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MYCOBURDEN OF TOMATO (*Lycopersicum esculentum* MILL), INOCULATION-INDUCED MYCOTOXIN PRODUCTION AND CONTROL BY PASSIVE MODIFIED ATMOSPHERE

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ABSTRACT

The effect of fungi of tomato fruits (*Lycopersicum esculentum*) were investigated at Ijebu-Ode, south western Nigeria. *Aspergillus niger*, *Fusarium oxysporum*, *Rhizopus stolonifer* and *Curvularia* species were isolated from deteriorating tomato and used for inoculating fresh and healthy tomato samples. The isolates (*Aspergillus niger, Fusarium oxysporum, Rhizopus stolonifer* and *Curvularia* species) were then inoculated separately into healthy tomato samples. Storage of samples in modified atmospheres and the effects of inoculation on quality changes were also evaluated. Modified atmospheres designed by packing the samples inside a polythene bag of 12µm thickness significantly maintained the quality of the samples compared with the controls. The isolates were then evaluated for mycotoxin production using thin layer chromatography and mycotoxigenic potential was assessed by animal feeding trial using rats of the wistar strain fed orally with different concentrations of the mycotoxin extracts. Mycotoxins were detected from tomato samples inoculated with *Rhizopus, Aspergillus* and *Fusarium* species, before and after autoclaving for 15 min at 121^{o}C. Experimental rats fed on mycotoxin extracts developed symptoms of neurotoxicity. Most of the fungal isolates showed great potential for mycotoxin production, which is of concern in public health. Modified atmosphere created by using sterile polythene bags of different thicknesses is therefore recommended for control of fungi infection of tomato fruits and extension of shelf life.

Key words: Deterioration, Modified atmosphere, Mycotoxin, Neurotoxicity, Tomato (*Lycopersicum esculentum*).

INTRODUCTION

Tomato (*Lycopersicum esculentum* Mill) is an economically important vegetable crop grown across the world. It is reported to have originated in South America (Kimura and Sinha, 2008). Tomato fruits are popular worldwide and are used in all kinds of stew/soups and also consumed raw in salads. Due to its importance as food, it has been bred to improve productivity, fruit quality, and resistance to biotic and abiotic stresses (Kimura and Sinha, 2008). It is grown in outdoor fields, greenhouses and net houses (Bihn and Gravani, 2006). Nigeria is second largest producer of tomato in Africa (Erinle, 1989).

In Nigeria, tomato plays a vital role in meeting domestic and nutritional food requirements, generation of income, foreign ex-

change earnings and creation of employment. The crop is grown for both fresh domestic and export market but there is increasing demand for processed tomato products. Tomato crop do well in warm climate with an altitude range of 0 – 2100 m above sea level. It requires rainfall ranging between 760 mm to 1300 mm and deep fertile loam soil that is well drained, with high content of organic matter and a pH ranging between 5-7 (Rice *et al*., 1994). Tomato fruits are used in salads or cooked as a vegetable, processed into tomato paste, sauce and puree. The nutritional value of tomato makes it a widely accepted vegetable by consumers. The fruits of tomato are rich in calcium, phosphorus, magnesium, copper, niacin, iron, folate, Vitamin A, B6, Vitamin E, Vitamin B2, Vitamin C, iron and carbohydrates (Wamache, 2005). Furthermore, the fruit has medicinal value as a gentle stimulant for kidneys, helping to wash off toxins that contaminate the body systems. It improves the status of dietary antioxidants (lycopene, ascorbic acid and phenols) in diet. Tomato juice is known to be effective for intestinal and liver disorders (Wamache, 2005).

Tomato production is limited by factors such as poor pre-harvest practices, adoption of poor production techniques, rough handling and moisture condensation causing pathogen infestation (Kader, 1992). Post-harvest deterioration/spoilage of plant produce has been substantially reported (Arinze, 1985; Data *et al.,* 1987; Onifade *et al.,* 2004). These deteriorations/spoilages are attributed to physical, physiological and microbiological factors. Mechanical damage during harvesting, storage or transportation has been implicated in fruit predisposition to storage deterioration (Snowdon, 1991). Pathogenic contamination through natural openings or wounds is considered the most critical factor in fruit decay (Udo *et al.,* 2000). The degree of pathogenicity varies and largely depends on storage conditions. Despite the present trend of discouraging the use of chemical fungicides to control post-harvest diseases of produce, they are still extensively used in many developing countries (Ogundana, 1993).Control of tomato fruit rot has been by application of synthetic chemicals. Several types of synthetic chemicals have been used successfully to control the post-harvest decay of fruits and vegetables (Adaskaveg *et al*., 2004). However there are three major concerns: the increasing consumer concern over pesticide residues on foods (Wisniewski and Wilson, 1992); the predominance of fungicide resistant strains due to excessive use of fungicides (Rosslenbroich and Stubler, 2000) and environmental pollution. There is increased crop production associated with use of synthetic chemicals for the management of plant pathogens, pests and weeds but with the negative effect of deterioration of environmental quality and human health (Onuegbu, 2002; Ramazani *et al*., 2002). However, consumers demand less use of chemicals and still want food devoid of microbial growth, toxins as well as other quality deteriorating factors (Lingk, 1991).

Post-harvest rot remains a major challenge in tomato production. The magnitude of post-harvest losses vary from one country to another, one season to another and even one day to another (Mujib *et al*., 2007). There are numerous microbes that cause post-harvest decay of tomatoes. Among these, fungi and bacteria are the most destructive. Most of the tomato fruits are also damaged after harvesting because of inadequate handling and preservation methods (Wills *et al*., 1981). Fruits, due to their low pH, high moisture

content and nutrient composition are very susceptible to attack by pathogenic fungi, which in addition to causing rots, may also make them unfit for consumption by producing mycotoxins (Stinson *et al*., 1981).

Mycotoxins are potential health hazards to man and animals and in most cases they are not easily unnoticed. Control of fruit rot also remains a major challenge in tomato production. Most microbes that infect plant tissues often produce secondary metabolite known as mycotoxins in their hosts which are known to be hazardous to animals including humans (Baiyewu *et al.,* 2007). Mycotoxins are naturally occurring toxic chemical (often of aromatic structures) compounds which are capable of inducing mycotoxicoses (toxic syndromes especially cancer) in humans following ingestion or inhalation. However, they differ in their degree and manner of toxicity (Samson and Van Reenen-Hoekstra, 1988; Efuivwevwere, 2000). Some of these metabolites include the ergotalkaloid by *Clavicep* species, zearalenone and vomitoxin by *Fusarium* species, ochratoxin A, produced by *Penicillium* species and aflatoxin produced by *Aspergillus* species (Prasad, 1992). Aflatoxins, which are a group of highly toxic, mutagenic and carcinogenic polyketide compounds, have been detected in fruits in South Western Nigeria (Baiyewu *et al.,* 2007).

Until recently, only tomato fruits without visible wounds or spoilage signs were retailed. The difficult economic situation in many developing nations, especially in Nigeria, may have changed all that, as tomato fruits with apparent spoilage signs and symptoms are now commonly displayed for sale in the open market. The potential hazard of storage rot/public health implications associated with these practices has been given little or no attention.

This study therefore was undertaken to investigate (i) the fungi associated with tomato rot, (ii) the pathogenicity of the fungal isolates, (iii) the effect of modified passive atmosphere storage on quality of tomato fruits and, (iv) Mycotoxigenic potentials of the fungal isolates.

MATERIALS AND METHODS

Source of tomato fruits

Tomato fruits were obtained from Central market (where they were displayed in the open under the sun for sale), at Ijebu-Ode, Ogun State, South western Nigeria. Twenty tomato fruits with lesions/rots and 66 healthy fruits were procured on two different occasions for this study.

Isolation of fungal pathogens from rotting tomato fruits

The infected tomato samples were first washed under a running tap, then dipped into 1 % Sodium Hypochlorite to surface sterilize for 3 minutes and rinsed thrice in sterile distilled water. They were then blot-dried with sterile blotting paper. Direct plating method was used for fungal isolation. A flame-sterile scalpel was used to cut 3 mm x 3 mm sections of tissue from the tomato moving from the healthy portions to the decayed portion where the pathogens are likely to be more active. The pieces were dried using sterile filter paper to dry the juice. The dried infected tissues were directly placed on potato dextrose agar (PDA, Difco) prepared following standard procedure. The plates were duplicated, incubated at 27 ± 2◦C and examined for fungal growth at 48hrs intervals for 6days.

Sub-culturing and identification of fun-

gal pathogens

Fungal colonies that developed on the plates were aseptically transferred onto freshly prepared PDA in new sets of petri-dishes and incubated 27 ± 2◦Cfor 5-7 days. The colony morphology and pigmentation of the isolates were recorded before sub-culturing for purification and storage under refrigeration (7◦C) until required. Identification was by comparison of fungal morphology and reproductive structures with earlier descriptions (Barnett and Hunter, 1972).

Pathogenicity test

Ten healthy tomato fruit samples were first washed under a running tap, then dipped into 1% Sodium Hypochlorite to surface sterilize for 3 minutes and rinsed in three changes of sterile distilled water. They were then blot-dried by using sterile blotting paper and a flame-sterile scalpel was used to cut 3 mm x 3 mm sections of tissue from the tomato. A 2mm disc cut from a 7-day-old culture of the test fungal isolate growing on PDA was introduced into the holes (2-3holes per fruits, depending on the size of the fruits) which were then sealed with sterile Vaseline. Control samples (two surface sterilized healthy tomato fruits) were treated in the same manner except that uninoculated PDA was used. The treated samples (eight surface sterilized healthy tomato fruits) and control were placed individually in sterile polyethylene bags and incubated at 27 ± 2◦C for 9 days. At 3 days intervals, the samples were sectioned through the site of inoculation and examined for lesion development. Infected or decayed portions were aseptically transferred onto PDA to confirm that the infection was caused by the inoculants.

Influence of passive modified atmosphere storage on the quality of inoculated tomato fruit samples

Twenty four (24) tomato fruit samples were surface-sterilized (as previously described above). They were then blot-dried by using sterile blotting paper and a flame-sterile scalpel was used to cut 3 mm x 3 mm sections of tissue from the tomato. Following inoculation (in duplicate) with *Aspergillus niger, Fusarium oxysporum* and *Rhizopus stolonifer* as described above, samples (total of eighteen tomato fruits), except for controls (total of six tomato fruits), were packed aseptically in sterile polyethylene bags (6, 9 or 12μm thick) and stored as indicated. Quality was evaluated by an 8-member panel familiar with the quality of tomato using a modified "assessment keys for plant diseases" (James, 1971; Sozzi and Fraschina, 1997). The key was rated 0-5 as follows:

0 - Fruit not showing any deterioration/ change in appearance
1 - Slight deterioration, up to 20% of the total surface area of fruit rotted
2 - 21-40% of total surface exhibiting rotting or showing deterioration signs
3 - Moderate degree of deterioration, 41-60% of total surface showing rotting or other forms of deterioration.
4 - High degree of deterioration, 61- 80% of total Fruit rotted, softened or showing other forms of deterioration
5 - Fruit completely rotted and hence unmarketable

Mycotoxin detection

Mycotoxin was detected essentially as previously described (Baiyewu *et al.,* 2007). Ten grams (10g) each of a set of sterilized (autoclaved at 121◦C for 15 min) inoculated tomato samples and another set of unsterilized inoculated tomato samples were extracted with chloroform (May and Baker Ltd.

England) and concentrated. Of the extracted samples, 5, 10 and 15 L were spotted on three different points on a ruled line of the thin layer chromatography (TLC) coated with plates of silica gel (Merck TLC grade 7749). Also 5, 10 and 15 L of the mycotoxin standards were spotted on another three points near the previous samples extract spotted points. These were then developed in TLC tanks containing the solvents (toluene, isoamyl alcohol and methanol) at a ratio of 3:3:2. When the solvent emigrated to about two-third of the plates, the plates were removed, air dried and examined under UV light at a distance of 365mm. The mycotoxin levels were semi-quantified based on comparisons with control levels.

Animal feeding trial

Fifty four albino rats of the wistar strain (males and females, age 7-8 weeks, weighing 22-28 gram)were used and divided into 6 groups of 2 (in duplicate), numbered and housed individually in wire-screen-plastic-bottom cages and fed with a conventional diet (Figure 1). Group 1, contained rats fed with sterile distilled water (0.1ml), this served as the control rats while groups 2, 3, 4, 5, and 6, contained rats fed with 10, 20, 30, 40 and 50 μg/100 μl of each extract from *Rhizopus, Aspergillus, Fusarium and Curvularia* species. Animals were fed *per oral* with mycotoxin extracts using 0.1 ml graduated syringe and kept off food and water for 24 hours. The animals were observed over a period of 24hours for onset of symptoms typical of neurotoxicity.

Statistical analysis

Data obtained were subjected to analysis of variance and means separated using the least significance difference (LSD) method where significant difference between means of treatment at p=0.05 were established (Steel and Torries, 1990).

RESULTS AND DISCUSSION

Fungi associated with tomato rots

Aspergillus niger, Fusarium oxysporum, Rhizopus stolonifer and *Curvularia* species were isolated from tomato fruits showing rot (Table 1). Colour, magnitude and texture of the symptoms varied with the organism. Comparable symptoms were observed following pathogenicity test but the symptoms became more pronounced with storage time.

Pathogenicity of the isolates

Different sizes of rots were induced by the isolates (Table 1) with *Aspergillus niger* inducing the most extensive lesions followed by *Rhizopus stolonifer,Fusarium oxysporum* and *Curvularia* sp. in that order (Table 1). On day 3, *Aspergillus niger* and *Rhizopus stolonifer* induced more extensive lesions, 2.3 ± 0.40 and 2.1± 0.20 respectively while *Curvularia* sp recorded the least lesion (1.2 ± 0.18). On day 6, the lesion size increased for all the isolates with *Aspergillus niger* recording the highest (3.1± 0.28) and *Curvularia* sp recording the lowest. Similar trend of rot extension were also observed on day 9 with *A. niger*, *Rhizopus stolonifer, Fusarium oxysporum* and *Curvularia* sp. recording, 4.0 ± 0.34, 3.4 ± 0.20, 3.3 ± 0.16 and 2.1 ± 0.27, respectively.

Table 1: Pathogenicity of fungal isolates inoculated into tomato fruit samples

Inoculum	Storage Time (days)		
	3	6	9
Aspergillus niger	2.3±0.40	3.1±0.28	4.0±0.34
Rhizopus stolonifer	2.1±0.20	2.9±0.31	3.4±0.20
Fusarium oxysporum	1.4±0.11	2.2±0.18	3.3±0.16
Curvularia sp.	1.2±0.18	1.7±0.22	2.1±0.27
Overall (mean)	1.8±0.22	2.5±0.24	3.2±0.24

Each value represents the mean ± standard deviation of two independent experiments.
Each value represents the overall means of lesions/rots induced by all the four isolated with storage time.

Mycotoxigenic potentials of the isolates

The thin layer chromatography (TLC) spot extracted from most of the inoculated tomato samples (sterilized and unsterilized) shows that *Aspergillus*, *Rhizopus* and *Fusarium* specie inoculated fruits and the standard mycotoxin, fluoresces and produced bluish spots of equal intensities indicative of mycotoxin while *Curvularia* specie did not fluoresce, thus lack production of mycotoxin (Table 2). Wistar rats fed with different concentrations of mycotoxin extracts, showed that three of the fungal isolates (*Aspergillus*, *Rhizopus* and *Fusarium* species) produced mycotoxin with consequent neurotoxic symptoms with varying severity characterized by ascending paralysis, convulsion and respiratory arrest within 24 hours of ingestion of the extracts. At concentration of 10-20 μg/100 μl, only extract from *Aspergillus* specie produced paralysis in rats and as concentration increased (30-40 μg/100 μl) paralysis occurred in rats fed with *Rhizopus* and *Fusarium* species extract. In contrast, *Aspergillus* sp. extract produced paralysis and convulsion at these concentrations, followed by a combination of paralysis, convulsion and respiratory arrest for *Aspergillus* sp. extract at concentration of 50 μg/100 μl (Table 3), whereas, animals fed on extracts from *Curvularia* specie did not develop neurotoxic symptoms and remained healthy after 24 hours, thus lack production of mycotoxin. *Aspergillus flavus* has been reportedly isolated from fruits in Nigeria and is probably the main producer of Aflatoxin in fruits and vegetables (Baiyewu *et al.,* 2007). Extracts from *Aspergillus* species showed the highest potency in this study. Previous studies reported that the most potent and best characterized mycotoxin is aflatoxin (B1, B2, B2a, G1, G2, and G2a) produced by certain strains of *Aspergillus flavus* and other fungi (Singh, 1983). Aflatoxins have been reportedly detected in grapes, tomatoes, and oranges (Sage *et al.,* 2002; Muhammad *et al.,* 2004). Out of 342 samples of different fruits and spices obtained from the stores of commercial centers

screened for aflatoxin, 95 of them were positive (Singh, 1983). It is probable that chronic ingestion of aflatoxin at low levels in moldy foods is a cause of hepatic disease in man in some parts of the world including Nigeria, where studies have linked the presence of high levels of aflatoxin in most common foods stored or retailed in unhygienic environments to the high incidence of primary liver cancer in young people under the age of 40 (Mbakwem-Aniebo, 2010). Aflatoxins are major inducers of neonatal jaundice (Sodeinde *et al.,* 1995). *Fusarium verticilliodes* recovered from African star apple (*Chrysophyllum albidum*) was confirmed to be mycotoxigenic (Efiuvwevwere, 2000). *Rhizopus oryzae* and *Rhizopus stolonifer* have also been revealed to be a potent mycotoxin producer (Smith *et al.,* 1994).

Although the obvious concern in mould-induced quality changes in tomato fruit is the economic loss due to consumer rejection or price reduction, an elusive and perhaps equally important issue is that of health safety, since many of the moulds isolated in this study have toxigenic effects. The production of neurotoxic symptoms, including, ascending paralysis, convulsion and respiratory arrest in wistar rats in this study confirms the mycotoxigenic potential of the concerned fungi, since these symptoms may be produced in humans if mycotoxins accumulate. The level of mycotoxicity is usually influenced by the age, sex, nutritional and immunological status of the host (Mbakwem -Aniebo, 2010). That most people, have not been diagnosed as having hepatoma or mycotoxicosis, does not mean that the toxic metabolite is absent in their body system (Muhammad *et al.,* 2004). Aflatoxin M1, for example, has been discovered in the urine of a Philippine woman that consumed peanut butter containing aflatoxin (Sage *et al.,* 2002).

Table 2: Mycotoxigenic potentials of fungal isolates inoculated into healthy tomato samples before and after autoclaving at 121°C for 15 minutes

Isolates (moulds)	Unsterilized Inoculated Samples	Sterilized Inoculated Samples
Rhizopus sp.	+	+
Aspergillus sp.	+	+
Fusarium sp.	+	+
Curvalaria sp.	-	-

Key: + = Mycotoxin detected, - = Mycotoxin not detected

Table 3: Effect of different concentrations of mycotoxin extract on severity of symptoms

Mycotoxin concentration (µg/100µl)					
Mycotoxin extracts	10	20	30	40	50
Aspergillus sp.	+	+	++	++	+++
Rhizopus sp.	-	-	+	+	++
Fusarium sp.	-	-	+	++	++
Control	-	-	-	-	-

Keys: - = No symptom observed, + = Paralysis only, ++ = Paralysis and convulsion, +++ = Paralysis,
convulsion and respiratory arrest

Quality of inoculated tomato fruit samples under passive modified atmosphere storage

Tomato samples inoculated and packed in polyethylene bags of different thicknesses showed lesions/rots of varying degrees (as revealed by sensory parameters; Table4). All samples inoculated with the fungi and packed in polyethylene bags of 12µm thickness showed the lowest scores (i.e., the best overall quality) while, control (unpacked) samples showed conspicuous lesions, discolouration and 'patchy' texture.

Table 4: Influence of Passive Modified Atmosphere Storage on Quality of Inoculated Tomato Samples

Inoculum	Thickness (µm) of packaging bag	Visual appearance	Firmness	Overall acceptability
Aspergillus niger	Control (unpacked)	4.1±0.01	4.4±0.04	4.5±0.05
	6	2.3±0.06	2.5±0.01	2.4±0.10
	9	1.0±0.11	1.1±0.08	2.3±0.16
	12	0.0±0.03	0.2±0.06	0.2±0.01
Fusarium oxysporum	Control (unpacked)	4.0±0.11	4.6±0.18	4.2±0.16
	6	2.1±0.13	2.3±0.10	2.2±0.12
	9	1.1±0.10	1.2±0.09	2.1±0.11
	12	0.1±0.06	0.1±0.14	0.1±0.03
Rhizopus stolonifer	Control (unpacked)	4.1±0.18	4.3±0.06	4.4±0.04
	6	2.2±0.01	2.3±0.02	2.3±0.01
	9	1.0±0.08	1.1±0.05	2.2±0.02
	12	0.1±0.03	0.1±0.01	0.2±0.01

Note: The lower values for quality indices of visual appearance and firmness indicate better quality. Values are mean ± standard deviation of two independent experiments.

The result of this study showed that *Aspergillus niger, Fusarium oxysporum, Rhizopus stolonifer* and *Curvularia* species were found associated with tomato rots/lesions (Table 1). The high rainfall pattern, high humidity and the temperature of between 19 and 31°C prevailing in the agroecology of southern Nigeria, favours the development of fungal diseases in field, market and in storage. The isolation of these pathogens confirms the studies of Data *et al.,* (1987) and Onifade *et al.,* (2004). Pathogenicity test revealed that four of the isolated fungi were highly pathogenic. *Aspergillus niger* and *Rhizopus stolonifer* induced the most extensive rots, *Fusarium oxysporum* was moderately pathogenic while *Curvularia* sp. was the least pathogenic. This suggests that, *Curvularia* sp. is not likely to be a pathogen of tomato but rather a contaminant. Tomato fruits have very low respiratory rates, consequently, the benefits of the 12μm thick polyethylene bags may be attributed to low water vapour build-up with moderate oxygen and carbon dioxide permeability, since gas barrier properties increase with increase in density of packaging material (Hui, 1992), thereby creating a suitable equilibrium modified atmosphere within the packs. The conspicuous and exacerbated lesions/discoloration observed in control (unpacked) samples could be attributed to the effects of enhanced pathogenicity of the isolates. The possible effect of high carbon dioxide and low oxygen levels in the packs is suppression of fungal growth, and this may have in part been responsible for the variations in the quality of the samples (Table 4). Similar findings have been reported (Hiu, 1992).The post-harvest rot of tomato has been demonstrated to be influenced by a number of factors, therefore; modified atmosphere storage will go a long way to minimize storage rots, thereby enhancing storage life.

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