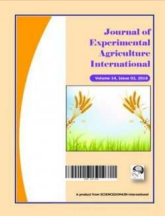

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Leaf Tip Die- Back of *Yucca elephantipes* by *Lasidiplodia theobromae* Pat. and Production of Phytotoxin in Filtrate and Infected Leaves

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Authors' contributions

This work was carried out in collaboration between all authors. Author BI designed the study and supervised the execution of the study. Author OFA conducted the experiments, collected the data, performed the statistical analysis and wrote the first draft of the manuscript. Author AOCC wrote the protocol, supervised the study, managed the literature, developed the manuscript and corresponded with the editorial board. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Leaf tip die-back is a foliar disease of *Yucca elephantipes* that significantly reduces its aesthetic value. Investigations were carried out on the pathogenicity of the causal organism.
Methodology: A Bioassay of the isolated phytotoxin produced by the pathogen was carried out on yucca, jatropha, cowpea and maize. The isolated fungus was grown on Czapek- Dox liquid medium and mycelia were harvested by filtration at 7-day intervals for 49 days. The filtrate was applied on leaves of test plants growing in pots arranged in Complete Randomized Design with four replicates.
Results: *Lasiodiplodia theobromae* was identified as responsible for the leaf tip die- back disease of yucca. Results of the pathogenicity test showed significant (p=.05) increase in the rate of

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development of necrosis and chlorosis with time. Significant increase in the necrotic and chlorotic lesions (p=.05) were observed among the test plants inoculated with 28, 35, 42, and 49 day old culture filtrate of *Lasidioplodia theobromae*. Extracts from infected leaves induced necrotic spots surrounded by a chlorotic halo ranging from 0.2- 1.2 cm. Evidence from this study demonstrated that *L. theobromae* is the causal organism of yucca leaf tip die back. The toxic principle can be isolated from 28 day-old cultures while the extract from infected yucca leaves produced all the symptoms as did the pathogen.

Keywords: Botryodiplodia; cowpea; filtrates; lasidioplodia; phytotoxin.

1. INTRODUCTION

The aesthetic value of plants in homes, avenues, lawns and hedges contributes to human pleasure and comfort. Their value cannot therefore be measured in quantitative terms [1]. The value of ornamentals is measured in terms of their quality such as colour, aroma or plant architecture. Their uses and benefits are wide and varied; In some countries it is a multi-million dollar business employing thousands of people and some sporting activities depend heavily on well manicured pitches and greens. Job specialties such as growers, maintenance personnel, seed specialist, potting media specialists, florists, sales persons etc are created in a thriving horticultural business environment [2]. In most developing countries however attention is not paid much to ornamentals as growers would rather produce food. Recently however, more attention is being paid to the aesthetic environment in developing countries such as Nigeria especially in large cities and state capitals [3]. Aesthetics and beautification of the immediate environment of private and public premises is developing into a good business venture in Nigeria. Many roadside gardens have sprung up in major cities of Nigeria where the lovers of ornamental plants and flowers can purchase these plants for their homes or offices. Many of these ornamentals are exotic plants, and are often prone developing diseases. Many diseases of ornamental plants go undiagnosed and unattended to because many people believe that ornamentals plants are resistant to diseases or that they are not important enough to receive 'treatment' [4]. The perception is that the necrotic spots and patches with or without chlorotic patches surrounding them are signs of age, over watering or soil problems hence, nothing is done about them until whole plant dies off.

Recent investigations carried out in parks, gardens and private homes showed that more than 60% of ornamental plants and flowers had at least one disease [4]. One of such ornamental plant is the Yucca plant, *Yucca elephantipes* Regel. Also known as the pineless yucca, *Yucca elephantipes*, originated from the arid regions of South America. Spineless yucca is a perennial plant which can be propagated from seeds or cuttings [5]. The plant is often used as a framing specimen at the side of a building or along walkways. It makes a striking presence in large landscapes, and are grown in containers and placed indoors. Many yucca plants including *Y. elephantipes* are used in phamaceuticals. Some saponins have been extracted from *Y. elephantipes* which showed moderate inhibitory activity against the growth of *Candida albicans* [6]. The plant however is frequently affected by a disease that causes the leaf tips to die back after which the leaf may eventually fall off. The disease symptoms include necrotic lesions of the tip of the leaves with non-invaded chlorotic zones which diffuse outward from the necrotic tip. The symptom advances until the whole leaf becomes necrotic, dries up and falls off the plant. The aim of this study was to identify the causal organism of the leaf tip die- back of *Y. elephantipes* in Nigeria and to investigate the mode of infection of the pathogen.

2. MATERIALS AND METHODS

2.1 Sources of Infected Plant and Planting Materials

Infected buds of Yucca plants were obtained from 24 horticultural Gardens in Oyo State. Test plants used for this study were cowpea (*Vigna unguiculata*), maize (*Zea mays*), jatropha (*Jatropha curcas*) and yucca (*Yucca elephantipes*). Seeds of cowpea, maize and jatropha and healthy buds of yucca were planted and maintained in sterilized soil in 5 kg pots on the Roof-Top Garden of the Department of Crop protection and Environmental Biology, University of Ibadan.

2.2 Isolation and Culturing of Causal Organism(s)

Infected leaves of yucca showing symptoms of the leaf tip die- back were collected from homes

and 24 commercial gardens in Ibadan, Nigeria. Infected leaves were removed from the plants and collected in paper envelopes and taken to the Plant Pathology Laboratory in the Department of Crop protection and Environmental Biology, University of Ibadan. In the Laboratory, the leaves were cut just below the point of infection and abaxial part of the infected leaves were scraped on the microscope slide containing a drop of lacto-phenol in cotton blue. The scrapings from each sample were covered with a microscope slide and observed under 10X magnification of a light microscope. The magnification was later increased to 40X for better viewing.

The infected leaf tips were cut into 2 mm pieces using a scalpel. The small pieces were surfaced sterilized by dipping in 10% solution of sodium hypochlorite for 30 sec and rinsed thrice with sterile distilled water to remove all traces of the disinfectant [7]. The leaf pieces were blotted dry on a sterile Whatman No. 1 filter paper. Each piece was placed on solidified Potato Dextrose Agar (PDA) in 9 cm- diameter Petri dishes and incubated at 28- 30℃ for 7 days. The Petri dishes were observed for the growth of developing organism(s) during 7 days of incubation. The growing fungi were sub-cultured and incubated at 27±2℃ until pure cultures were obtained. The pure cultures were incubated and observed for fourteen days for the sporulation of the pathogen.

2.3 Identification of Causal Organism

An inoculating needle was used to pick a little of the growing fungi from each culture and placed on separate clean microscope slides. A drop of lacto-phenol in cotton blue was added to the fungus on the slide, and the mounted needle was used to gently tease the fungus, and was covered with a glass cover slip. The slides were observed under 10X and 40X magnifications and identified by matching morphological characteristics to descriptions in identification manuals [8,9].

2.4 Pathogenicity Test of *Lasidiplodia theobromae* on Healthy Yucca

Healthy yucca buds were planted in sterilised soil contained in 5 kg pots. The plants were inoculated four weeks after planting with 7-day old culture of the fungus. Inoculation was done by applying *L. theobromae* agar plugs to leaves or with sterile PDA (control). Inoculated leaves were incubated by covering with transparent polyethylene bags for 24 hrs. Plants were observed for 7days for symptom expression.

2.5 Reaction of Yucca Plants to Inoculation with Filtrate from Infected Leaves

Infected leaf tissue of yucca plants made up mainly of chlorotic portions were weighed, cut into smaller pieces and washed in running tap water. The pieces were then surface sterilized by shaking for 30 sec in 10% solution of sodium hypochlorite, after which they were rinsed thrice with sterile distilled water and allowed to dry on sterile filter paper (Whatmann No. 1) [7].

The surfaced sterilized infected tissue was transferred into a Warring® blender containing 700 ml of cold (4℃) sterile distilled water and macerated at high speed, five times for 10 seconds each time. The blended tissue was filtered through 8 layers of muslin cloth and re-filtered using No 1 Whatmann filter paper. The filtrate was then poured into a vacuum evaporator and placed on ice. The contents were evaporated to 20 ml which was poured into a 50 ml Erlenmeyer flask and stored in a refrigerator at 4℃. The same procedure was conducted for asymptomatic leaves to serve as control. The concentrated filtrates of both infected and uninfected yucca leaves were inoculated separately on healthy yucca plants and observed at the 12-hr intervals for 48 hours.

2.6 Investigation of Causal Fungus for Toxin Production

Test plants used for this study, cowpea (*Vigna unguiculata*), maize (*Zea mays*), jatropha (*Jatropha curcas*) and yucca (*Yucca elephantipes*) were planted in 5 kg pots filled with sterilized soil. Plants were inoculated at relatively uniform stage of 6-8 leaves per plant. Buds of yucca were planted in wood shaving for 14 days to attained 6- 8 leaf-stage before transplanting into 5 kg pots. Planting was done specifically for each filtrate inoculation at 7 day intervals for 7 weeks. Pots were arranged in a Completely Randomized Design with four replicates. A total of 140 test plants were evaluated in the experiment.

A sterile cork- borer of 5 mm- diameter was used to cut discs from the actively- growing, seven

day- old pure cultures of causal organism growing on PDA. One disc each was aseptically inoculated into seven separate 250-ml Erlenmenyer flasks containing sterile peptone-broth. The peptone- broth medium made up of 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 10.0 g peptone, 1.0 g yeast extract, and 10.0 g dextrose, was prepared according to Ikotun's method [10]. The Erlenmeyer flasks were incubated at 28- 30℃ for 7, 14, 21, 28, 35, 42 and 49 days. At the corresponding culture age, the appropriate flask was centrifuged at 4000 rpm for 20 minutes. The supernatant was poured into sterile test tubes through sterile 8- layer cheese cloth to obtain filtrates of 7, 14, 21, 28, 35, 42 and 49 day-old cultures. The filtrates were then used to inoculate test plants. Control plants were inoculated with sterile broth medium.

A micro pipette was used to deliver the filtrate in 0. 1 ml of the filtrate discretely on to the leaf surfaces of the yucca, maize, cowpea and jatropha plants in their vegetative stage. The tip of the pipette was used to lightly scratch the leaf surface through the filtrate. The inoculated plants were observed at 12 hrs intervals for 48 hours for plant reaction to the filtrate.

2.7 Extraction of the Suspected Phytotoxin Produced in the Infected Yucca Leaf

According to the procedure of Amusa et al. [11] two hundred grams of infected Yucca leaves were cut into 1-2 cm pieces and blended in a warring blender in 1L methanol as the solvent. The suspension was agitated with a mechanical shaker for 8 hrs. The suspension was filtered using an 8-ply muslin cloth and the methanol was removed by fractional distillation. Sterile distilled water (10 ml) was added to the residue and shaken to homogenous suspension. The suspension was tested for biological activity on leaves. The homogenous suspension (0.1 ml) was dropped on the test plants with a syringe while bruising the leaves gently at their vegetative stages. Control leaves were inoculated with sterile distilled water.

2.8 Statistical Analysis

Data was analysed using Analysis of variance (ANOVA) using SAS [12]. Means were separated using Least Significant Difference (LSD) at 5% level of significance.

3. RESULTS AND DISCUSSION

Infected leaves of *Y. elephantipes* that were scraped unto slides directly showed pycnida containing spores of *Lasidiplodia theobromae* Pat. associated with the dried necrotic portion of the leaf tip while the surrounding chlorotic portion of the leaves did not contain propagules of the fungus. Typical symptoms of most plant diseases revealed the involvement of phytotoxic metabolites, which therefore suggest a role for toxic metabolite secreted by the pathogen in the disease development. Plant reaction to both filtrates was similar to disease symptoms on infected plant metabolites of many fungi may have adverse or stimulatory effects on plants [13].

Fungal mycelia growing from PDA plates were white for the first 48 h after plating but later turned grey and then black 7 days after plating (Plate 1a). The fungus was fast growing and filled the 90 mm-diameter Petri plates by 5 days after inoculation. Seven days after plating, conidia (Plate 1b) which were identified as those of *L. theobromae* after due comparison of the mycelia and conidia.

Lasidiplodia theobromae has been shown to be present on pods of cocoa infected by *Phytophthora megakarya* and *P. palmivora*, as a secondary pathogen [13]. It has also been implicated in yam storage rot causing a dry, black pulverulent rot [14]. This same fungus was reported as causing die-back and bark canker of pear [15,16]. It is therefore interesting to find that *L. theobromae* is the primary invader of the leaves of yucca plants and the cause of leaf-tip die back. This is a first report of the occurrence of the pathogen on yucca.

Amusa et al [11] reported that *L. theobromae* was the causal agent of stem canker disease of Eucalyptus in India. Several other reports of *L. theobromae* causing disease of fruit trees include those on mango [17-20] apple [21], and peach [22]. It is now certain that *L. theobromae* is a confirmed pathogen of many fruit trees all over the world causing die-back disease [18].

Application of the filtrate extracted from infected yucca leaves, to healthy leaves also produced necrotic spots at the point of inoculation surrounded by a chlorotic halo. The symptom induced was similar to that induced by 35 day-old culture filtrates of *L . theobromae*. Plant reaction

to both filtrates was similar to disease symptoms on infected plants (Plates 2a and 2b). Cowpea, jatropha and yucca leaves gave the largest sizes of necrotic and chlorotic spots (Table 1).

Plants did not react to the culture filtrates from 7, 21, 24 day old cultures of *L. theobromae*. The result obtained for those days were similar to those obtained for the control. However, leaves of jatropha, maize and yucca plants reacted to exposure to the culture filtrate of 28 day-old cultures (Table 2). The point of inoculation of the culture filtrate became necrotic 24 hrs after inoculation turned black and was surrounded by a chlorotic halo (Plate 3a), similar to symptoms of the disease on the field the control plants merely showed the pin prick injury of inoculation and did not develop necrosis or chlorosis (Plate 3b).

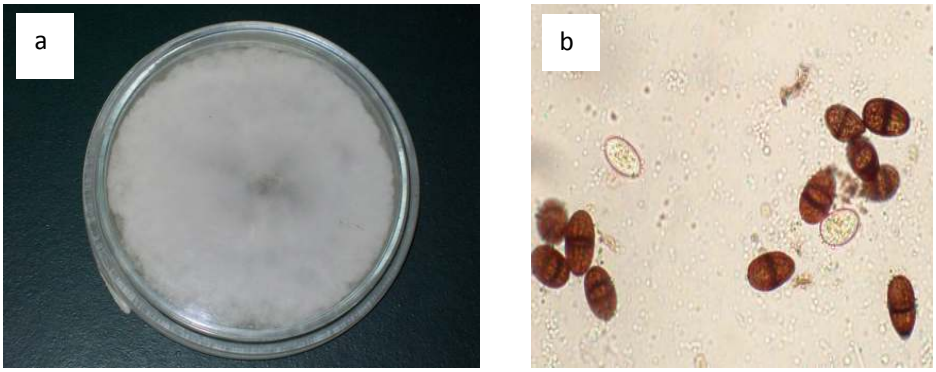

Plate 1. Radial growth of *L. theobromae* (a) mature and immature spores of *Lasidiplodia theobromae* 8days after inoculation (b)

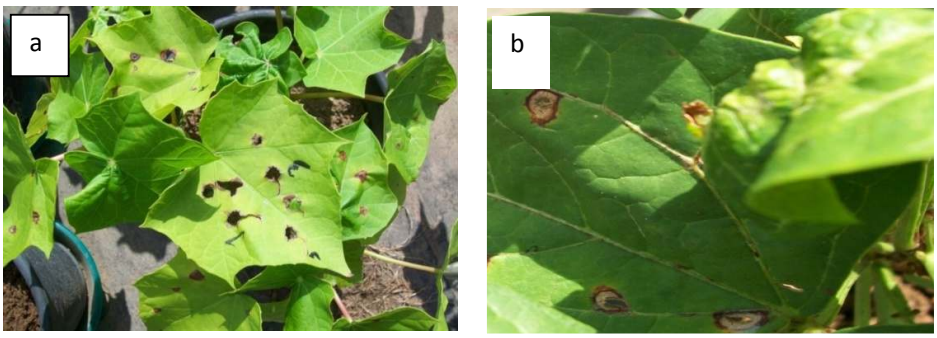

Plate 2. Necrosis Jatropha (a) and cowpea (b) leaves with filtrate from infected plants

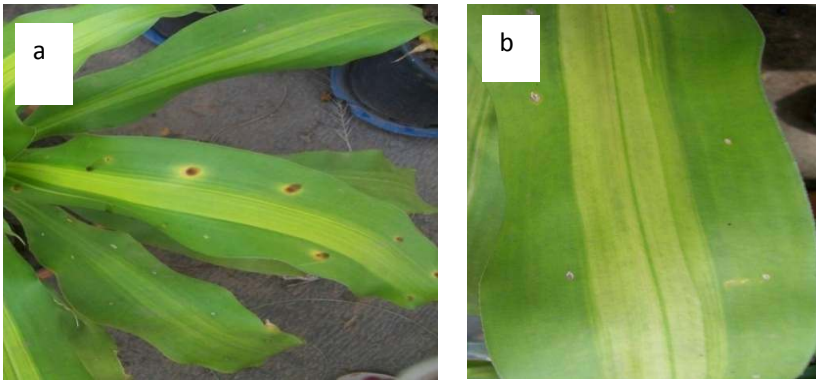

Plate 3. Necrosis induced on Yucca leaves surrounded by a chlorotic halo (a) with 28 day old culture filtrate and Control Yucca leaves (b)

Table 1. Leaf spot induced on test plants after inoculation with extract from incubated culture filtrate and infected leaves

Treatment	Infected leaves extract (mm)	Culture filtrate extract (mm)
Control	0	0
Cowpea	3.4	5.0
Jatropha	3.4	7.0
Maize	3.1	2.3
Yucca	4.3	6.7
LSD (*P* = .05)	1.5	1.2

Effect of the culture filtrate was most severe on Jatropha from 28, 35, 42 and 49 day old cultures followed by the effect on yucca. Cowpea leaves showed necrotic halo with inoculation of filtrates from 35, 42 and 49 day old cultures (Table 2, Plates 4a and 4b). The appearance of symptoms without active pathogen infection shows that an active principle in the filtrate reproduced the symptoms on yucca plants caused by *L. theobromae*. The fact that infected yucca leaves extract induced symptoms similar to those caused by the pathogen on yucca plants indicates that a phytotoxin was produced by the pathogen.

A phytotoxin is a substance produced by a pathogenic organism that is injurious to the host plant [23]. In this study, a phytotoxin was suspected to be involved in the appearance of the chlorotic portion of the leaves of infected plants. The chlorotic portion occurred between the necrotic portion and the healthy leaf. The pathogen was not isolated from the chlorotic portion but its pycnidia were found mostly in the necrotic portion of the leaf.

It seems, in this study that *L. theobromae* acted as a hemibiotroph; one that kills host cells in advance of pathogen [24]. No growth was observed when only the chlorotic part of the leaf tissue was plated, implying that the toxin rather than the pathogen caused the chlorosis, further confirming the possible hemibiotrophic nature of *L. theobromae* on infected yucca plants. This study has demonstrated that the toxic principle was not host-specific or host selective [24]. This was supported by the fact that both the cell-free culture filtrate and the comminuted infected plant tissue contained the toxic principle when they were applied to healthy yucca, cowpea, jatropha and maize leaves. The implication of this is that filtrate of culture of this strain of *L. theobromae* can be further developed for use as a nonselective contact herbicide in future.

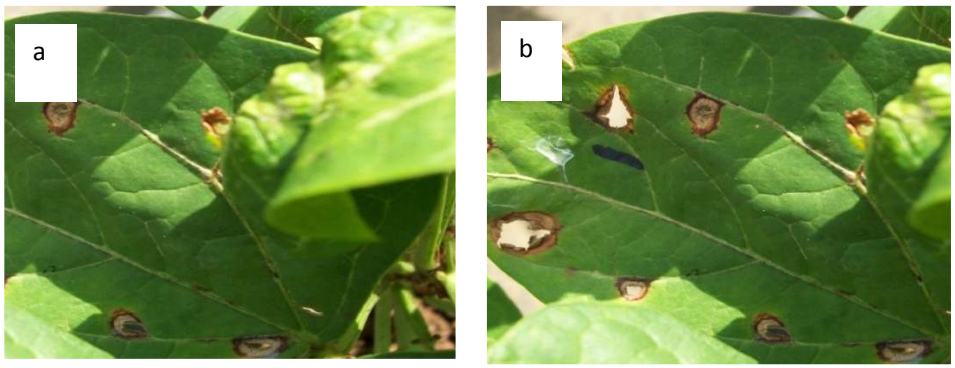

Plate 4. Necrosis induced on Cowpea leaves with 35 day old culture filtrate (a) and 49 day old culture filtrate (b)

Table 2. Size (cm diameter) of leaf spot induced by culture filtrate of *Lasidiplodia theobromae* on test plants

	Age of culture filtrate (Days)						
Test crops	**7**	**14**	**21**	**28**	**35**	**42**	**49**
Cowpea	0.0	0.0	0.0	0.0	0.3	1.0	1.3
Jatropha	0.0	0.0	0.0	0.3	0.4	0.6	1.1
Maize	0.0	0.0	0.0	0.2	0.2	0.3	0.5
Yucca	0.0	0.0	0.0	0.3	0.3	0.5	1.0
LSD (*P* = .05)	NS	NS	NS	0.1	0.1	0.1	0.2

4. CONCLUSION

This study demonstrated that *L. theobromae* is the causal organism of yucca leaf tip die back and is the first report of the pathogen on yucca. The toxic principle could be isolated from 28 day-old culture filtrate. The fact that the host and non-host plants reacted to the toxic principle indicates that the toxic principles is not a host- specific or host selective toxin.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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