HISTOPATHOLOGY OF *LASIODIPLODIA* ROT OF SWEET ORANGE FRUIT

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ABSTRACT

This study was conducted to investigate histopathologically, the peel of fruit of Citus sinensis infected with Lasiodiplodia spp. IMI 503248 by light microscopy. The suspension used to inoculate the orange fruits contained 6.05 x 10^6 spores $m1^{-1}$. The fruits were injected with the spore suspension 1mm deep at the equator. The suspension was agitated before and during inoculation in order to maintain uniform spore distribution. Histopathological analysis was carried out on the infected peel tissue after 5 day of inoculation. Conidium was not observed in the photomicrograph peel section of healthy non-infected fruit. However, photomicrograph peel section of the infected fruits tissue revealed the presence of conidium in the peel section and showed hyphal structures that were observed inside the tissue of the diseased orange. Infected tissue showed an extensive distribution of the fungus. The fungal hyphae were found crossing the cell walls, growing both transversely and longitudinally, occupying extensive areas of the interior of cells. The activities of the hyphae of the fungus might have resulted into disruption of the cell wall boundaries and rottenness of the orange fruit.

Keywords: *Lasiodiplodia spp.*, Citrus sinensis, sections, microscopy, rot

INTRODUCTION

Fruits are of great nutritional value and Citrus is one of the World's most important fruit crops. Sweet orange constitutes approximately 65% of the world's citrus production followed by mandarins at 19%, lemons and limes at 11% and grape fruit at 5% (Ismail and Zhang, 2004). Worldwide per capital consumption is estimated at 12.2kg/year for oranges, 1.8kg for lemons and limes and 0.7kg for grapefruit (Anonymous, 2003). Among the citrus cultivars grown in Nigeria, sweet orange (*Citrus sinensis* (L.) Osbeck) is by far the most widely cultivated in Nigeria and the bulk of the oranges produced is always distributed to prospective consumers through open sales at market stalls and road stands where good quality and marketability of the fruits is often lost due to excessive post harvest spoilage of the fruits which consequently leads to loss of market appeal (Babarinsa *et al.*, 2002). Rind breakdown has also been observed during post harvest storage and this causes considerable reduction in external fruit quality and is responsible for large reduction in the amount of exportable fresh fruit (Agusti *et al.*, 2001).

However, one of the greatest problems encountered during post harvest storage of sweet oranges is infection by microorganisms. *Lasiodiplodia* sp IMI 503248 is a common post harvest fungus disease of citrus known as stem-end rot. The fungus usually infects at the stem- end of the citrus fruit, leading to development of soft brown to black decay symptoms at both ends of the fruit. Therefore, this research investigated the histopathology of sweet orange fruit infected with *Lasiodiplodia* sp IMI 503248, an area where information is lacking, to better understand the colonization process of this pathogen.

MATERIALS AND METHODS

Source of fruits and inoculation

Mature, green healthy orange fruits were harvested from a commercial orchard in Igbatoro, Akure North (7° 5¹ 3N; 5° 2¹ 46E) Nigeria. Fruits of uniform size and colour were selected.

Before treatment, the fruits were washed with fresh water, disinfected superficially for 10 minutes in 10% sodium hypochlorite, rinsed with water and allowed to air-dry at 26°C.

The *Lasiodiplodia* sp. IMI 503248 isolate used was obtained from decayed oranges. The organism was identified based on the structural features as seen on culture plates as well as microscopic characteristics after staining with lactophenol in cotton blue using the method of Markson *et al.* (2005). The fungus was then identified based on colonial morphology, mycelia structure and spore morphology according to the keys of Mohali *et al.* (2005) and Alexopoulous *et al.* (1996). The fungus was later confirmed to be *Lasiodiplodia* sp. IMI 503248 by DNA sequence analysis using the FASTA algorithm with the fungus data base from European Molecular Biology Laboratory (EMBL). A Ten day old agar slant

culture of *Lasiodiplodia* sp. IMI 503248 used for inoculum was obtained by inoculating the isolate on already prepared malt extract agar slants. Sterile water was poured into the slant bottle and shaken vigorously to dislodge the spores from the vegetative hyphae. The wash water was collected in a sterilized beaker. One milliliter of the suspension was spread on an area of $1cm^2$ and allowed to dry on a clean microscope slide before counting using the high dry (x40) objective. The spore concentration of the suspension was determined by using the formula of Breed Direct Counting Technique (Ogundana, 1989):

Number of spore $m1^{-1}$ = Average number of spores x MCF (about 50 fields)
MCF = Microscope correction factor = Area of smear x dilution factor Area of Microscope field
Area of microscope field = $\pi r^{2,}$

Histopathology of orange fruit infected with Lasiodiplodia sp. IMI 503248

The suspension used to inoculate the orange fruits contained 6.05 x 10^6 spores $m1^{-1}$. The fruits were injected with the spore suspension 1mm deep at the equator. The suspension was agitated before and during inoculation in order to maintain uniform spore distribution. Histopathological analysis was carried out on the infected peel tissue. Peel was carefully cut from the equatorial zone of inoculated fruit with a new razor blade and dehydrated in different percentages (50, 70, 80, 90 and 100%) of alcohol for 1½ hours each (Lamb, 1981). After dehydration, they were cleared with 100% xylene and were left for 2 hours to remove any remnant alcohol and were later impregnated in molten paraffin wax overnight for embedding. The embedded tissues were sectioned to form ribbons (sections) using HM 325- microtome. The ribbons were made to float on the surface of water bath (XH 325 model) of warm water (45^0C) and gently mounted on a slide and dried in an oven (GallenKamp model) at 40^0C for 2hours and the section was now ready for staining.

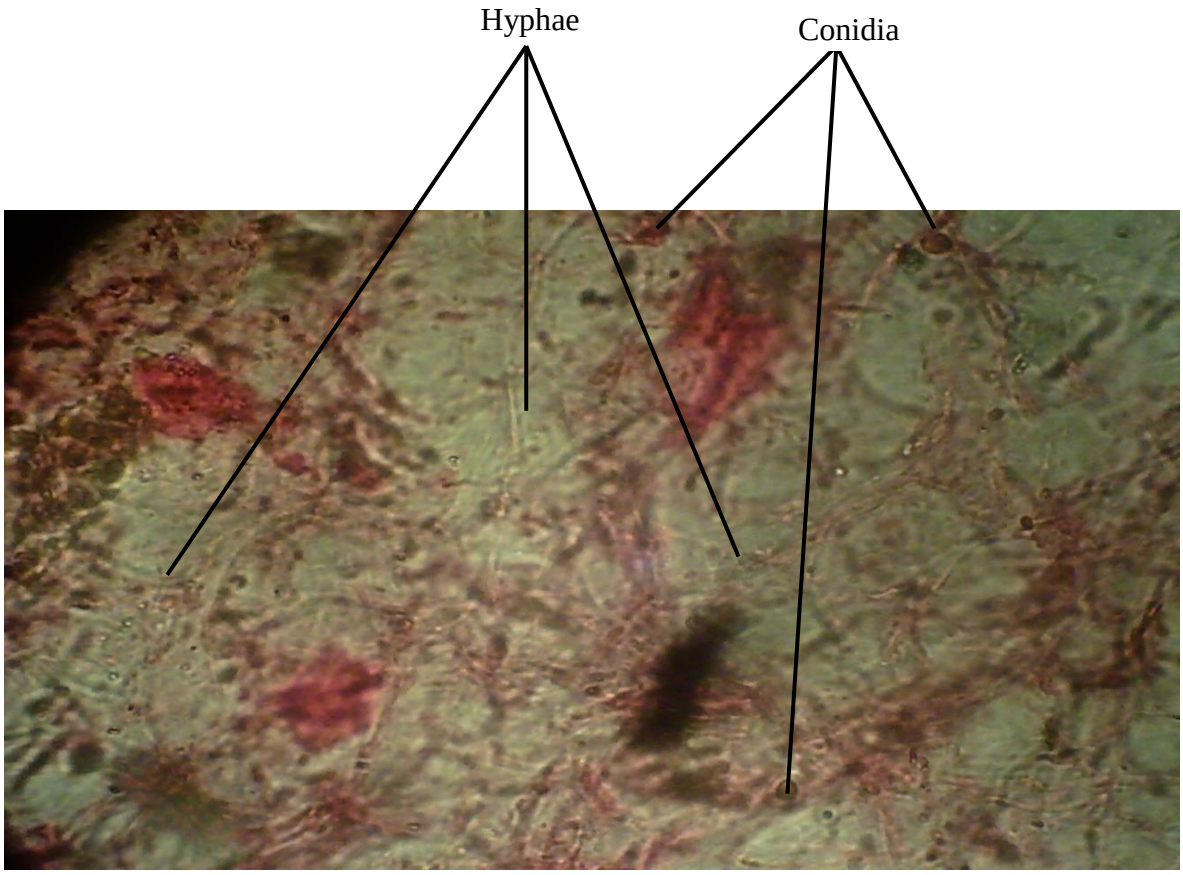
Before staining, section was cleared 100% xylene and treated with different percentages of alcohol (50, 70, 80, 90 and 100%) to remove the wax, and finally washed in running tap water (hydration) to remove the alcohol. The hydrated section was stained first with haematoxylin for 4 minutes. Excess stain was removed by washing in running tap water and the stained section differentiated in 1% acid alcohol and washed again in running tap water before counter staining in eosin for 2 minutes. The counterstained section was washed in running tap water and dehydrated in different percentages of alcohol (50, 70, 80, 90 and 100%) and then cleared in 100% xylene. After clearing in xylene, Canada balsam was added and cover slip placed on the slide. The preparation was left in the oven at 40^0C and then observed under photomicrograph microscope (Olympus CX40 model) with a digital camera connected to a computer system and photographs were taken. The procedure was also repeated on healthy orange fruits which served as control (Lamb, 1981).

RESULTS

Histopathology

Photomicrograph of the peel of the infected orange fruit showed that hyphal structures were observed inside the tissue of the diseased orange. Infected tissue showed an extensive distribution of the fungus. The fungal hyphae were found crossing the cell walls, growing both transversely and longitudinally, occupying extensive areas of the interior of cells. The cells were seen with large masses of homogenous groups of hyphae showing both regular and irregular septate arrangement (Figure 1). There was cell wall disintegration and full hyphae dispersal. The activities of the hyphae and conidia of the fungus resulted into disruption of cell wall boundaries and eventual cell death. These observations showed that the fungus caused necrosis on the fruit destroying its cell boundaries and causing cell death.

Also, sections of healthy orange fruits showed that the epidermal cells of the flavedo (rind) appeared intact with no sign of collapse (Figure 2) while the organization of the epidermis is lost in diseased tissue due to the breakage in its cell walls. The plastids and other cellular contents were lost and the neighbouring cells got separated from each other (Figure 3).



X1000

Figure 1: Photomicrograph peel section of Orange Fruit showing the presence of conidia and hyphae of *Lasiodiplodia* sp in infected tissue.

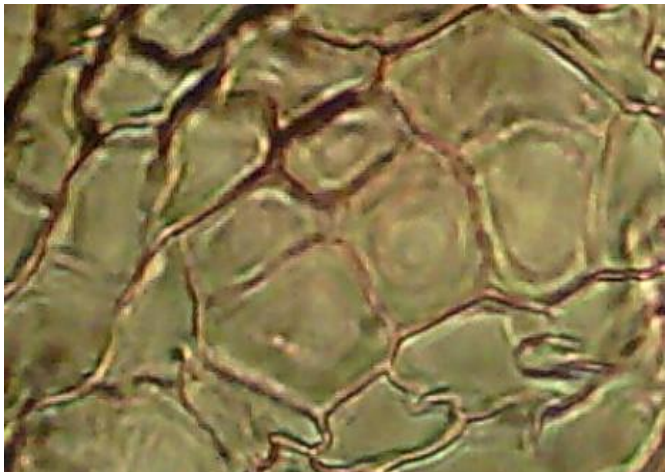


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Figure 2: Photomicrograph of peel section of healthy orange fruits showing intact cell structure, with no conidium and hyphal structures

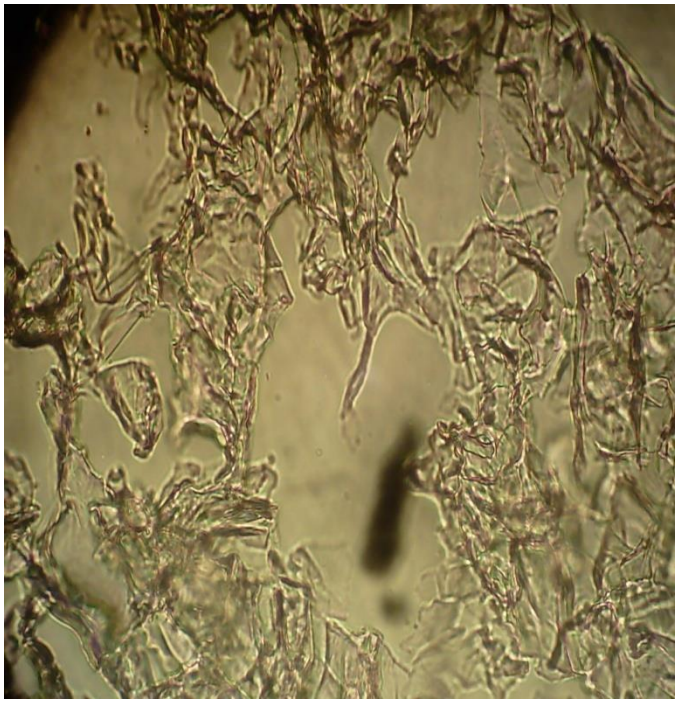

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Figure 3: Photomicrograph of peel section of *Lasiodiplodia* sp. infected orange fruit showing disrupted cells of the tissue

DISCUSSION

In this study, it was observed that the test pathogen entered the orange fruit tissue through the wound created at the time of inoculation. This observation was buttressed by the report of Mehrotra and Ashok (2003) that many pathogens are not able to penetrate the host surface in the absence of a wound. All bacteria, most fungi, some viruses and all viroids can enter plants through various types of wounds. In fact, injuries sustained by citrus fruit during harvest or handling, according to Dorria *et al*.(2007) allow the entry of wound pathogens , including green and blue moulds. Having penetrated the wound, the spore of *Lasiodiplodia* sp. IMI 503248 was seen in the fruit tissue as shown by the histopathological photomicrograph of the infected orange fruit. Fungal invasion of host plant is chiefly by spore germination and growth of the resulting germ tube on the surface of the host plant. The germ tube from the spore grows into appresorium for direct penetration (Mehrotra and Ashok, 2003) and according to Emmett and Parbery (1975), the initiation, formation and action of appresoria are integral parts of the infection process of many parasitic fungi.

In citrus, the rind consists of two tissues, the exocarp or outer rind (flavedo) and the mesocarp or albedo (Agusti *et al.*, 2001). A structural comparison of cross-sections of the rind from infected and healthy fruits shows morphological characteristics of sweet orange fruits. Infected fruits had distorted the

structure of the fruit tissue while healthy fruits showed intact cell wall structure. The cytoplasm of these infected cells was disorganized and appeared as a mass of collapsed material. Affected cells seemed devoid of contents and had twisted and squashed walls (Storey and Treeby, 1994). This was also supported by Arpaia *et al.* (1991) who reported that collapsed cells formed a mass of tissue with very large irregular spaces, thus showing irreversible damage.

These observed differences in the cell wall structures of the infected fruits marked the predisposition of the fruit to decay. No doubt, fruits with distorted cell wall structures deteriorated rapidly during storage while fruits whose cell wall structures remained intact had extended shelf life during storage.

REFERENCES

Agusti, M., Almela, V., Juan, M., Alferez, F., Tadeo, F. and Zacarias, L. (2001)**.** Histological and phsyicological characterization of rind breakdown of 'Navelate'sweet orange. *Annals of Botany* 88:415-422.

Alexopoulous, C.J., Mims,C.W. and Blackwell , M. (1996). Introductory Mycology, John Wiley and sons Inc., New York. 869pp.

Arpaia, M. I., Kahn, T. I., Elotmani, M., Coggings, C. W., Demason, D. A., O' Connell, N.V. and Pehrson, J. E. (1991). Pre-harvest rind stain of 'Valencia' Orange: Histochemcial and developmental characterization. *Scientia Horticulturae* 46:261-274

Babarinsa, F. A., Williams, J. O. and Omomukuyo, A. (2002). Effect of skin-coating materials on the storage of Valencia oranges. *Post-harvest Science* 1:7-11.

Dorria, M. A., Omalma, M. H. and Mohei, E. S. (2007). Integrated control of postharvest decay on blood orange fruits by curing, hot water and sodium carbonate applications. *Research journal of Agriculture and Biological Sciences.* 3(6):590-598.

Emmett, R. W. and Parbery, D. G. (1975). "Appresoria", Ann. Rev. Microbiol; 9:297-318 Ismail, M. A. and J. Zhanj, (2004). Post-Harvest citrus diseases and their control in: *Outlooks on Pest Management News Letters*, Florida Department of Citrus, Citrus Research and Education Centre, USA. 2:29-35.

Lamb, G. M. (1981)**.** Manual of Veterinary Laboratory Techniques. CIBA-GEGY Publisher, Kenya, 134 – 149pp.

Markson, A.A; Madunagu, B.E., Umana, E.J. and Udo, S.E. (2005). Survey and bio control of fruits rotting fungi of tomato. *Nigeria Journal of Plant Protection*. 22:122-131.

Mehrotra, R. S. and Ashok, A. (2003). Plant Pathology. Second Edition. Tata MC

Graw- Hill Publishing Company Limited, New Delhi, 64 – 80pp.

Mohali, S., Burgess, T. I and Wingfield, M.J. (2005). Diversity and host association of the tropical tree endophyte *Lasiodiplodia theobramae* revealed using simple sequence repeat markers. Forest Pathology 35:385-396.

Ogundana, S.K. (1989). Introductory Microbiology. 1st Edition. Obafemi Awolowo University Press Ltd., Ile-Ife, Nigeria. 215pp. Storey, R. and M. T. Treeby, (1994). The morphology of epicuticular wax and albedo cells of orange fruit in relation to albedo breakdown. *Journal of Horticultural Science* 69:329-338

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