plays an important role in alleviating food problems, because it thrives and produces stable yields under conditions in which other crops fail. Cassava is a versatile crop and can be processed into a wide range of products such as starch, flour, tapioca, beverages and cassava chips. Cassava is also gaining prominence as an important crop for the emerging biofuel industry and, as documented by Ziska *et al*. (2009), is a potential carbohydrate source for ethanol production. A well planned strategy for the development and utilization of cassava and cassava products can provide incentives for farmers, crop vendors and food processors to increase their incomes. It can also provide food security for households producing and consuming cassava and cassava products (Plucknett *et al*., 2009).

The production of cassava is hindered by storage problems since it is a highly perishable commodity and can be easily contaminated by fungi and bacteria (Okigbo *et al*., 2009d). Cassava is also subject to sprouting due to increased metabolic activity (Okigbo *et al*., 2009a; Christopher, 2009). In order to minimize these problems, processing is required. One way is to process roots into dried cassava chips. The processing of cassava into chips is a common traditional activity in Nigeria during the dry season, especially in the Southern and Western regions of the country. Chips are subject to attacks by fungi of which the most important are *Aspergillus, Fusarium* and *Penicillium* (Bassa *et al*., 2001; Wareing *et al*., 2001). Fungal contamination can lead to the discolouration of the chips, mould taste, odour (Gwinner *et al*., 1996) and possibly the production of mycotoxins that are harmful to animals and humans who consume them. Another very important impact of spoilage and postharvest deterioration of chips by fungal pathogens is the production of various types of mycotoxins (Christopher and Daniels, 2010). Mycotoxins are low molecular weight toxic secondary metabolites from some species of fungi. They are dangerous in minute quantities and present extreme toxicity due to their ability to withstand heat (Okigbo, 2004; Shukla *et al*., 2012). The

mycotoxins of most agricultural importance are aflatoxins, fumonisins, ochratoxin A, zearalenone and deoxynivanelol (Bankole and Adebanjo, 2003).

Rot is a major factor limiting the post-harvest life of farm produce and losses can be very high. Losses due to post-harvest rot significantly affect farmers'/ traders' income and food security. The quality of food materials are affected by rots, which makes them unappealing to consumers. Several methods are used to control microbial spoilage which includes: fungicides treatment, gamma irradiation and hydro warming. These methods were found to have intermediate impacts in controlling spoilage and enhancing shelf life of harvested farm produce (Anukwuorji, *et al*., 2013), but several authors have reported that these methods have some challenges which includes but not limited to, not readily available and affordable and there were cases of development of resistance in target organisms (Anukwuorji *et al*., 2016; Okigbo and Nmeka, 2005), also the overzealous and indiscriminate use of these non-botanical methods have created different types of environmental and toxicological problems (Nzekwe, 2010; Malkhan *et al*.,2012). However, in the context of post harvest rot management, botanical pesticides/ plant extracts has been reported to play a very significant role in prolonging the shelf life of stored plant products in developing countries, hence the popularity of botanical pesticide is increasing. In the recent past, many botanicals have been extensively researched on and proved to posses antimicrobial properties, hence myriads of reports have been documented stating the use of plant extracts to control plant diseases. Some plants tested, with very high satisfactory results as botanical fungicides include: *Cymbopogon citratus, Chromolena odorata, Ocimum gratissimum* and *Zingiber officinale, Newbouldia laevis, Vitex doniana* (Ekpo and Asiedu, 2009; Okigbo and Nmeka, 2005; Okigbo *et al*., 2009a; 2009b; 2009c; 2009d).

The present study seeks to investigate the various fungi responsible for post-harvest spoilage of cassava chips and compare the antifungal efficacy of *Newbouldia laevis* and *Vitex doniana* at different concentrations in the control of the casual organisms and determine the most suitable extraction medium for effective activity on the test organisms with the view of providing a cheap and readily available source of control to improve the market value of this very important food in Nigeria. Also efforts were made to isolate and identify the various fungi associated with cassava chips and their frequencies of occurrence from three markets in Umuahia.

Materials and Methods

This study was conducted in the laboratory College of Crop and Soil Sciences of Michael Okpara University of Agriculture Umudike, Abia State, located in the south east geopolitical zone of Nigeria, with elevation of 152m above sea level and population of 4, 222, 476 (NRCRI, 2008).The method of Adeniji *et al*. (2007) was adopted in sampling cassava chips. The chips were sampled using simple random sampling technique from traders in Ubani, Ndoro and food village markets, all in Umuahia. A sample was obtained by collecting a 150g of cassava chips that represented the whole and collecting 50g from different parts of the bag to form a composite sample. Fresh leaves of *Newbouldia laevis* and *Vitex doniana* were collected from Mmaku in Awgu Local Government Area of Enugu State. The plants were identified and authenticated by the Horticulture Unit of National Root Crops Research Institute, (NRCRI) Umudike, Abia State, Nigeria.Potato Dextrose Agar (PDA) was used for microbial isolation and maintenance. The preparation of the medium was according to the specifications of the manufacturer thus: 39g of PDA powder dissolved in one liter of distilled water in a flat bottom flask. This was shaken and melted by boiling in a heater, then sterilized with the aid of autoclave at a temperature of 121^{o}C for 15 minutes. The medium was cooled to a temperature of 50^{o}C and later poured into sterile Petri dishes where it was allowed to solidify (Downes and Ito 2001).

Isolation and Identification of Fungi from Cassava Chips

Isolation of microorganisms was done by using laboratory blender to mill samples of cassava chips obtained from markets in Umuahia, and then 1g from each milled sample was weighed using sensitive weighing balance. Dilution plating was carried out by suspending 1g sample into the first test-tube (10^{-1}) shaken together. 1ml was again taken from (10^{-1}) dilution and transferred to the second test tube (10^{-2}). The dilutions continued till the ninth tube (10^{-9}). Each test tube was shaken vigorously before transferring into culture Petri dishes. All inoculated plates were incubated at 27°C for 5 days. All plates were examined daily for mycelia growth of fungi. Fungal colonies were transferred to fresh PDA plates to obtain pure cultures.Pure culture was obtained by transferring hyphal tips from the colony edge of the mixed cultures to fresh plates of PDA using flame sterilized blades. After purification, the resulting pure cultures were used for characterization and subsequent identification of the fungi isolates. Identification was done based on the structural features as seen on the culture plates and slide viewed under the low power microscope. Generally, structural features of colony, colour, extent of growth, presence or absence of mycelia, spores and the nature of colony surface were observed. Each organism was identified with the aid of compound microscope and identification guide (Barnett and Hunter, 2003). All the instruments and laboratory wares were sterilized according to the methods described by (Cheesbrough, 2000; Jawetz *et al*., 2004). The glass wares were surface sterilized to remove surface contaminants with 70% ethanol and thoroughly rinsed with sterile distilled water. They were placed in racks to dry and all iron instruments like inoculation loop, forceps, knives and scissors were also packed into the Autoclave for sterilization at a temperature of 121^{o}C for 15 minutes.

Preparation of Plant Extracts and Applications

Fresh leaves of *Vitex doniana*, and *Newbouldia laevis*, were thoroughly washed with tap water and then rinsed

with sterile distilled water and sun dried until they were dry enough for milling. The dried samples were separately ground using a laboratory blender to obtain a fine powdered processed sample used for the extraction. Extracts were obtained from the processed plant samples using cold solvent extraction method (Doughari *et al*., 2007). A 20g and 10g portion of each processed sample was soaked in 100ml of water. The mixture was shaken in a mechanical shaker for 1 hour and then allowed to stand for 6 hours at room temperature. The antimicrobial effect of different concentrations: 10% and 20% of botanical on fungal pathogens of cassava chips was determined. The method of food poisoning technique by Sangoyomi, (2004) was adopted for the inhibition of mycelia growth. The plates were gently rotated to ensure even dispersion of the extracts. The agar/extract mixture was allowed to solidify and then inoculated at the center with a 5mm diameter mycelia disc obtained with a cork bore (5mm) from the colony edge of 7-day old pure cultures of each of the fungi to be tested. Each treatment was replicated thrice. The negative control was agar plate with no extract inoculated with the test fungi as described. All the plates were incubated at 25 ± 20°C for 7-10 days and examined daily for growth and presence of inhibition. Colony diameter was taken as the mean growth along two directions on two pre-drawn perpendicular lines on the reverse side of the plates. The effectiveness of the extracts was recorded in terms of percentage inhibition, which was calculated according to the method described by Whips (1987).

Percentage inhibition = $\frac{R1 - R2}{R2} X \frac{100}{1}$

Where R1 is the farthest radial distance of Pathogen in control plate,
R2 is the farthest radial distance of pathogen in extracts incorporated agar plates.

Experimental Design/Statistical Analysis

The design of experiment used was a 3x 2 x 3 factorial in CRD (Complete Randomized Design). All the data obtained was subjected to statistical analysis of variance and means separated using Least Significant Difference (LSD) at 5% level of probability.

Results and Discussion

Fungal Isolates from Samples of Milled Cassava Chips and number of colonies

Results of the fungi pathogens that were consistently isolated from milled cassava are presented in Table 1. Fungal organisms isolated include: *Aspergillus niger*, *Fusarium solani, Aspergillus flavus, Aspergillus fumigatum, Rhizopus stolonifer* and *Penicillium digitatum*. The frequency of occurrence varied significantly ($P<0.05$) with different fungi associated with the milled cassava chips. The most frequently occurred pathogens were *Rhizopus stolonifer* and *Aspergillus niger* with 86 and 74 number of colonies respectively, while others had lower number of colonies for *Aspergillus flavus* and *Fusarium solani* , 12 and 19 respectively.

Table 1: Fungal Isolates and number of colonies

Fungal Isolates	Number of colonies
Aspergillus niger	74
Aspergillus flavus	12
Fusarium solani	19
Rhizopus stolonifer	86
Penicillium digitatum	2

This study revealed that fungitoxic compounds were present in *Newbouldia laevis and Vitex doniana,* since they were able to inhibit the growth of the test fungi, this result is in consonance with the earlier reports of several research reports but on different fungal organisms (Ameinyo and Ataga, 2007; Sangoyomi *et al*., 2009; Okigbo *et al.*, 2009d; Suleiman, 2010), hence the two plant extracts used have the potential application in the protection of cassava chips against fungi. However, the efficacy of the extracts differed with the plant material, concentration, solvent of extraction and with each test fungus. The presence of bioactive substance has been reported to confer resistance to plants against bacterial, fungal and pest (Srinwasan *et al*., 2001). This therefore explains the demonstration of antifungal activity by the plant extracts used in this study, hence the antifungal properties of these plant extracts is probably due to the presence of phytochemicals which are anti microbial agents (Okwu and Joshia, 2006), that are inhibitory to the growth of these pathogens (Okigbo and Ajalie, 2005).

Newbouldia laevis can also be used as a second option because they exhibit a moderate fungitoxic activity on the test organisms. However, the results of this study has gone a long way in providing better alternative to the over dependence on synthetic fungicides. The use of plant extracts in controlling rot causing organisms and pests could reduce over reliance on one source of agricultural chemicals to the farmers, that are reported to predicate long term harmful consequences on environment, man and wildlife, and reduce production cost, hence the antimicrobial activity of the extracts were comparable to those of the antibiotics (Suleman,

2010). The demonstration of activity against the test fungi produces scientific base for the local use of these plants in controlling microbial rot, since these plants are locally available, less expensive, environmental friendly with easy extraction method. It can be exploited in the control of cassava spoilage. Therefore the cogent data on the antimicrobial potentials of plant extracts deserve multi-institutional attention as early as possible. The prospects of relatively cheaper means of controlling rot inducing organisms could then be brighter, particularly for the numerous peasant farmers across the globe and Nigeria in particular.

Effect of Botanicals on the isolated Fungal Contaminants

Table 2 shows the effect of concentrations of extracts on the test organisms was significant ($P < 0.05$). Colony diameter of the inhibition increased as the concentration of the extract increased (10% and 20%). The interaction of extraction medium and concentration of extract was also significant ($P<0.05$) on the inhibition of all the three test fungi (*Aspergillus niger*, *Rhizopus stolonifer*, and *Penicillium digitatum*).

Table 2: Effect of botanicals on the isolated fungal contaminants

Plant Extracts	Concentration	% inhibition
Vitex doniana	10	88.37
Newbouldia laevis	10	88.13
Control	10	0.367
Newbouldia laevis	20	91.7
Vitex doniana	20	89.13
Control	20	0.500
Newbouldia laevis	0	82.97
Vitex doniana	0	81.5
Control	0	0.500

Aqeous extracts of *Newbouldia laevis* gave the highest inhibitory effect of *Aspergillus niger* by 91.067%, followed by *Vitex doniana* (89.133%). The inhibitory effect of *Newbouldia laevis* was significantly ($P<0.05$) higher than that of *Vitex doniana*. For plant extract concentration, there was significant ($P<0.05$) difference between all the levels/rates, 20% of *Newbouldia laevis* showed the highest inhibitory effect (91.67%), followed by 20% of *Vitex doniana* (89.133%). The least inhibition was recorded by 10% concentration of *Newbouldia laevis and Vitex doniana* which gave inhibition percentages of 88.367% and 88.133% respectively. However, there was significant difference ($P<0.05$) among all the values recorded by the different concentrations. For their interactions, *Newbouldia laevis* at 20% extract concentration gave the highest inhibitory effect (91.7%), this was significantly different ($P<0.05$) from the inhibitory effect of 83.07% recorded by *Vitex doniana* at 20% extract concentration. The aqueous extracts of *Newbouldia laevis* had the highest inhibitory effect on *Pennicillium digitatum* (82.97%), followed by *Vitex doniana* (81.5%). The inhibitory effect of *Newbouldia laevis* (91.7%) was significantly ($P<0.05$) greater than the rest , which was on the same hand significantly ($P<0.05$) greater than the inhibition showed by *Vitex doniana* at 10%. The control showed no inhibition on growth of the fungus. Table 2 also shows aqueous extracts of *Newbouldia laevis* had the highest inhibitory effect on*Rhizopus stolonifer* (82.967%), followed by *Vitex doniana* (81.467%). The inhibitory effect of *Newbouldia laevis* was significant ($P<0.05$) and greater than the inhibition showed by *Vitex doniana* at 10%. The control showed a non inhibited growth on the fungus.

Table 3 summarizes results of mean % frequencies in culture. The test fungal pathogens that were constantly isolated from the cassava chips include: *Aspergillus niger, Rhizopus stolonifer and Penicillium digitatum.* The frequency of occurrence varied with different fungi associated with the cassava chips. The most frequently occurred were *Rhizopus stolonifer and Aspergillus niger* (61.63% each), while *Pennicillium digitatum* has the lowest frequency of 49.36%.

Table 3: Frequency of occurrence of Fungi Pathogens in Culture

OrganismsMean	Frequency (%)
Rhizopus stolonifer	61.833
Aspergillus niger	61.633
Pennicillium digitatum	49.36
LSD	**6.9707**

Marketers and consumers are faced with problems of contamination of cassava tubers after harvest especially when processed into chips (Okigbo *et al*., 2009a). Another very important impact of spoilage and postharvest deterioration of chips by fungal pathogens is the production of various types of mycotoxins.

The invasion of cassava roots by microbial pathogens, especially fungi is considered most critical factor in root decay (Okigbo *et al*., 2009c). Several studies have reported the isolation of many different types of fungi from rotted cassava roots in storage. Some of the fungi found to be pathogenic on cassava roots after re-inoculation include: *Sclerotium rolfsii* (IITA, 1990); *Rosellinia necatrix* Prill*, 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*., 2009d). These fungi pathogens may cause infections either singly or in combination with several others (Sangoyomi, 2004).

Fungi on their own cannot penetrate healthy intact roots, hence wound agents such as pests through their feeding activities, and more important, damages arising during harvesting, storage, transportation and handling are sufficient to provide entry points for these fungal pathogens (Sangayomi, *ibid*). Environmental factors such as temperature and relative humidity are also very important in the rate of disease development in storage (Anukwuorji *et al*., 2012). Studies have shown the extent of decay in root and tuber crops caused by three storage fungi: *Penicillium oxalicum*, *Aspergillus niger* and *Botrydiplodia theobromae*. There was negligible decay in the roots inoculated with these fungi stored for four weeks at 15°C. *Penicillium oxalicum* was less aggressive (above 25%) but *Aspergillus niger* produced greater decay at 35°C.

One factor constraining the output of root is the difficulty in extracting it from the hard ground during the dry season which results in artificial scarcity of cassava products like gari (FIIRO, 2006). Physical losses of about 10% (by weight) were quite typical of conventional starch storage; these were probably caused by starch degradation since increase in soluble sugars has been detected. Storage of starch with acetic acid (2%) eliminated this weight loss. In addition to this 10% loss of product with conventional storage and processing, an additional 10% of the starch was lost during re-washing and sedimenting before final sun-drying (Jato, 2007). When artificially acidified stored starch was processed, this loss was reduced to 3.75% (Marder *et al*., 1996; Graffham *et al*., 2005). Since the Federal Government promulgated the policy on addition of 10% cassava flour into baking flour by flour mills, the demand for cassava by industries would increase and this may affect the supply of the product to local food processors (FIIRO, 2006). These peculiar problems can be minimized by processing the fresh roots into shelf stable intermediate product (dried chips) which can be converted to other cassava based products as the need arises. Ekwu and Ugwuonah (2007) and Oluwole *et al*. (2004) have demonstrated that dried cassava chips can be converted into gari of good chemical and functional quality.

This present research aimed at reducing surface contamination of dry cassava chips by applying some plant extracts as control measure will help both markets and consumers to solve the long-term problem of saprophytes and fungi contamination associated with cassava chips in markets (Doughari, 2010). From the results obtained in this study, it is obvious that *Newbouldia laevis* and *Vitex doniana* possess potential inhibitory activity against rot-inducing fungi to varying degrees, hence the demonstrated antifungal potential of these plant extracts on cassava chips spoilage causing fungi. Therefore, this research recommends their use as natural fungicide on cassava chips in storage.

Recent studies on the use of plant extracts have opened a new opportunity for the control of plant pathogenic diseases. Plant extracts have been reported to be safe, non-toxic to man, but effective against plant pathogens (Shivpuri *et al*., 1997; Okigbo *et al*., 2009). In Nigeria, plant extracts have been used to control fungal diseases of plants such as cowpea (Amadioha *et al*., 2000), banana (Okigbo *et al.*, 2004), yam (Onifade, 2000; Okigbo *et al.*, 2005), sweet potato and maize (Amienyo *et al.*, 2007, Anukwuorji *et al*., 2012) have been sparsely used in the control of cassava diseases. Botanicals such as *Newbouldia laevis, Vitex doniana, Garcinia kola, Azadirachta indica* and *Allium sativum L*. (garlic; *Alliaceae*), are used for treatment of cough and chest pain. Groppo *et al*., (2007) reported that fresh garlic shows good antimicrobial activity on oral streptococci. Similarly, *Rhizopus stolonifer* and *Fusarium oxysporium* amongst other pathogens were also reported inhibited by extracts from *Chromolaena odorantum*, *Azachirachta indica, Vernonia amygdalina* and *Tridax procumbens* in eggplants and tomato (Ijato *et al*., 2011). Not only have the mycelial growth of the pathogenic species been reported retarded by the several extract formulations, they have also been noted to inhibit myco-pathogenic spores germination. For example, investigators have reported the inhibition of the germination of the conidia of the fungus *Fusarium oxysporium* (Rongai *et al*., 2012). Those of *Colletotrichum destructivum* as well have been observed to be inhibitory and have been reported (Enyiukwu *et al*., 2011). *Ocimum gratissimum* L. (African basil) is grown in gardens and its leaves are used as a tea for fever relief (INIARCR, 2003; Okigbo *et al.*, 2006).

Conclusion

The challenges of cassava as a result of post-harvest spoilage is obvious and as such requires urgent attention if cassava must not be pushed further down the ladder to the status of a minor vanishing crop. With respect to the plants used, further pharmacological evaluation, toxicological studies and possible isolation of the

therapeutic antifungal from these plants are the future challenges; hence it is recommended that further investigations should be done on the chemical nature of the active principles of the plants. Further investigations can combine the plant extracts for possible synergistic effect. Further research involving invivo assay would be needed to investigate the fungistatic effects of these botanicals on the fungal inducing spoilage of cassava chips that are not included among the test fungi in this study. It is essential to devise good storage facilities to prolong the shelf life of cassava chips after processing.

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