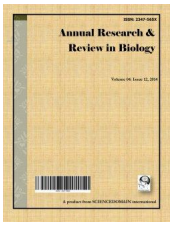

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Histological Studies of Cassava (*Manihot esculenta* Crantz) Root Infection Using *Aspergillus niger Rhizopus stolonifer*, *Aspergillus niger*, *Aspergillus flavus*, *Botryodiplodia theobromae* and *Trichoderma viride*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Histological studies were carried out to investigate the impact of some pathogenic organisms on infected cassava root after different periods of incubation. Cassava roots inoculated with *Aspergillus niger, Rhizopus stolonifer*, *Aspergillus flavus*, and *Trichoderma viride* showed progressive depletion of the starch grains in the cells and tissue maceration of the cassava root as the period of incubation increased. Cassava root inoculated with *A. flavus* showed tissue necrosis and slight fragmentation of the starch grains whereas, cassava tissues infected with *A. niger* showed tissue necrosis and general maceration of the starch grains within the tissue thereby creating empty spaces within the

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cells with no clear arrangement of the starch grains. Inoculation of the cassava tissues with *R. stolonifer* resulted in rapid loss of the starch grains and erosion of surrounding cell boundaries. Cassava tissue colonized by *T. viride* showed tissue necrosis and maceration of the starch grains. Healthy (uninoculated) cassava root tissue showed intact cells packed with large starch grains suggesting that in the host-parasite relationship, the pathogens established themselves inter and intracellularly in the parenchymatous cells of the cortex of the cassava root clearing them of starch grains, and resulting to poor yield and nutritional value of cassava and also economic loss.

Keywords: Cassava root; pathogens; starch grain depletion; tissue maceration; necrosis.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) belongs to the family *Euphobiaceae* and it is an important food and cash crop that contribute to the survival of human beings and livestock by providing a ready and cheap source of carbohydrate for food, feed and raw material for industries [1-4]. Root crops are 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 [5-8]. In addition to human consumption, cassava root is used for the production of bioethanol, and starch for industrial products hence an appropriate commodity for feature economic development of developing countries of the world [9,10].

Despite the importance of cassava in the world, its potential is still undermined by the activities of various disease agents which constitute serious production challenge that greatly reduce yield, eating quality and market value of the crop [11-13]. The cassava root rot disease is one of the main constraints to cassava production in many developing countries of the world [14-17]. Root rot apart from reducing cassava yield can also reduce the quality of cassava tubers harvested. Cassava root rot diseases occur as dry, soft or wet rots and enormous postharvest losses have been attributed to fungal deteriorations caused by *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 [18-20,12,13]. The histological studies on the impact of *A. niger R. stolonifer*, *A. flavus*, *B. theobromae* and *T. viride* on depletion of starch grains and tissue maceration of infected cassava root have been analyzed and presented in this paper.

2. MATERIALS AND METHODS

2.1 Culture Medium

The culture medium used was Potato Dextrose Agar (39g) dissolved in one liter of distilled water in a conical flask, mixed thoroughly and heated in an electric water bath until the agar melted before autoclaving at 120°C for 15 minutes. The sterile medium was allowed to cool (46°C) and 15ml dispensed into sterile Petri-dishes and allowed to solidify.

2.2 Isolation of Fungal Pathogen

Rotted cassava root was washed with tap water, surface sterilized with 70% ethanol solution and rinsed in sterile distilled water and then blotted with filter paper. A sterile scalpel was used to cut the cassava open to reveal the boundary portion between the rotted and healthy parts from where five pieces of the tissue (3 mm diameter) were taken and separately placed with the aid of a sterile forceps on the culture medium. The inoculated plates were transferred into the incubation chamber set at 26°C. The plates were examined daily for mycelial or colony growth. The emerging different fungal colonies were sub-cultured to obtain pure cultures of the isolates.

The percentage fungal occurrence was determined [18]. The number of occurrence of each isolate in the eight different samples of rotten cassava roots were calculated and recorded as a percentage of the total number of occurrence.

Percentage occurrence (%) =

$$\frac{T}{N} \times \frac{100}{1}$$

Where,

T = Total number of each organism in all the samples.

N = Total number of the entire organism in all the samples screened.

2.3 Pathogenicity Test

The pure fungal isolates obtained from the rotted cassava tuber were tested for its ability to cause the same rot condition in a healthy (uninfected) cassava root. The method of [21] was adopted where fresh healthy cassava roots were washed under tap water, surface sterilized with 70% ethanol and rinsed in sterile distilled water. Cylindrical discs (3mm diameter) were removed from the cassava root with a sterile 3mm cork borer. With a sterile inoculation needle, a disc (3mm diameter) of a seven days old pure cultures of each of the test fungi was separately introduced into the hole created in the tubers, then covered with the cylindrical disc previously removed from the cassava root, after cutting off 2mm to compensated for the thickness of the fungal disc. The point of inoculation was sealed completely with vaseline to avoid the entry of extraneous microorganisms. The control set-up consisted of cassava tubers that were similarly bored into but inoculated with sterile PDA discs of 3mm diameter, and covered with vaseline. All inoculated cassava roots were enclosed in polyethylene bags moistened with sterile cotton wool soaked with sterile distilled water to maintain a high relative humidity and incubated at 28±2^{o}C for 7 days. At the end of the incubation period, they were cut open at the point of inoculation and observed for rot development. 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. Those that caused rot of the cassava roots were regarded as pathogens and were then identified and characterized as the causal organism of root rot of cassava whereas those that did not cause any rot were discarded as saprophytes.

Assessment of rot development was done using destructive sampling of cassava roots. The rotted area of the treated cassava tuber and the control were measured with a ruler and expressed as the percentage of the total area using the method of [22]:

$$\text{Percentage rot (\%)} = \frac{\text{Area of rotted tissue}}{\text{Total surface area}} \times \frac{100}{1}$$

$$= \frac{\text{ART - ARC}}{\text{TSA}} \times \frac{100}{1}$$

Where; ART - Area of rotted tissue of the treated tuber, ARC – Area of rotted tissue in the control. TSA – Total surface area of inoculated tuber.

2.4 Identification and Characterization of Pathogenic Organisms

Macroscopic and microscopic observations were carried out on the pure cultures of the identified pathogens. In the macroscopic observation, the physical characteristics of the mycelia such as the colour and structure were recorded. In the microscopic observation, wet mount method of [23] was used in viewing the purified micro organisms under the microscope. The pathogens were each mounted on a separate glass slide containing a drop of cotton blue in lactophenol. A portion of the mycelial growth was carefully picked with the aid of sterile inoculating needle and placed on the slide. The glass slide was covered with a cover slip and examined under the microscope, first with x10 and then with x40 objective lens. Some morphological structures identified include septation, presence/absence of sporangiophores, fruiting bodies and other special organs like the rhizoids. The identification and microscopic characteristics of the microorganisms were matched with mycology manuals of [24] and [25].

2.5 Histological Studies on Inoculated and Uninoculated Cassava Roots

The method of [1] was used. Samples of inoculated and uninoculated (healthy) cassava roots were cut into slices and fixed with Formalin Acetic Acid for 24 hours. The fixed tissues were washed with sterile distilled water and sectioned at 10 mm depth using Reichert Rotary microtome. The sectioned tissues were then stained with safranin for 3 minutes and dehydrated using pure xylene. These dehydrated sections were then mounted on a glass slide and covered with the cover slip and then observed under the microscope fitted with a camera for their photomicrographs.

3. RESULTS

3.1 Isolation of Fungal Isolates

The isolation of fungal pathogens from the rotted cassava roots (Fig. 1) showed that *Rhizopus*

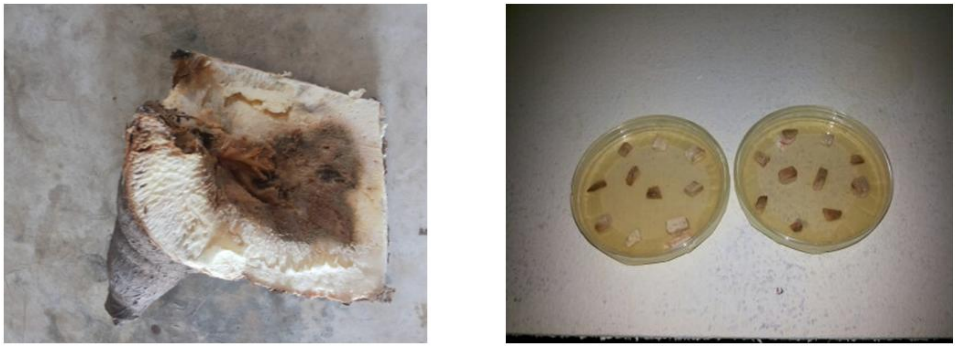
stolonifer, *Aspergillus niger*, *Aspergillus flavus*, *Mucor sp*, *Botryodiplodia theobromae* and *Trichoderma viride* were consistently associated with the rot tissues of the cassava roots. *Aspergillus niger* showed the highest percentage prevalence of 28.6% followed by *Rhizopus stolonifer* with 21.4% and the least *Botryodiplodia theobromae* with 7.1% (Table 1).

3.2 Pathogenicity Test

The pathogenicity test of the fungal isolates carried out on healthy cassava roots revealed that only *Aspergillus niger, Aspergillus flavus*, *Rhizopus stolonifer* and *Trichoderma viride* induced rot on inoculated healthy cassava root after 7 days, hence considered pathogenic (Fig. 2). *A. niger* had the highest rot incidence of 75% and was considered the most virulent followed by *R. stolonifer* with rot incidence of 62.5% whereas the other two organisms; *A. flavus* and *T. viride* depicted a lower pathogenic effect with rot incidence of 45.7% and 33.3% respectively (Table 2).

Table 1. Frequency of occurrence of pathogenic organisms

Pathogen	Frequency of Occurrence (%)
Aspergillus niger	28.6
Rhizopus stolonifer	21.4
Aspergillus flavus	17.9
Botryodiplodia theobromae	7.1
Trichoderma viride	14.3
Mucor sp	10.7



X 1/2

A **B**

Fig. 1. Isolation of fungal pathogens from the rotted cassava root. A : Infected Cassava root ; B :Inoculated Plates

X 1

A **B**

Fig. 2. Pathogenicity Test: Inoculated Cassava roots after 7 days with: A = *Aspergillus niger*; B = *Aspergillus flavus*; C = *Trichoderma viride*; D = *Rhizopus stolonifer*; E = Control

Table 2. Mean percentage rot of cassava root by fungal pathogens

Fungi	Percentage Rot (%)
Aspergillus niger	75.0
Rhizopus stolonifer	62.5
Aspergillus flavus	45.7
Trichoderma viride	33.3

3.3 Characterization of Pathogenic Organisms

3.3.1 *Trichoderma viride*

The morphological characteristics of *T. viride* include: rapid growth, sparse mycelium, initially colourless, but rapidly turns green with age. The conidiophores are pyramidal and branched with profuse green conidia (Fig. 3).

3.3.2 *Aspergillus flavus*

The morphological characteristics of *A. flavus* include: moderate to rapid growth, sparse mycelium which turns yellow to grayish green with age. The hyphae are branched and septate with globose to sub-globose conidiophores and almost smooth conodia (Fig. 4).

3.3.3 *Rhizopus stolonifer*

The *A. stolonifer* has a very rapid growth with abundant cotton-like mycelium, colourless when young and becomes deep gray or black with age. The hyphae are non-septate or sparsely septate, rhizoids with round to ovoid sporangiophores, and round shaped sporangia (Fig. 5).

3.3.4 *Aspergillus niger*

The characteristics of *A. niger* include: rapid growth, dense mycelium which turns dark brown to black with age. The hyphae are branched and septate with globose conidiophores and rough surfaced conidia (Fig. 6).

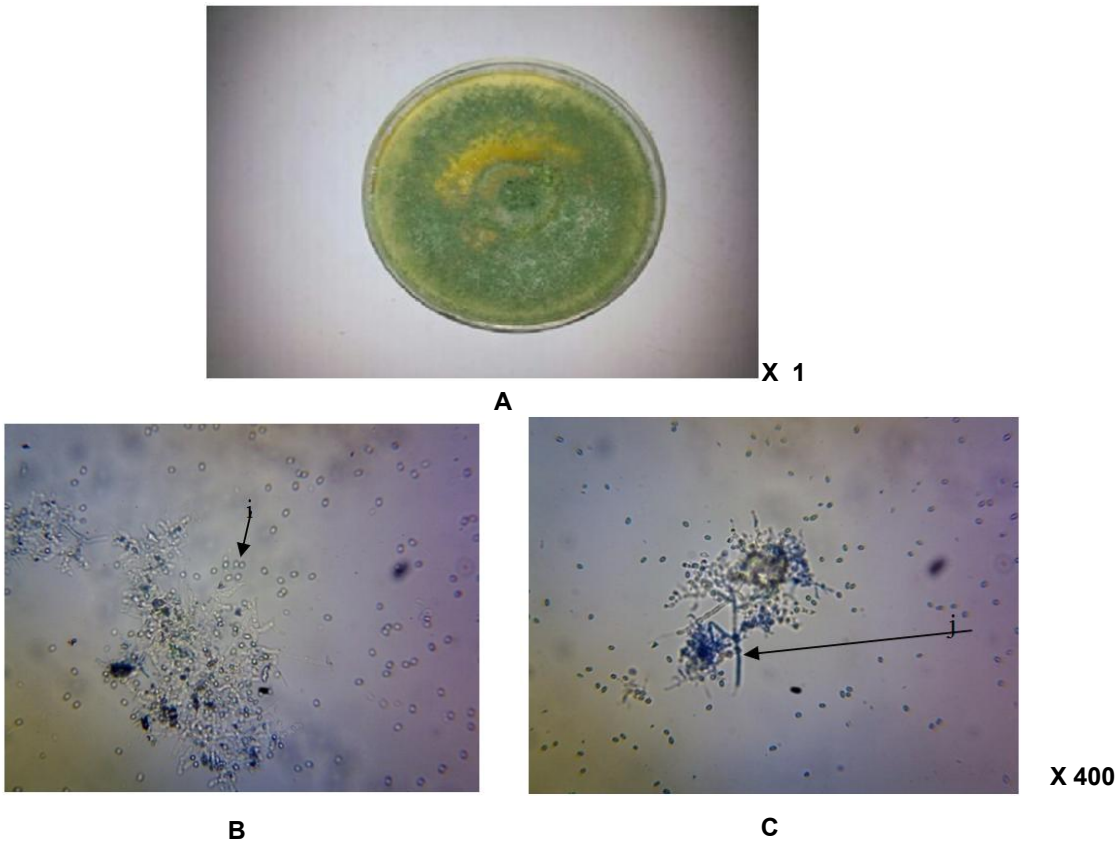


Fig. 3. *Trichodrema viride* : A = Pure culture in a Petri dish (bright green in appearance) B and C = Photomicrograph: i - the conidia and j – the conidiophores

3.4 Impact of Pathogens on Tissue of Cassava Root

Impact of test fungal pathogens on the nutrient content of infected cassava root after 3 days of incubation (Fig. 7a – e) showed that cassava root inoculated with *A. flavus* had tissue necrosis and slight fragmentation of the starch grains (Fig. 7a) whereas, cassava tissues infected with *A. niger* showed tissue necrosis and general maceration of the starch grains within the tissue thereby creating empty spaces within the cells with no clear arrangement of the starch grains (Fig. 7b). Inoculation of the cassava tissues with *R. stolonifer* resulted in rapid loss of the starch grains and erosion of surrounding cell boundaries (Fig. 7c). Cassava tissue colonized by *T. viride* showed tissue necrosis and maceration of the starch grains (Fig. 7d). Uninoculated cassava root tissue showed intact cells packed with large starch grains which were somewhat arranged in regular pattern and the tissue was generally uniform in colour (Fig. 7e).

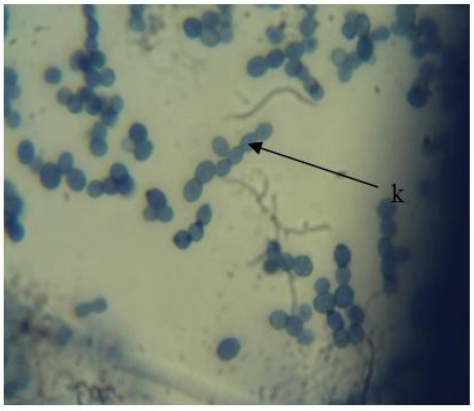
The impact of the test pathogenic organisms on cassava roots after 6 days of incubation shown in Fig. 8a-e indicated that invasion of starch grains in inoculated cassava roots was intense with *A. flavus* (Fig. 8a) and cassava tissues inoculated with *A. niger* showed intense maceration of the starch grains and invasion of the cell boundaries (Fig. 8b). Inoculation of the cassava tissues with *R. stolonifer* resulted in intense breaking of the starch grains into smaller fragments (Fig. 8c) and cassava roots inoculated with *T. viride* caused a clear erosion of the starch grains and cell boundaries (Fig. 8d) whereas uninoculated cassava root tissue (control) showed intact cells with large starch grains (Fig. 8e).

The cytological studies of the tissue of cassava root infected with the test pathogenic organisms after 9 days period of incubation is illustrated in Fig. 9a– e. Cassava roots inoculated with *A. flavus* revealed cells with pockets of starch grains and infection of the cell wall boundaries (Fig. 9a). Cassava tissues infected with *A. niger*

showed intense eroding of cell wall boundaries and severe depletion of starch grains (Fig. 9b) whereas those infected with *R. stolonifer* showed intense tissue necrosis and depletion of starch grains which became smaller in size (Fig. 9c). Cassava root inoculated with *T. viride* recorded intense erosion of the cell walls and massive depletion of starch grains (Fig. 9d) whereas the uninoculated cassava tissues showed a loosely arranged starch grains but still large in size (Fig. 9e).

X 1

A



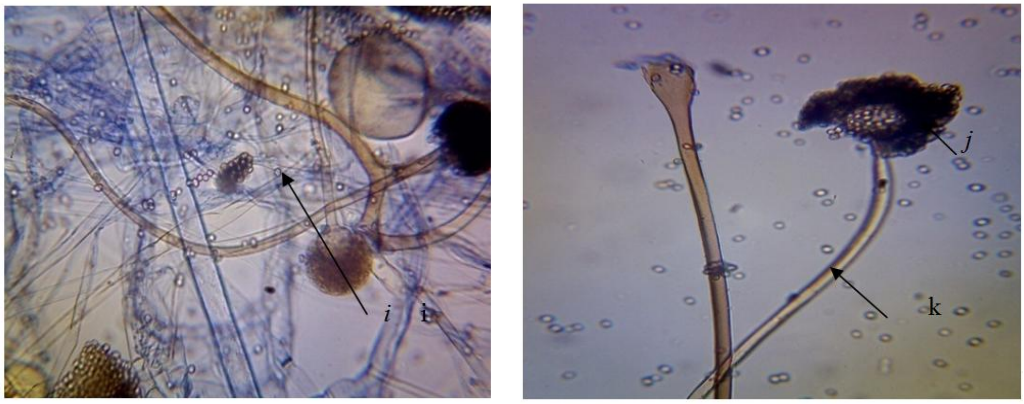
X 400

B C

Fig. 4. *Aspergillus flavus*: A = Pure culture of *A. flavus* in a Petri dish (pale green in appearance); B & C= Photomicrograph; i - the conidiophore which is hyaline, j - coarsely roughened, radiate conidial head and k - the globose to subglobose conidia

X 1

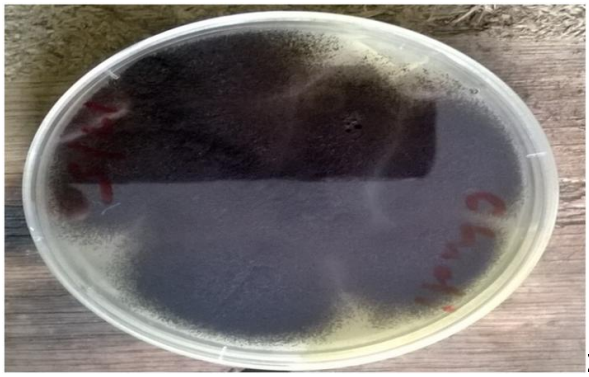
A



X400

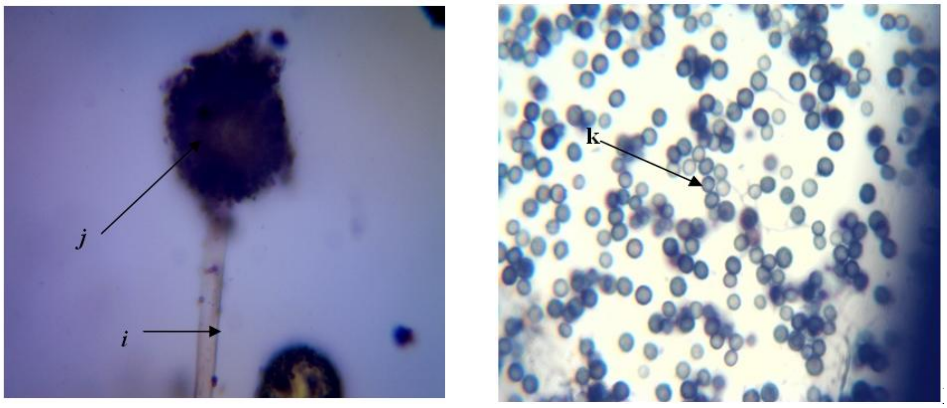
B C

Fig. 5. *Rhizopus stolonifer*: A = Pure culture in a Petri dish (grey to dark in appearance). B and C = Photomcrograph: B - rhizoids (i); C – sporangiophore (k) and the sporangium bearing the spores (j)



X 1

A



X 400

B C

Fig. 6. *Aspergillus niger*: A = Pure culture in a Petri dish (dark in appearance). B and C = Photomicrograph: B - the smooth walled conidiophores (i) and large globose conidial head (j); C - loosed globose conidia (k)

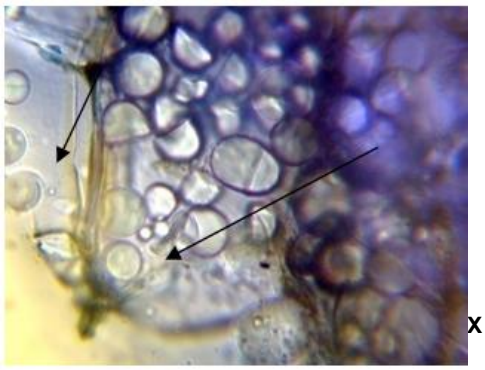


Fig. 7a. *Aspergillus flavus*

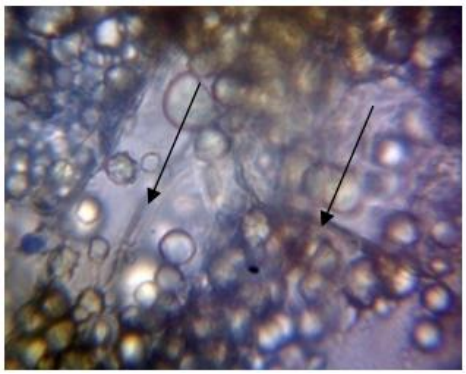


Fig. 7b. *Aspergillus niger*

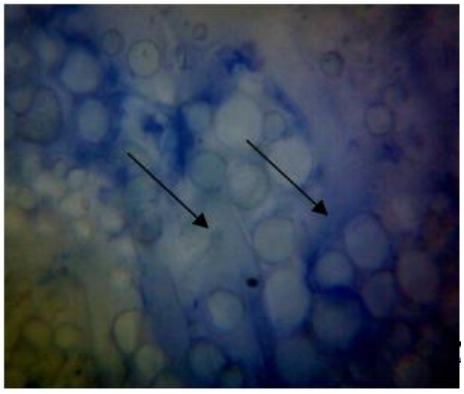


Fig. 7c. *Rhizopus stolonifer*

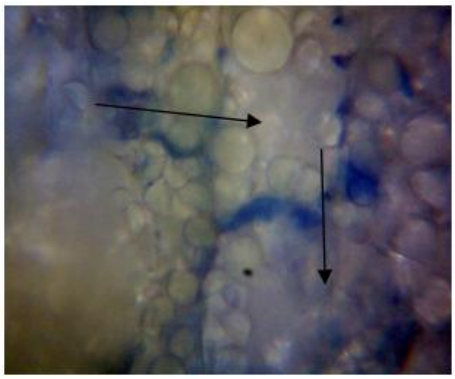


Fig. 7d. *Trichoderma viride*

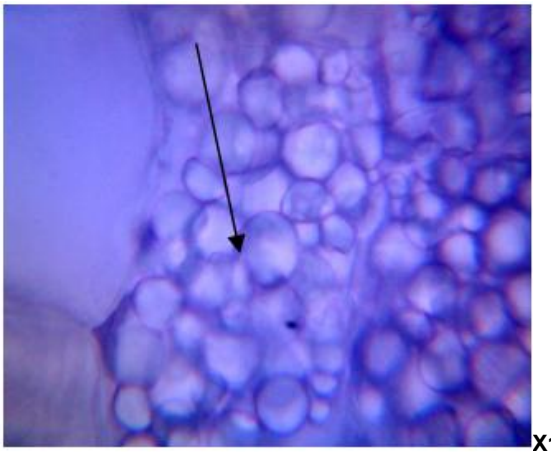


Fig. 7e. Control

Fig. 7. Intact (control) and depletion of starch grains in cassava tissue by pathogens 3 days after inoculation

Massive depletion of starch grains and tissue maceration were recorded in cassava roots inoculated with the test pathogenic organisms after incubation period of 12 days (Fig. 10a – e). Cassava tissue infected with *A. flavus* showed a severe invasion of the starch grains in the cells (Fig. 10a). Action of *A. niger* left the cassava tissues with huge reduction of starch grains

leading to the cell collapsing into small disjointed empty irregular shaped cells (Fig. 10b). Cassava tissue infected with *R. stolonifer* showed more intense disintegration of the cell wall boundaries and starch grains (Fig. 10c) whereas the effect of *T. viride* on the cassava tissues showed a near total maceration of the cell walls with their boundaries broken and twisted and total depletion of the starch grains (Fig. 10d). In the uninoculated cassava tissue, there was a gradual reduction in the size and amount of starch grain (Fig. 10e).

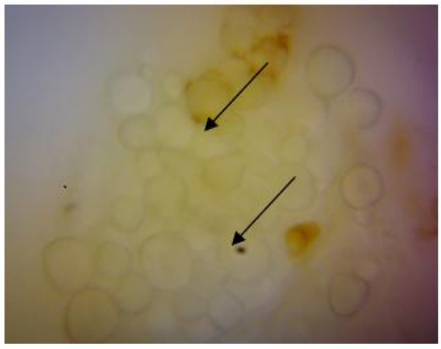


Fig. 8a. *Aspergillus flavus*

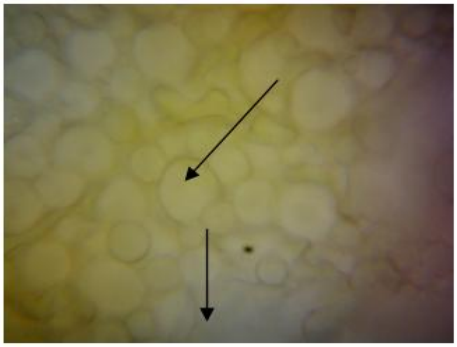


Fig. 8b. *Aspergillus niger*

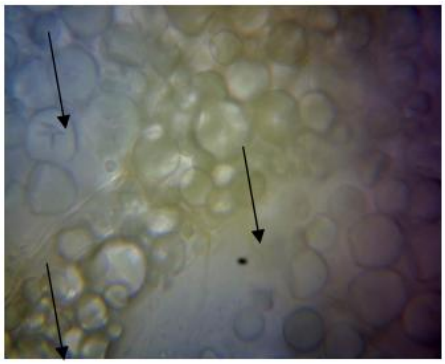


Fig. 8c. *Rhizopus stolonifer*

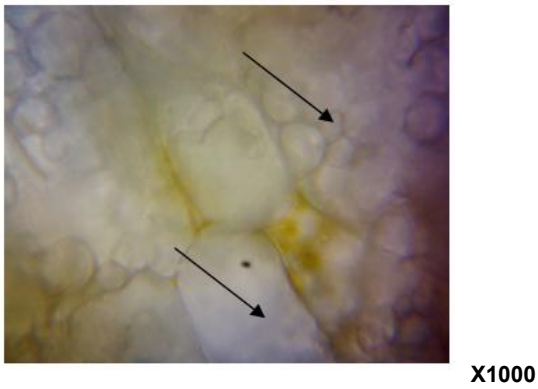


Fig. 8d. *Trichoderma viride*

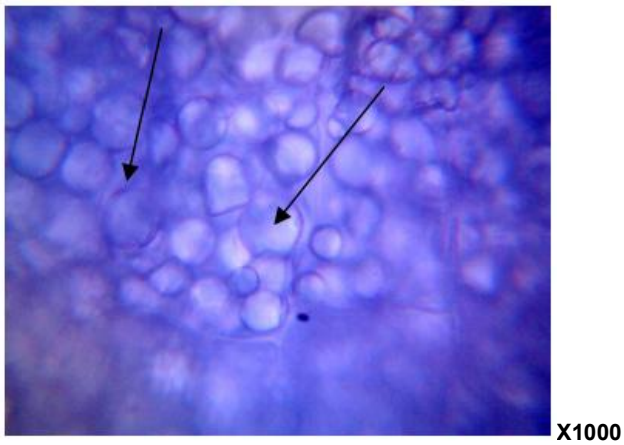
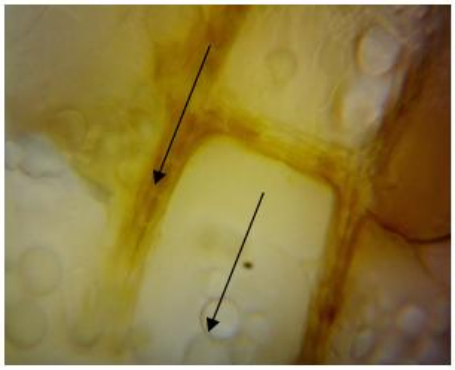


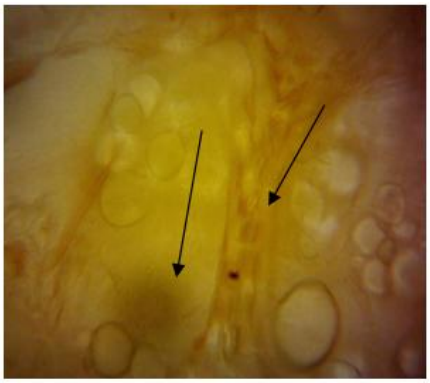
Fig. 8e. Control

Fig. 8. Depletion of starch grains and tissue maceration by pathogens after 6 days of incubation



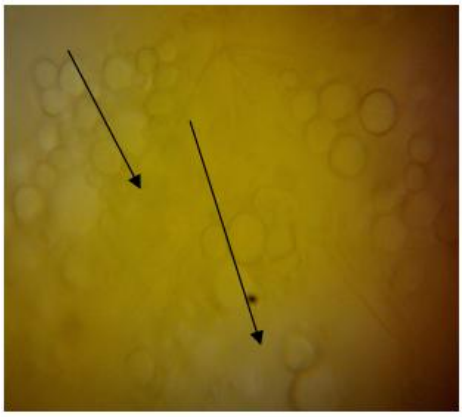
X 1000

Fig. 9a. *Aspergillus flavus*



X 1000

Fig. 9b. *Aspergillus niger*



X 1000

Fig. 9c. *Rhizopus stolonifer*

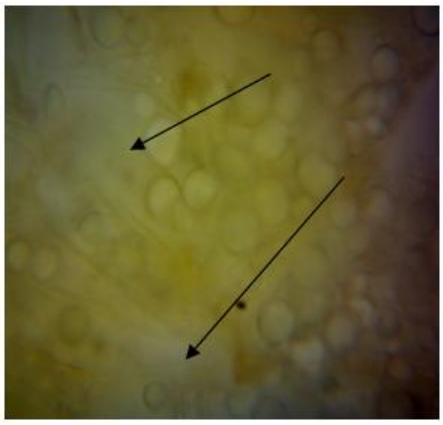


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Fig. 9d. *Trichoderma viride*

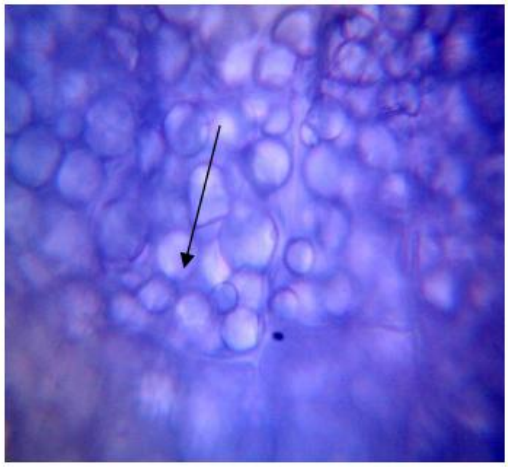

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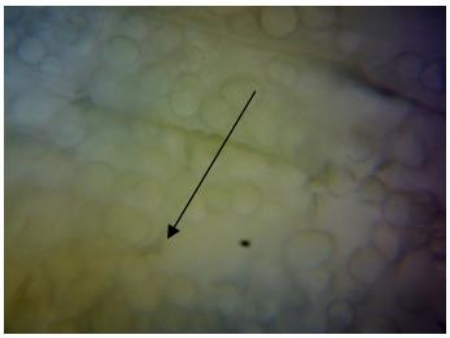
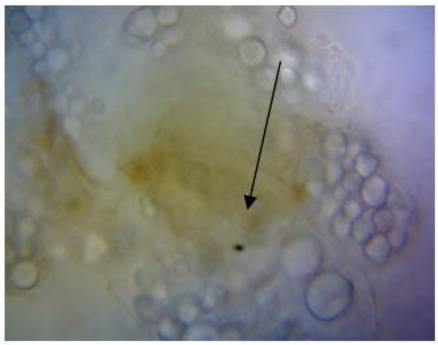
Fig. 9e. Control

Fig. 9. Depletion of starch grains and infection of the cell wall boundaries by pathogens 9 days after incubation



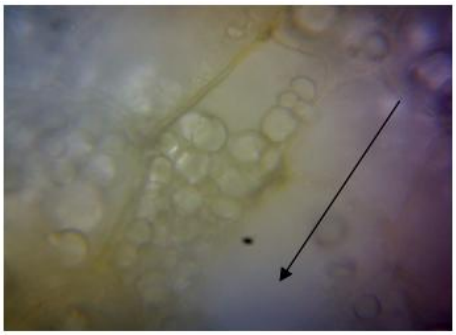
X 1000

Fig. 10a. *Aspergillus flavus*



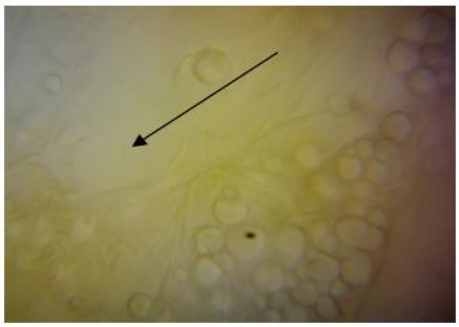
X 1000

Fig. 10b: *Aspergillus niger*



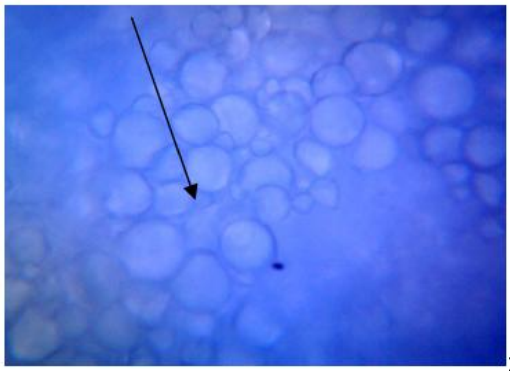
X 1000

Fig. 10c. *Rhizopus stolonifer*



X 1000

Fig. 10d: *Trichoderma viride*

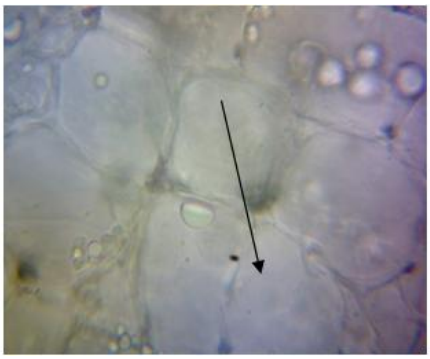


X 1000

Fig. 10e. Control

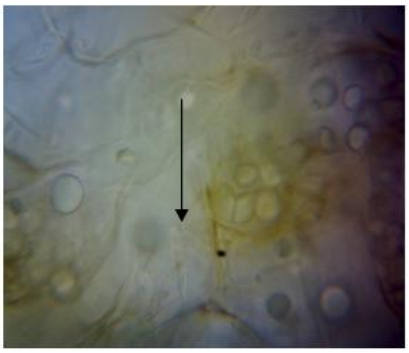
Fig. 10. Maceration of the cell walls and total depletion of the starch grains after 12 days period of incubation

Fig. 11a – e showed cassava roots inoculated with test fungal pathogens after 15 days incubation period. Inoculation with *A. flavus* left the cassava tissue with a clear visible near empty cells containing few traceable starch grains (Fig. 11a). Infection of cassava tissue with *A. niger* resulted in disjointed cells with near empty starch grains (Fig. 11b) whereas *R. stolonifer* caused huge depletion of starch grains with disintegration of cell wall boundaries (Fig. 11c). Cassava tissues with *T. viride* revealed severe erosion of the cell wall boundaries and a remarkable depletion of the starch grains (Fig. 11d). Cassava root tissues that were not infected revealed sparsely distributed starch grains which were still large in sizes (Fig. 11e).



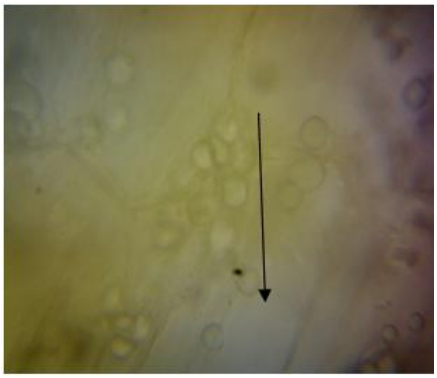
X 1000

Fig. 11a. *Aspergillus flavus*



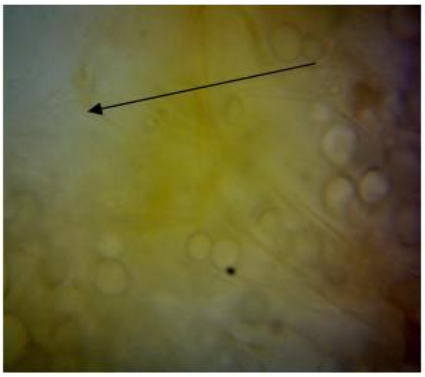
X 1000

Fig. 11b. *Aspergillus niger*



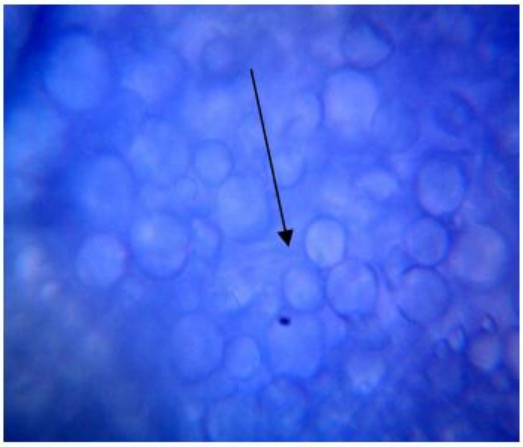
X 1000

Fig. 11c. *Rhizopus stolonifer*



X 1000

Fig. 11d. *Trichoderma viride*



X 1000

Fig. 11e. Control

Fig. 11. Huge depletion of starch grains and disintegration of cell wall boundaries 15 days after inoculation with the test pathogens in comparison with intact starch grains in the control

4. DISCUSSION AND CONCLUSION

The pathogenic organisms in this study, *A. niger, A. flavus, R. stolonifer,* and *T. viride* have been linked with storage rot of cassava root and tubers of yam and cocoyam (Okigbo *et al*., 2014; Okigbo *et al*., 2009a, b; Amadioha and Markson 2007a, b). Of all these rot causing organisms, *A. niger* and *R. stolonifer* had the highest percentage frequency of occurrence of 28.6% and 21.4% as well as highest percentage mean rot of cassava root, 75.0% and 62.5%

respectively implicating them as most virulent. These findings corroborates the report of [18] that *A. niger* could be the leading cause of postharvest fungal root rot of cassava in South–East Nigeria.

The histological studies on cassava roots inoculated with the test pathogenic organisms revealed that starch grains were progressively depleted from the cells of the cassava roots as the period of incubation increased. However, the rate of depletion of starch grains and tissue maceration differed across the pathogens tested suggesting that the pathogens utilized the starch grains at varying degrees for their establishment and growth within the cassava tissue. Similar observations were made by [1,7] on cassava root and they attributed the disappearance of the starch grains to the ability of the pathogen to secrete carbohydrate – degrading enzymes. *T. viride* was found to be more aggressive in starch grain depletion followed by *R. stolonifer, A. niger* and *A. flavus* in that order. The differences in starch grain fragmentation and depletion and tissue maceration across the pathogens may be due to differences in their mode of attack or colonization of cassava tissues [3]. The test pathogens also caused necrosis of the tissues which is likely a function of toxins produced by these pathogens during pathogenesis [26,6]. In the uninoculated cassava tissues (control), starch grains were large and fully packed within intact cells but reduced slightly as the period of incubation increased due to the normal physiological processes of dormant but active cells of the cassava root. The findings of this study have shown that pathogenic organisms are the major cause of postharvest losses of stored food products and their control through sustainable plant disease management is imperative for actualization of food security in cassava producing countries of the world.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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