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International Journal of Current Research
Vol. 4, Issue, 04, pp.099-104, April, 2012

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

TISSUE MASCERATION AND STARCH DEPLETION CAUSED BY SOME ROT PATHOGENS OF CASSAVA (*Manihot esculenta* Crantz)

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ARTICLE INFO

Article History:
Received 26th January, 2012
Received in revised form
15th February, 2012
Accepted 14th March, 2012
Published online 30th April, 2012

Key words:
Tissue maceration,
Starch depletion,
Rot pathogens,
Cassava.

ABSTRACT

Investigations on the anatomical damage of cassava caused by *Botryodiplodia theobromae, Rhizopus stolonifer* and *Penicilliun expansum* was carried out in Calabar, Cross River State, Nigeria. Inoculated and uninoculated cassava tissues were treated, sectioned weekly for four weeks and photomicrographs taken. The plates revealed massive cell wall macerations and depletion of starch grains in the samples infected with test pathogens. All the pathogens caused fragmentation of starch grains accompanied by minimal damage to infected cells and slight loss of starch grains in the first one week of incubation. The pattern of starch grain depletion adopted by *P. expansum* was from the lumen of the cell towards the cell boundaries thereby, creating empty spaces at the central portion of the affected cells. From the 3rd week of infection, massive cell wall degradation and starch grain depletion was evident. *B. theobromae* and *P. expansum,* in addition to starch depletion caused massive collapse of cell wall structure. *R. stolonifer* caused extensive necrosis, starch depletion and minimal collapse of cell wall structure particularly in the 4th week of incubation. Of the three pathogens studied, *B. theobromae* was the most aggressive in causing tissue damage and starch depletion in cassava tissues.

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INTRODUCTION

Cassava (*Manihot esculenta* Crantz) also known as tapioca, manioc or yucca is a dicotyledonous perennial shrub belonging to the family Euphobiaceae. Cassava is one of the most important food crops in the humid tropics because of its drought-resistant ability and capacity to thrive well in conditions of low nutrient availability (Burrell, 2003). It is a major staple food (Stephens, 1994, FAO, 1999) and a ready source of industrial raw materials for the teaming world population (Tonukari, 2004, Pattron, 2008, Westby, 2002, Hankoua and Bessong, 2008). According to Tonukari (2004), cassava ranks very high among crops that convert the greatest amount of solar energy into soluble carbohydrates per unit of area. Nutritionally, cassava tuber is very rich. It is reputed to be the cheapest source of calories for both human nutrition and animal feeding as it produces carbohydrate which is 40% higher than rice and 20% more than maize. Other nutrient components of cassava tuber include, starch (31%), dietary fibre (2%) and protein (0.53-1.5%). Minerals [magnesium and sodium (3%)] and vitamins (riboflavin, thiamin and nicotinic acid) are present in high amounts while iron and vitamin A are found to be low (Westby, 2002). The usefulness of cassava tuber goes beyond its food value. The leaves of the bitter variety of cassava have been used successfully to treat headache, hypertension and pain. Industrially, cassava has found a lot of uses. Cassava starch when treated appropriately, makes a good adhesive and remoistening gums (on stamps and envelops). Cassava starch functions to give correct viscosity and water-holding capacity in bores for drilling of oil wells. They are useful in paper and textile industries. In the wood furniture industry, cassava starch is useful in the production of particle board. As dusting powders, starch is applied to the skin to reduce friction. They are also incorporated into plastics to improve biodegradability and are employed as stain removers in fabrics. They are also used as bio-fuels, particularly, in the production of ethanol. Cassava starch costs 15-30% less to produce per acre than corn starch making cassava an attractive and strategic source of renewable energy (Hankoua and Bessong, 2008).

With the ability to survive harsh environmental conditions like drought and frost, cassava is massively produced in the tropical and subtropical regions of the world (FAO, 1994). Nigeria stands as the world's leading cassava- producing country, recording 3.27 and 3.39 million tons in 1999 and 2000 respectively and only followed by Brazil with 2.07 and 2.41 within the same period (FAO, 1994). At the continental level, Africa is rated the biggest producer of cassava with an estimated 92.7 million tons in 2000 (Food market exchange, 2002-2003). World cassava use is estimated to increase from

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172.7 to 275 million tons within the period 1993 to 2020 using the International Food Policy Research Institute (IFPRI's) baseline data (Westby, 2002). A higher prediction of demand and production growth puts the 2020 production at 291 million tons (Scott *et al.,* 2000). World consumption of cassava for food (fresh and processed) is concentrated in the developing countries (FAO, 1999). In Africa, about 70% of cassava produced is used as food in forms like foofoo, gari, lafun and attiéké. In Latin America and the Caribbean, between 35 and 40% of the cassava produced is consumed by humans. According to Scott *et al,* (2000), Sub-Saharan Africa is expected to experience the most rapid growth in food demand in root and tubers averaging 2.6% per year through 2020. The contribution from cassava to meet this demand is put at 66% of the total and the demand for cassava is expected to grow by 2.0% annually for food and 1.6% for feed in developing countries while total production is projected to reach 168 million tons by 2020 based on the current production rate. With the afore x-rayed statistics, it is clear that cassava is a crop that has occupied a central position in the world's industrial and economic revolution as well as being a key factor to achieving food security for the teaming human population in the world. These expected laudable projections may not be achievable if the issue of microbial spoilage of cassava tubers is not addressed. Over 30 genera of rot pathogens, including *Rhizopus stolonifer*, *Botryodiplodia theobromae* and *Penicillium spp.* have been isolated from rotting cassava tubers (Booth 1977). Among them, *B. theobromae* was reported as the most virulent in causing extensive tissue rot compared with other pathogens isolated. Similar reports of virulence by this pathogen has been documented in yam (Markson *et al.,* 2010, Okoro and Nwankiti, 2004). To adequately tackle the issue, the knowledge of the gravity of damage must be appreciated. This paper is intended to give insights to the cellular damage and loss of starch grain (the very component that gives value to the cassava tuber) in the cassava tuber resulting from attacks by some rot pathogens.

MATERIALS AND METHODS

Sample collection and pathogen identification

Infected and healthy (uninfected) cassava (*Manihot esculenta*) tubers were collected from open market stalls in three markets in Calabar, Cross River State, Nigeria. The markets were Akim, Marian and Watt. Tissues (about 5mm in diameter) from the symptomatic and asymptomatic cassava tubers were removed following surface sterilization with 70 % ethanol for 10 s, blotted dry with sterile paper towel, and plated onto chloramphenicol-amended Potato Dextrose Agar (PDA). After three days of incubation at 28^0C, microbial growth was assessed by microscopy. Cultures of the isolates were transferred to new PDA-containing plates, from where axenic cultures were generated (Gevens *et al*., 2008). Identification of the isolates was based on morphological characteristics, described in the 1995 illustrated genera of fungi by Barnett and Hunter and with literature on identification of pathogenic fungi by Rossman *et a*l, (1997). Confirmation was made by comparing with cultures identified by International Mycological Institute, Egham, UK.

Koch's postulates and pathogenicity test

To confirm the pathogenicity of isolates from cassava tuber tissues, axenic cultures of these isolates were used to inoculate three cassava tubers with 5mm-diameter mycelial agar plugs of a 4-day-old culture. On appearance of symptoms, the tissues at the margins of the healthy and diseased parts were surface-sterilized, excised and plated onto molten PDA for incubation at 28^oC for four days. At the end of this period, morphological characteristics and growth patterns observed in each case were compared with the ones of the original isolates (Markson *et al.,* 2010).

Anatomical studies

The method used was the modification of the method described by Arinze *et. al.* (1975) to determine the mode of entry by the pathogens into the host tissues. Two cassava tubers were peeled and surface-sterilized by dipping them in calcium hypochlorite (3 % available chloride) for 3mins rinsed with several changes of distilled water and allowed to dry naturally. With a sterilized kitchen knife about 1 cm thick slices were obtained. These were placed in sterilized glass Petri dishes and inoculated with spore (conidia) suspension. The spore (conidia) load was estimated using haemacytometer. The inoculum load was determined using the formula.

$$\text{Spore Load} = \frac{N \times v}{V}$$

Where

N = mean number of spores counted in the chosen square (total amount of spores counted: number of squares).

V = volume of the mounting fluid (sterile distilled water)

v = volume of the mounting solution between the cover glass and above square counted (area of square × depth of the chamber).

A spore (conidia) load of 5.0×10^4/ml of sterile distilled water was used in all the experiments for each test pathogen except when otherwise stated. Inoculated slices of tubers in the Petri dishes were incubated at 30^0 C and then sectioned after a week, then at interval of every other week for a period of 4 weeks. Uninoculated slices (control) were similarly sectioned.

Preparation of tissue sections of inoculated and uninoculated yam

Sectioning was done by first fixing the inoculated and the uninoculated slices of cassava tissues with F.A.A (1:1:18) for 24 – 28 hrs then washed with distilled water. Tissue sectioning was done at 10 mm depth using Reichert Rotary microtome. The sections were then stained with safranin for 2 – 3 minutes and dehydrated using pure xylene. These dehydrated sections were then mounted in Canada balsam on a glass slide. The slide was dried over a hot plate at 35 – 40^0C for 1min. Photomicrographs of the prepared slides were taken using Leitz Weitzler Ortholux microscope fitted with a Vivitar – V335 camera (Markson *et al.,* 2010).

RESULTS AND DISCUSSION

Sample collection and pathogen identification

Three rot-causing organisms (*Botryodiplodia theobromae, Rhizopus stolonifer* and *Penicillium expansum*) of cassava which were very virulent were isolated. On PDA, cultures of *B. theobromae* were initially white, fluffy and feathery, becoming grey and eventually black. The growth was radial in

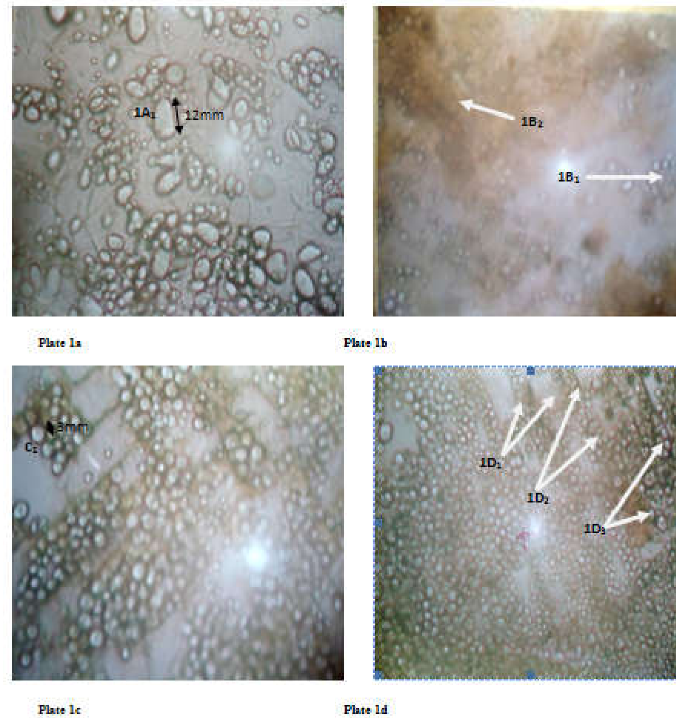

Plate 1a Plate 1b

Plate 1c Plate 1d

Plate 1 shows the impact of the test pathogens on the cassava tubers after one week of inocultion in comparison with the untreated (control). (a) Fresh and uninoculated cassava tuber tissue showing intact cells packed with large starch grain which are somewhat oval-shaped clearly visible and arranged in a regular pattern. The tissue is generally uniform in colour. (b) cassava tuber tissue 1 week after inoculated with *B. theobromae* showing marked reduction in the sizes (**$1B_1$**) of the starch grains, tissue necrosis [brown coloration (**$1B_2$**)] and general maceration of the tissue with no clear arrangement of the starch grains. (c) Inoculation of the cassava tissues with *R. stolonifer* resulted in breaking the starch grains into smaller spherical fragments about 3mm in size (**$1C_1$**)with clear indication of erosion of some cell boundaries; the tissues appeared slightly brownish. (d) In the cassava tissues, *Penicillium expansum* caused fragmentation of the starch grains and aligning them with the cell boundaries (**$1D_1$**) and creating empty spaces (**$1D_2$**) within the lumen of the cells; the tissues became necrotic (**$1D_3$**).

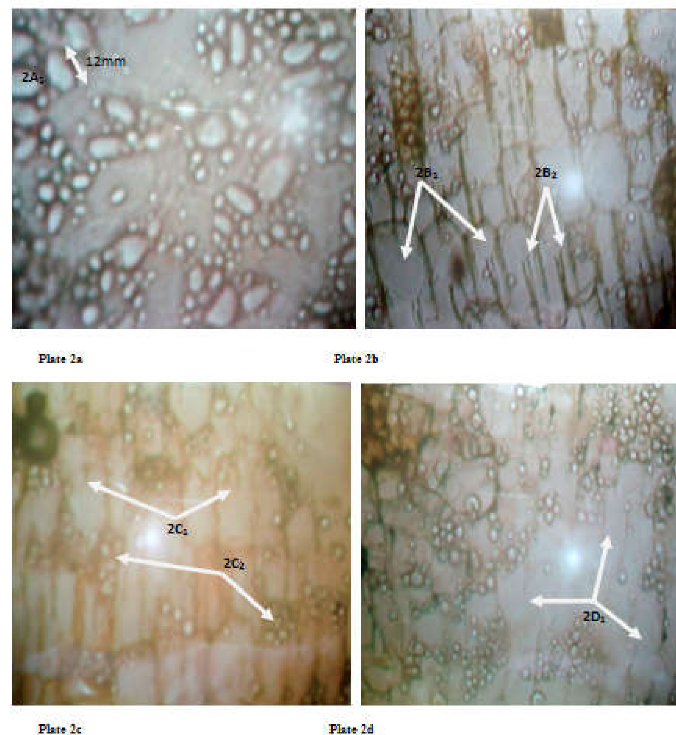

Plate 2a Plate 2b

Plate 2c Plate 2d

Plate 2 shows the cytological state of the cassava tubers two weeks after inocultion with the test pathogens in comparison with the untreated (control). (a) After two weeks, the fresh uninoculated cassava tissues showed a loosely arranged starch grains but still large in size (**$2A_1$**). (b) Tissues inoculated with *B. theobromae* revealed cells depleted of starch grains (**$2B_1$**) and broken cell walls boundaries (**$2B_2$**), two weeks following inoculation. (c) Within two weeks of incubation, *R. stolonifer* reduced the intact cells of the cassava tuber tissue to macerated necrotic structure (**$2C_1$**) with pockets of starch grains (**$2C_2$**) attached to eroded remains of cell walls. (d) after two weeks of incubation, *Penicillium expansum* recorded a near complete degradation of the cell walls (**$2D_1$**) and massive depletion of starch grains.

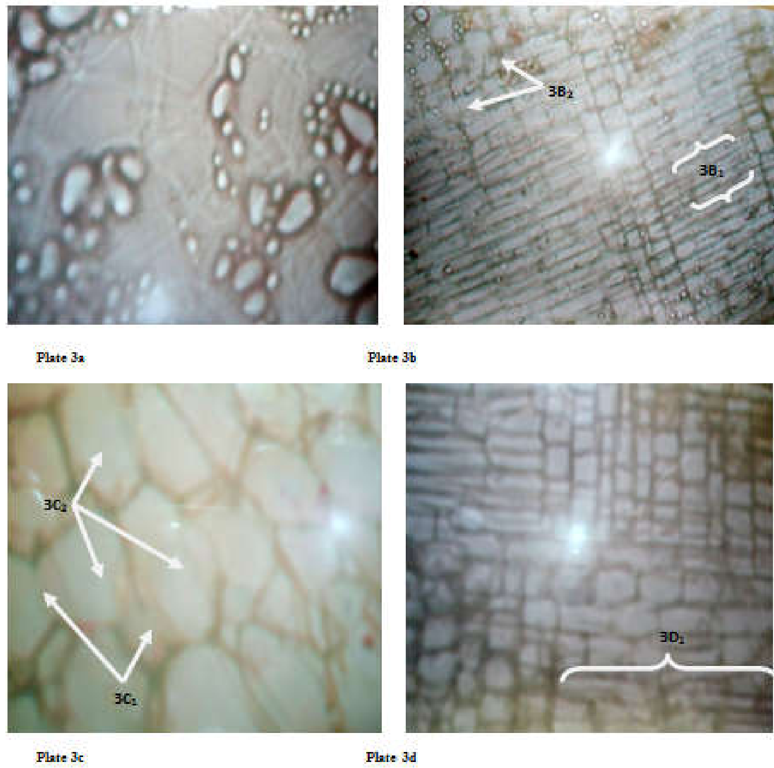

Plate 3 shows the cytological state of the cassava tubers three weeks after inocultion with the test pathogens when compared with the untreated (control). (a) In the fresh and uninoculated cassava tissue, there is a gradual reduction in the amount of starch grains, however, the cell walls were still intact.(b) Effect of *B, theobromae* on the cassava tissues after three weeks of incubation is clearly visible by the near total depletion of starch grains and total maceration of the cell walls which collapsed into smaller cell sizes (**$3B_1$**) with their boundaries broken and twisted (**$3B_2$**). (c) With *R. stolonifer*, there were aberrations of the cell wall boundaries (**$3C_1$**) and complete removal of starch grains (**$3C_2$**). (d) Action of *Penicillium expansum* left the cassava tissues totally devoid of starch grains; the cell collapsed into small disjointed empty irregular shapes (**$3D_1$**).

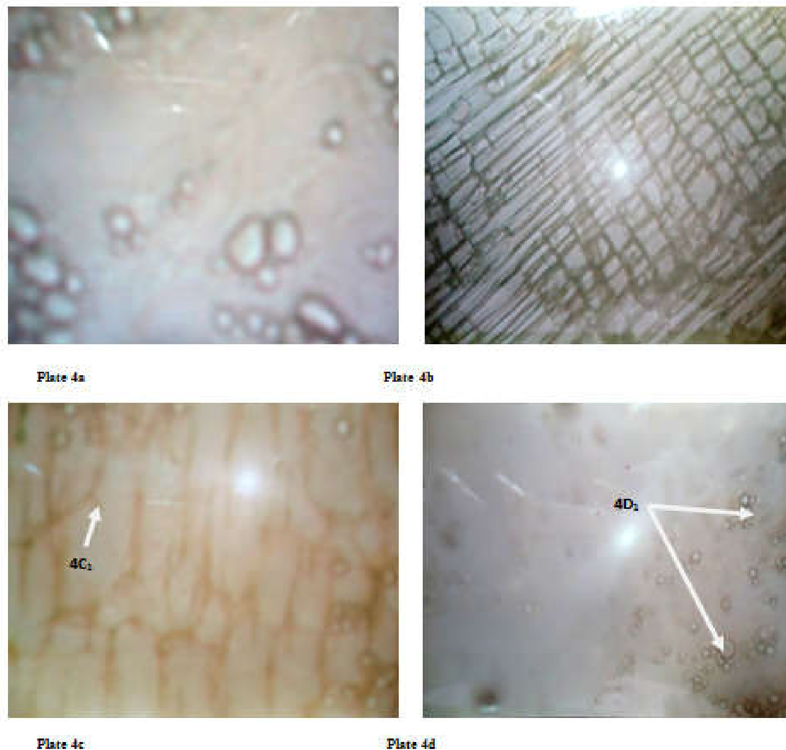

Plate 4 shows the cytological state of the cassava tubers four weeks after inocultion with the test pathogens in comparison with the untreated (control). (a) Fresh tuber tissues revealed sparsely distributed starch grains which are still large in sizes. (b) Infection of *B. theobromae* left the cassava tissue with no trace of starch grains after 4 weeks. (c) *R. stolonifer* caused total disintegration of cell wall boundaries and depletion of starch grains. (d) Cassava tissues, 4 weeks following infection with *Penicillium expansum* revealing total degradation of cells and depletion of starch grains, except for

pattern from the centre of the plate outwards. *R. stolonifer* exhibited profused growth with their whitish thread-like mycelia dotted with grey coloured sporangiospores after three days of growth. The colony of *P. expansum* was greenish in colour and appeared crust-like on the growth medium. Literature on identification of pathogenic fungi (Rossman *et al*., 1997) corroborates these observations and the appearance of this fungus (*B. theobromae*) fitted the description of *Botryodiplodia* Pat. (=*Lasiodiplodi theobromae* (Pat.) Griff and Maubl.) given by Marley (1998). The true identity of each of these three fungi was confirmed by comparing their cultures with those identified by International Mycological Institute, Egham, UK.

Koch's postulates and pathogenicity test

The *B. theobromae, R. stolonifer* and *P. expansum* isolates were pathogenic on cassava tubers used for the test. Symptoms of decay (rot) caused by *B. theobromae* were seen as black rot. *R. stolonifer* produced soft rot symptoms and *P. expansum* caused dry rot on the cassava tubers tested.

Anatomical studies

The figures below show the photomicrographs of sectioned tissues of cassava tubers (healthy and infected). The infected samples were incubated for four weeks and sectioning carried out after every one week. Results obtained revealed that starch grains were progressively removed from the cells of the cassava by the test pathogen during the course of the experiment, suggesting that the pathogens utilized them for its successful establishment, cellular growth, reproduction and survival within the tissues of the cassava tubers. In the fresh uninoculated portions (1a, 2a, 3a 4a), starch grains were seen fully packed within the cells, especially within the first one week of incubation as compared with the slight reductions observed in the infected samples. Similar observations were made by Markson *et al,* (2010) on yam. They reported a progressive removal of starch grains from the cells of the yam by the test pathogen (*Botryodiplodia theobromae*) in treated samples whereas, in the fresh uninoculated portions (head, middle and tail), starch grains were seen fully packed within the cells. They also reported slight reductions in starch grains and minimal maceration of the cell walls 1 week following inoculation as compared with massive starch depletion and cell wall maceration that occurred in the 3rd and 4th weeks. The minimal damage evident in the affected tissues in the first week of incubation may have resulted from the action of polygalacturonase which is the cell wall degrading enzyme reported to be the first to be secreted by rot pathogens (including *B. theobromae.*) during tissue invasion (Cooper, 1987). This corroborates the findings of Markson *et al.,* (2010) who reported slight loss of starch grains from tissues of white yam infected with *Botryodiplodia theobromae* in the first seven days of incubation. Within this period, they also reported minimal maceration of the cell walls of the affected tissues. Uninoculated samples were not affected.

They correlated the disappearance of starch with the depletion of carbohydrates from the tissues and attributed this to the ability of the pathogen to secrete carbohydrate – degrading enzymes. In this study, it was observed, in addition to slight reductions in starch grains and a minimal maceration of the cell walls, one week after inoculation (Plate 1b, 1c and 1d), there was fragmentation of the starch grains into smaller sizes by the test pathogen. Reports of starch grain fragmentation have been documented in tubers of *Ipomea batatas* attacked by *Rhizopus stolonifer* (Markson *et al.,* 2012 unpublished). The slight difference in the mode of attack may be due to differences in the chemistry of the starch grains of these two tubers. A comparative study of the physicochemical properties of cassava starch with other starch-containing tubers and cereals carried out by Nuwamanya *et al.,* (2011) revealed a wide array of differences that may have influenced the mode of attack by *B. theobromae* on the two tuber crops in question. Salami and Akintokun (2008) have also reported of difference in reaction of different cultivars of cassava to different pathogens and their enzymatic activities. *Penicillium expansum,* in addition to starch grain fragmentation caused alignment of the fragmented starch grains with the cell boundaries (**$1D_1$**) and creating empty spaces (**$1D_2$**) within the lumen of the cells. The pathogen also caused necrosis of the tissues. This is likely a function of toxins produced by the pathogen during pathogenesis (Isaac, 1992). In the second week of incubation, maceration of cell wall became more visible and from the 3rd week, cell degradation became massive in addition to the near depletion of starch grains. *B. theobromae* caused total maceration of the cell walls which collapsed into smaller cell sizes (**$3B_1$**) with boundaries broken and twisted (**$3B_2$**). *R. stolonifer* caused aberrations of the cell wall boundaries (**$3C_1$**) and near total removal of starch grains. Similar reports of depletion of carbohydrates have been documented in two cultivars of potato (Irish cobbler and Red Pontiac) infected with *Rhizopus stolonifer.* In Red Pontiac, the total carbohydrate content decreased from777.8mg/g to 291.1mg/g dry weight and from 777.8mg/g to 165.4mg/g in Irish cobbler within ten days of incubation (Amadioha, 1998). Attacks by *P. expansum* resulted in the collapse of cells into small disjointed empty irregular shapes (**$3D_1$**). By the 4th week of incubation, *B. theobromae* reduced the cassava tissue to a mass of irregular shapes devoid of starch while tissue attacked by *R. Stolonifer* appeared necrotic littered with strands of cell wall tissues. In tissues colonized by *P. expansum,* no cell wall boundaries were visible except for few starch grains scattered within the empty tissue. The massive cell wall degradation and cell necrosis observed from the 3rd through the 4th suggests the activity of extra cellular hydrolytic enzymes that are capable of degrading such cell components in the course of pathogenesis (Arinze, 2005, Arinze, 1985, Amadioha, 1993, Amadioha, 1994). Salami and Akintokun (2008) reported that the activity of cell wall degrading enzymes produced by *B. theobromae* and *R. stolonifer* increased with incubation period with their activity peak evident between the 4th and 6th day of incubation for three different cultivars of cassava. Generally, the three pathogens assessed in this study demonstrated different levels of aggressiveness and pathogenic abilities probably depending on the amounts of the different hydrolytic enzymes produced in the course of pathogenesis. It is logical to conclude that the aggressiveness and virulence of *B. theobromae* may be a function of the amounts of extra cellular hydrolytic enzymes it produces within the shortest time during pathogenesis. Cassava has, for ages, continued to contribute to the survival of humans and animals by providing a ready source of carbohydrate and raw materials for industries. However, the major nutrient (carbohydrate) provided by cassava is also the carbon source for the growth of fungi.

Hence the infection of cassava tubers by these pathogens invariably makes unavailable carbohydrates for human use. The insights provided by this paper are to encourage researches aimed at developing methods effective against field and storage rot pathogens of cassava.

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