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Isolation and Assay of the Toxic Metabolite(s) Produced by *Colletotrichum gloeosporioides* on *Jatropha curcas* (L.)

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Abstract: Anthracnose on *Jatropha curcas* leaves is caused by *Colletotrichum gloeosporioides*. On infected leaves, it is associated with a characteristic dark brown to black necrotic lesions that are irregularly shaped and appear on the lamina and center of the leaf and are surrounded by a yellow halo, which indicates a role played by toxic metabolites secreted by the pathogen. Six test plants which include jatropha, maize, cassava, melon, sorghum and cowpea were grown in five kg pots and arranged in a Completely Randomized Design (CRD). Pure culture of *C. gloeosporioides* was grown in a modified Richard's B medium. Mycelia were harvested by filtration at 7 day- intervals and discarded. Chlorotic and necrotic symptoms were observed on plants inoculated with 35, 42, 49, 56, 63 and 70 day-old culture filtrates, but not on control plants. The results from this study suggest the presence of toxic metabolite(s) in the culture filtrates of *C. gloeosporioides* as symptoms induced by the filtrates on leaves were similar to those produced by the pathogen under the natural infection conditions.

Key words: *Colletotrichum gloeosporioides* · Anthracnose · *Jatropha curcas* · Culture filtrates · Assay · Toxic metabolites

INTRODUCTION

Jatropha curcas L. is a deciduous, monoecious large shrub of 5-8m tall; it belongs to the family Eurphorbiaceae, genus Jatropha. It is a native of Central America and Mexico where it occurs naturally in the forests of coastal regions [1, 2] and was introduced to Asia and Africa by Portuguese traders [3, 4]. *J. curcas* is a drought resistant plant which is widely cultivated in the tropics as a living fence [2]. *Jatropha* is cultivated globally since the biofuel world market is steadily growing as more countries want to participate because of environmental and security aspects [5, 6]. *Jatropha* is very effective in preventing soil erosion both concerning wind and water. The parts used for medicinal purpose are root, stem, bark, leaves, seeds and oil. Its branches are used as chewing stick in Nigeria [7]. The latex has antimicrobial properties against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Streptococcus pyogenes* and *Candida albicans* [2, 8]. *Jatropha* has been thought to be resistant to pests and diseases as they had always appeared to be clean and healthy, recent observations have shown that the plant is susceptible to many pests and diseases. Some of the pests are*:* Leaf miners**,** Shield-backed bug, Flea beetle, *Pinnaspis strachani* (cushion scale) die-back of branches, Wooly aphid [2]. Some of the diseases are: Bacterial wilt, Bacterial leaf spot, Rusts, Yellow mosaic virus, Powdery mildew, Stem canker and die back*,* damping off, Root rot [2], *Colletotrichum* species are known to be hemibiotrophs, requiring living host cells initially but soon causing the death of the host cells in advance of the pathogen [9]. In many cases, death of the host cells is caused by the production of toxins or enzymes or a combination of both. It is conjectured that these toxic metabolites may be responsible for the causation of the chlorotic halo surrounding the leaf spots produced by *Colletotrichum* on *Jatropha*, hence the need to investigate the phenomenon. Hence, in this study an attempt was made to isolate and identify the pathogen associated with leaf spot on *Jatropha curcas*, determine

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the pathogenicity of the pathogen, obtain cell-free culture filtrate of the isolated pathogen and assay the phytotoxin produced by the isolated causal pathogen on *Jatropha* and other crop varieties.

MATERIALS AND METHODS

The study was carried out in the Plant Pathology Laboratory and Roof top garden, Department of Crop Protection and Environmental Biology, University of Ibadan. Oyo state, Nigeria. Six test plants which include jatropha, maize, cassava, melon, sorghum and cowpea were grown in five kg pots and arranged in a Completely Randomized Design (CRD).

Isolation and Identification of the Pathogen: Infected leaf tissues were cut into pieces of about 2-3cm from the advancing margin of the lesions, using sterile forceps, surface-sterilized in 10% of commercial sodium hypochlorite (JIK) for 30 seconds, rinsed in 5 changes of sterile distilled water. Pieces of the sterilized leaf tissues were placed on the gelled agar at 4 peripheral points on the plates which were incubated at 28°C for 3 days. The mycelial growth and acervuli development around leaves were observed, conidial masses picked from colonies which were suspected to be *Colletotrichum,* were sub-cultured on potato dextrose agar (PDA) for 7 days at 28°C for further purification and sporulation of the fungal isolate.

Pathogenicity Test: Pure cultures of *C. gloeosporioides* were obtained from single spore culture maintained on PDA. The conidial suspension was prepared by flooding the plates each with 10ml of sterile distilled water and the fungal suspension was filtered using 4-layered sterile gauze. Spore density was adjusted to $2.0x10^6$ spores/ml, using a haemocytometer. A drop of Tween 20 was added per 10ml of fungal suspension prior inoculation to assure adhesion of the spores on *Jatropha* leaves under surface [10]. Three healthy plants were sprayed each with 10ml of the spore suspensions, while two healthy plants were sprayed with 10ml of sterile distilled water served as control. Inoculation was done by spraying the conidial suspension on 28 days (4-5 leaves stage) old *Jatropha* plants.

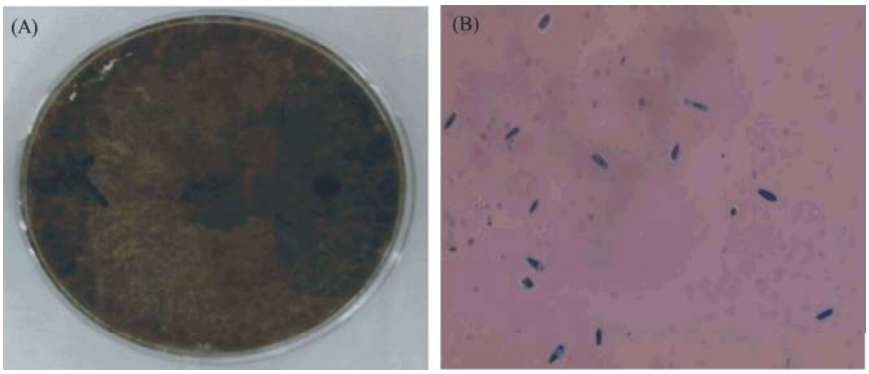

Plate 1: (A) Pure culture of *C. gloeosporioides* on PDA (B) Spores of *C. gloeosporioides* under the photomicrograph X40.

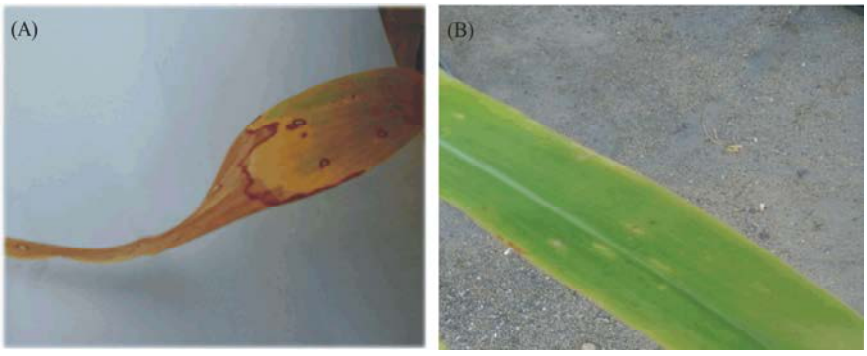

Plate 2: (A) Severe marginal necrosis induced on Sorghum leaf inoculated with 28 day-old culture filtrate. (B) Control Sorghum leaf inoculated with broth (not containing pathogen).

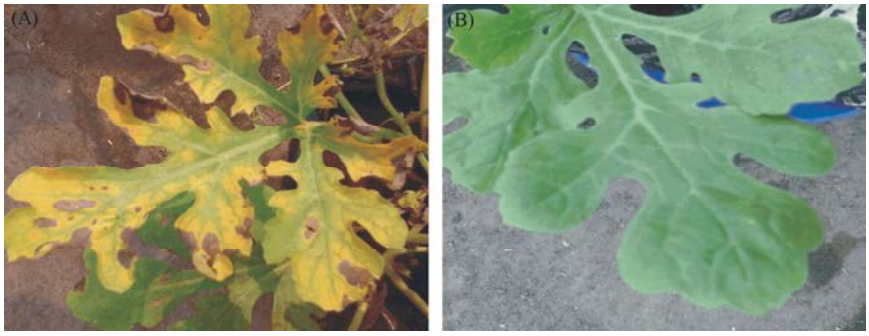

Plate 3: (A) Severe necrosis induced on Melon leaf inoculated with 35 day-old culture filtrate. (B) Control melon leaf inoculated with broth (not containing pathogen).

Plate 4: (A) Severe blight patches induced on Maize leaves inoculated with 42 day-old culture filtrates. (B) Control maize leaf inoculated with broth (not containing pathogen).

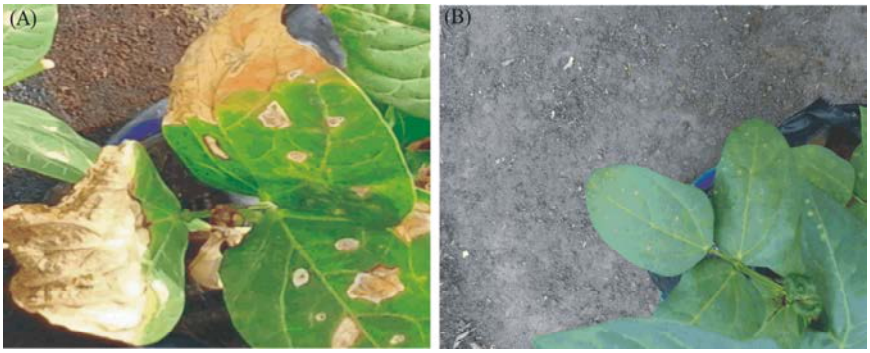

Plate 5: (A) Tip die back with necrosis induced on Cowpea leaves inoculated with 49 day-old culture filtrate. (B) Control Cowpea leaves inoculated with broth (not containing the pathogen).

Plate 6: (A) Severe marginal necrosis induced on Cassava leaves inoculated with 56 day-old culture filtrate. (B) Control Cassava leaves inoculated with broth (not containing the pathogen).

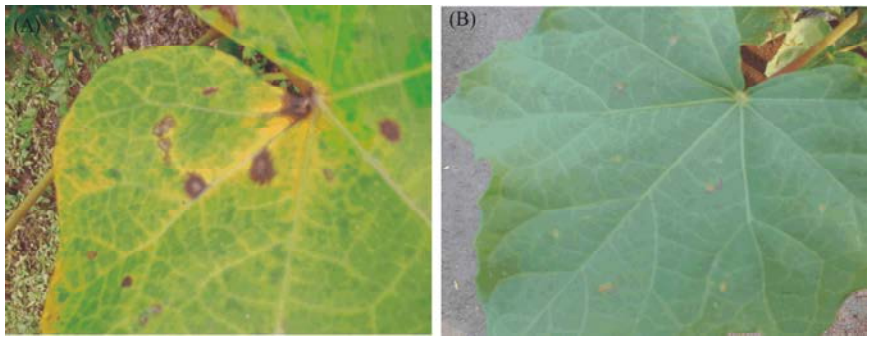

Plate 7: (A) Necrotic spots surrounded by halo induced on Jatropha leaf inoculated with 63 day-old culture filtrate. (B) Control Jatropha leaf inoculated with broth.

Table 1: Necrosis (cm) induced by culture filtrates (days) on test plants in a potted experiment

Treatment	7 DOCF	14 DOCF	21 DOCF	28 DOCF	35 DOCF	42 DOCF	49 DOCF	56 DOCF	63 DOCF	70 DOCF
Control	0.00	0.00	0.00b	0.00d	0.00d	0.00d	0.00e	0.00e	0.00d	0.000c
Cowpea	0.00	0.00	0.00b	0.12a	0.13ab	0.15a	0.18a	0.18a	0.20a	0.20a
Maize	0.00	0.00	0.00b	0.11ab	0.12b	0.14b	0.15b	0.17b	0.20a	0.20a
Cassava	0.00	0.00	0.00b	0.08bc	0.09c	0.12c	0.14c	0.16c	0.18bc	0.20a
Jatropha	0.00	0.00	0.00b	0.08bc	0.09c	0.12c	0.14c	0.17b	0.17c	0.19ab
Melon	0.00	0.00	0.00b	0.08bc	0.10bc	0.13bc	0.13d	0.15d	0.19b	0.20a
Sorghum	0.00	0.00	0.05a	0.07c	0.14a	0.15a	0.17ab	0.18a	0.20a	0.20a
	ns	ns								

Ns: not significant

DOCF: Day old culture filtrate

Means with same letter (s) in a column are not significantly different at 95% level of probability by Duncan Multiple Range Test (DMRT).

Production and Assay of the Cell Free Culture Filtrates of *C. Gloeosporioides* on Jatropha and Other Crops: The broth was prepared following slight modifications of Ikotun [11], the medium which was composed of (1g K_2HPO_4, 0.5g KH_2PO_4, 1g $(NH_4)_2NO_3$, 0.5g $MgSO_4.7H_2O$, 10g Peptone, 10g Dextrose, 1g Yeast extract, 1000ml Distilled water), was sterilized in an autoclave for 15 minutes at 121°C and allowed to cool. Seven days old pure culture of *C. gloeosporioides* was inoculated into the medium using a 3mm diameter agar plug containing the fungus and allowed to grow for 7 – 70 days at 28°C. Culture filtrates of *C. gloeosporioides* were obtained from the medium from 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 days of incubation. The culture was made cell-free by centrifuging it at 4,000 revolution per minute for 15 minutes, the supernatant was tested for biological activity by placing 0.1ml in a rubber wells on Jatropha, cassava, maize, melon, sorghum and cowpea leaflets through the epidermis at their vegetative stage. Symptoms development was observed and recorded after seven days, the control leaves were inoculated with the broth not containing the pathogen and sterile distilled water.

Analysis of Variance (ANOVA) was carried out on observations recorded using SAS, 2004 and Duncan Multiple Range Test (DMRT) at 5% level of probability was used to separate the means.

RESULTS

The symptoms of anthracnose disease on Jatropha leaves are dark brown to black necrotic lesions that are irregularly shaped and appear on the lamina and center of the leaves and are surrounded by a yellow halo, which is an indication of the role played by the toxic substances secreted by the pathogen.

Pathogenicity Test and Identification: Symptoms of the disease on leaves were observed 7 days after inoculation. Jatropha leaves inoculated with the conidial suspension showed an irregular necrosis 7-10 days after inoculation which later coalesced along with a yellow halo. The infected leaves fell off from the plant at 14 days after inoculation was done while the control plants remained healthy. On Potato Dextrose Agar, a snow-white mycelial growth was observed 3 days after incubation which became dark grey at 5-7 days after incubation. The fungus began to produce spores at 10-14 days after incubation with abundant masses of conidia. Its spores when viewed under the microscope were hyaline, one-celled, non-septate and ovoid to oblong, slightly curved or dumbbell shaped conidia with setae as described by Barnett and Hunter [12].

Assay of the Culture Filtrates for Biological Activity on Jatropha and Other Plants: Culture filtrates of 7 and 14 day old did not produce any symptom of infection on sorghum, the plant appeared healthy as the control plants. Slight reaction was observed to the culture filtrate of 21 day old, as necrosis was observed on inoculated leaves 3 days after inoculation that ranged from 0.1-0.4 cm in diameter. 28 day old culture filtrate caused necrotic spots which were surrounded by a yellow halo on sorghum leaves, the spots enlarged beyond the point of inoculation 48 hours after inoculation, the diameter of the spot that ranged from 0.2-0.8cm at 7 days after inoculation. Culture filtrates of 35, 42, 49, 56, 63 and 70 days caused severe necrosis surrounded by a yellow halo, necrotic spots that ranged from 0.3-1.2 cm in diameter after 7 days of inoculation, the spots later coalesced causing blights after 10 days, with the yellow halo which diffused towards the leaf lamina causing wilting and drooping of the leaves 14-18 days after inoculation. There were no signs of infection on the control plants as there were neither necrotic spots nor chlorosis on the inoculated leaves.

Melon leaves inoculated with culture filtrates of 7, 14 and 21 day-old did not show any sign of infection. However, on leaves inoculated with culture filtrate of 28 days old, small brown spots were observed at 7 days after inoculation which ranged from 0.1-0.3 cm in diameter, 35 day-old culture filtrate induced necrosis on inoculated leaves that ranged from 0.1-0.3 cm in diameter at 3 days after inoculation which later enlarged to 0.2-0.4 cm in diameter at 7 days after inoculation. Culture filtrates of 42-70 day old caused severe dark spots surrounded by halo that ranged from 0.4-0.9 cm in diameter at 7 days after inoculation, which later advanced to marginal necrosis on inoculated leaves, the necrosis that was observed on melon leaves diffused towards the leaf lamina which later coalesced causing tip die back and wilting of leaves, which later fell off from the mother plant at 14 days after inoculation. There were no signs of infection (uninfected) on the control plants as there were no necrotic spots or chlorosis on the inoculated leaves.

Maize leaves inoculated with culture filtrates of 7, 14 and 21 day-old did not show any sign of infection, but leaves inoculated with culture filtrate of 28 days old, induced severe chlorotic patches 7 days after inoculation, which later became necrotic 10 days after inoculation that ranged from 0.1-0.4 cm in diameter, while culture filtrates of 35-70 day old culture filtrates induced necrotic spots that ranged from 0.2-0.8 cm in diameter alongside with severe chlorotic patches at 7 days after inoculation. The necrotic spots coalesced which resulted into blight, the inoculated leaves wilted, which later fell off from the plant 18 days after inoculation. Necrosis, chlorosis and blights were not observed on the control leaves.

Cowpea leaves inoculated with culture filtrates of 7, 14 and 21 day-old caused no infection as there were no chlorosis nor necrosis on the leaves inoculated. However, leaves inoculated with 35 and 42 day-old culture filtrates induced dark brown spots that ranged from 0.2-0.3 cm in diameter 3 days after the leaves were inoculated. The area inoculated enlarged rapidly, the spots coalesced causing marginal necrosis, while culture filtrates of 49-70 day-old culture filtrates induced severe marginal necrosis ranging from 0.5-1.0 cm in diameter at 7 days from the point of inoculation, diffusing towards the leaf lamina which coalesced and surrounded by a chlorotic halo which later resulted to wilting of the inoculated leaves. The wilted leaves fell off from the plant of 14 days after inoculation. Control leaves were unaffected, no necrosis, blights and tip die back.

Cassava leaves inoculated with culture filtrates of 7, 14 and 21 day-old did not produce any sign of infection, while leaves inoculated with culture filtrate of 28 days old, induced brown spots 7 days after inoculation that ranged from 0.1-0.3 cm in diameter, while culture filtrates of 35-70 day old culture filtrates induced necrotic spots that ranged from 0.2-1.0 cm in diameter at 7 days after inoculation. The necrotic spots coalesced, resulting into blight and tip die back of the inoculated leaves, wilting was also observed. The inoculated leaves later fell off from the plant of 21 days after inoculation. Necrosis, chlorosis and blights were absent from the control leaves. The control leaves remained unaffected, no necrotic spots on the leaves.

Culture filtrates of 7, 14 and 21 day-old failed to cause infection on Jatropha leaves as there was no necrotic spots on the leaves. While necrotic spot surrounded by yellow halo was observed on leaves inoculated with culture filtrates of 28-70 day-old that ranged from 0.3-1.0 cm in diameter 7 days after inoculation, which advances from the point of inoculation surrounded by a yellow halo. No signs of necrotic spots on control leaves inoculated with culture filtrates void of the pathogen.

DISCUSSION

The bioassay of the phytotoxic metabolites of *Colletotrichum gloeosporioides* were found to induce necrotic lesions of varying sizes on all the test crops as reported by Jayasankar *et al*., [13] that phytotoxins of all *Colletotrichum* are non-host specific. This is in agreement with the report of Amusa and Ikotun [14] that

toxic metabolites produced in culture by the *Colletotrichum* species exhibited a wide host range spectrum like the producing pathogen. The cell-free culture filtrates caused brown necrosis with variation in the size of necrotic spots among the test plants. The toxic metabolites of *C. gloeosporioides* were able to infect and induce necrosis which extended beyond the point of inoculation in Jatropha and other crops. Test plants inoculated with culture filtrates of 7, 14 and 21 days old had no chlorotic or necrotic effect on the leaves, which indicates that *C. gloeosporioides* might not have produced any toxic metabolite in that short time or the amount of toxic metabolite produced might be insufficient to express symptoms on the leaves of the test plants [15]. Marginal necrosis, blights, tip die-back and wilting of the inoculated leaves which later fell off 21 days after inoculation was evident in all the test plants inoculated with 28-70 day old culture filtrates. Wood [16] stated that when a toxin is continuously produced in small amounts by a pathogen in an infected host, its effect may be first stimulatory, then inhibitory and eventually lethal. This agreed with the activity of the culture filtrates on the test plants. But the time taken for symptom expression varied, the necrosis was observed as the spots gradually enlarged and clear chlorotic zones were formed around them 3-5 days after inoculation, as reported by Gauri *et al*. [17] that symptoms induced by the filtrates were similar to those caused by the pathogen under the natural infection conditions. As the age of the culture filtrates increased, the time required to express symptoms decreased, indicating increased amount of toxic metabolite in the medium, but from the 56th day, the time remained constant. This could be due to the growth of the fungus in the liquid medium having attained the peak of toxin production. After this, the mycelia could have started degenerating resulting in death of the organism. The culture filtrates induced several symptoms ranging from mild to severe chlorosis, necrosis, marginal necrosis and blights on the weeds sprayed as reported by Amusa and Ikotun [14] that wilting and blighting associated with the broad leaved weeds sprayed with the toxic metabolites is probably as a result of the damaging effect of the metabolites on the cells of the shoots and roots.

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