Efficacy Of Leaf Extract Of Neem (Azadirachta Indica) Against Shoot Dieback Disease Of Two Species Of Ficus In Atbara Town-Sudan

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Abstract: A widespread stem canker and dieback diseases threatened different plant genera in Sudan. It was highly spread among different Ficus species. This research was carried out in Atbara town north Khartoum. The causal agents of this disease were Isolated from the infected shoot system of Ficus bengalensis and F. retusa. Morphological identification and relative densities of the isolated fungi were carried out with the help of authentic manuals of fungi. Biocontrol experiment was conducted for the most prevalent pathogenic fungus using aqueous neem extract in both cold and hot water. The results showed that the fungal isolates belong to three genera, Aspergillus spp. (Aspergillus niger, A. terrus and A. flavus) , Botryosphaeria sp. and Botrytis sp. The most prevalent mycoflora was Botryosphaeria sp. (relative density range 35-45%), the second one was A.niger 20%, then Botrytis sp. 15%, A. flavus 10% and A.terreus 2-8%. The efficacy of aqueous cold and hot extracts of neem leaf on the growth of Botryosphaeria sp. showed that the hot extract is effective than the cold one and the inhibition percentage were 100 and 65% respectively on the 14th days.

Keywords: Ficus bengalensis, F. retusa, dieback disease, Botryosphaeria sp.

Introduction:

Ficus often grown as an attractive ornamental tree found mostly outdoors and is frequently encountered along city streets lining parkways, medians and sidewalks. Under Sudanese conditions, Ficus trees have special importance in parks and in the newly established cities to reduce the impact of the desert environment. Ficus trees are usually affected by several diseases particularly in warm and moist conditions where the disease can lead to heavy defoliation; trunk and branch canker [1]. For several years ago we noticed that Individual Ficus trees (Ficus bengalensis and F. retusa) were lining on the streets, losing their leaves and undergoing canopy dieback. Trees cankers were investigated by several plant pathologists such as [2]. Many fungi were isolated from Ficus infected leaves, branches and trunk canker such as, Botryosphaeria sp. from leasevs bloch of Ficus religiosa [3]. Other fungal species (Neosyctalidium dimidiatum and Scytalidium dimidiatum) has been identified as the causal pathogens of Ficus dieback and dying in Southern California and Sudan[4], [5] and [6].) The fungus Nattrassia was considered the main causative agent of wilt and dieback diseases of Ficus benjamina and many trees in the tropics and subtropics [1]. To control the causal agents of this disease many agrochemicals were tried. The extensive use of this chemicals especially fungicides, caused carcinogenic risk and may give rise to undesirable biological effects on animals and human beings [7]. Now major challenge is felt in the field of plant pathology to introduce some ecofriendly and safe alternative control strategies for agriculture, which led researchers to turn their attention to plants and microorganisms as sources of biocontrol agents. Plant extracts and essential oils show antifungal activity against a wide range of fungi [8] and Alkhail [9] showed that aqueous extracts of plants viz., Allium sativum, Cymbopogon proxims, Carum carvi, Azadirachta indica and Eugenia caryophyllus had strong antifungal activity against fungi viz., Fusarium oxysporum, Botrytis cinerea and Rhizoctonia solani. This study was undertaken with the aim of determining the nature of the disease and its causal agent on two Ficus spp. trees and to draw attention to the causal agent in Sudan. Part of this research was conducted to evaluate some of the available neem leaf extract against pathogenic fungi to control the disease through in vitro applications.

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Materials and methods:

Survey and incidence of infection

A survey of infection by the branch wilt and trunk canker disease in Ficus bengalensis and F. retusa was carried out at different location in Atbara town north Khartoum. Isolation and identification of mycoflora was carried out from every tree showing a single and/or a combination of chlorosis, necrosis, wilt of foliage, twigs or branches, canker and presence of the characteristic black sooty layer of conidia under the bark .

Collection of plant material samples

Random samples from F. bengalensis and F. retusa that developed the diedack symptoms were collected from the, woody bark which were taken by means of an increment borer which was inserted inside the stem to a depth of 5 cm after being surface sterilized by swabbing with 95% ethanol. All samples were kept inside sterilized polythene bags and kept in a fridge for further analysis.

Isolation of mycoflora:

Plant materials from infected trees was cut into smaller pieces which were surface sterilized by immersion into 1% sodium hypochlorite for 1 minute, then washed thoroughly in two changes of sterile distilled water and dried on filter

paper. All samples were cultured in Petri dishes containing sterile PDA medium. Cultures were then incubated at 30 ± 1°C. Daily examination of fungal colonies was done under a stereoscopic binocular microscope which was further verified by isolation and identification of mycoflora. The relative density (RD)of the isolates were calculated according to [10] as follows:

RD (%) = No. of isolated genus or species x 100
Total No. of isolated fungi

Identification of fungal Isolates:
Starting at 48 hours and during the following 8 days the plates were observed and the isolated colonies were identified. Identification was based on the macroscopic and microscopic morphology of the colonies according to the Illustrated Manual on Identification of Some Seed-borne Aspergilli, Fusaria, and their Mycotoxin [11] and [12].

Biological control of pathogenic Fungi:
Azadirachta indica leaves were used in this experiment as following:

Collection of plant materials: Fresh and healthy leaves of species Azadirachta indica were collected from Nile Valley university in Atbara town north Khartoum. For surface sterilization the leaves were soaked in 5ml ethanol (95% v/v) for 5 minutes. Then washed thoroughly with sterilized distill water and the leaves were dried in an electric oven at 30oC for 72 hours and crushed to make powder.

Preparation of aqueous extract:
Twenty grams of dried leaf powder was soaked in 100 ml of sterilized cold distilled water for 24 hours and another 20 g. were soaked in hot distilled water at 90^{o} C for1 hour. Extract was filtered through a double layered muslin cloth followed by Whatman No.1 filter paper and used in the further experiment immediately. PDA medium was prepared and 0.5 g of neem powered was added to 100 ml medium
.

Results:

The incidence of infection of the Ficus bengalensis and Ficus retusa was presented in table1, which revealed that three different fungal genera were isolated from the tested samples with different relative densities. They were designated as Bt, B2, A1, A2 and A3. The most prevalent fungal isolate was Bt (RD. 35-45%) on the two Ficus spp. (F.retusa and F.bengalensis respectively), whereas A2 recorded the lowest relative densities (2-8%). The morphological characteristics of Aspergillus isolates (A1, A2 and A3) were presented in Table (2). A1 Colony colour in CZ agar plates from obverse side was reddish brown to blackish and from reverse side was creamish yellow, to yellow orange. The head was globose and the stipe was long and smooth with hyaline to brownish colour. The vesicle was globose thick-walled, brownish in colour. The metulae were present and were characterized by being long closely packed and brownish in colour. Phialides were short ampulliform while conidia were globose to subglobose. The conidia had rough surfaces and were of dark to black colour.

Table (1): *percentage Relative Density of Funal Isolates of Ficus bengalensis and Ficus retusa*

Samples	Fungal Isolates	Relative Density %
Ficus bengalensis (F1)	Bt	45
	B2	15
	A1	20
	A2	8
	A3	10
Ficus retusa (F2)	Bt	35
	B2	15
	A1	20
	A2	2
	A3	10

Isolate A2 was characterized on CZ agar plate was cinnamon from the obverse side and yellowish brown from the reverse side. The head was columnar, while the stipe was short and smooth. The vesicle was hemispherical and fertile in the upper one-third area. The metulae were present almost parallel to the stipe axis and cylindrical in shape. The phialides were ampulliform and thin, while the conidia were globose in long chains and of uniform diameter. A3 On Czapek dox agar, colonies are granular, flat, often with radial grooves, dark yellow-green with age. Conidial heads are typically radiate, mostly biseriate but having some heads with phialides borne directly on the vesicle. Conidiophores are hyaline and coarsely roughened, the. Conidia are globose to subglobose, pale green and conspicuously echinulate. The isolate Bt showed black chlorotic areas on the trunk of the tested samples which is black fruiting bodies. These ascomata were solitary (diameter 1-1.5 mm), with papillated ostiole. Also asci were clavate, bitunicate with numerous pseudoparaphyses. Ascospores were hyaline and aseptate .Initially, a colony on malt extract agar showed blackish white to slight grayish color with an evenly dense mat and after 10 days colony changed to black colour (Fig.1). The colony developed in culture medium showed two types of conidia; hyaline, aseptate and mature brown septate conidia . while B2 colony on malt extract agar were compact, cottony, radial or in concentric rings . Colonies grayish, or hyaline at first but soon becoming light grey, dark grey to dark brown green. Conidia were solitary and attached to the ampulla by fine denticles Fig.2. They were hyaline or pale brown. Conidia ovate, ellipsoidal, sometimes globose to subglobose or flat in one part. They were smooth, often with a slightly

protuberant hilum and usually unicellular but occasionally 1- or 2-septate conidia was observed. Conidiophores were more or less straight, septate, branched towards the apex often dichotomously. They were brown becoming paler near the apex with the ends of the branches often quite colourless
.

Table 2: *Morphological Characteristic of different Aspergillus isolates*

Characteristics	A1	A2	A3
Phialides			
Shape	A	A	A
Neck type	-	-	-
conidia			
Shape	G.S.	G.S.	G.
Texture	Sm.	Sm.	Sm.
Color	H. L.b.	D.b.	H. L.b.
Colony diameter cm			
CZ	5.6	6.5	6.0
CYA	7.0	7.0	8.0
MEA	4.4	4.7	4.0
Colony color in agar Petri- dishes			
Obverse side	R. b.	B. b.	B.b
Reverse side	C.y	C.y	Y. G.
Head shape	G	G	G
Stipe			
Length	+	+	+
Texture	Sm.	Sm.	Q
Vesicle			
Shape	G	G	
Fertile area	A.O.	U.O.T.	U.T.T.
Metulae	Pre.	Pre.	Pre.

Ampuliform = A

Hyaline to light brown = H. l.b.

Hyaline to light yellow = H. L.b.

On Upper OneThird = U.O.T

On Upper twoThird = U.T.T.

All over = A.O.

Blackish brown = B. b.

Pyrifor = P

Dark brown = D.b.

Present = Pre.

Reddish brown = R. b

Green yellow = Y. G.

Creamish yellow = C. y.

Globose to sub g = G.S.

Very broad =V.b.

Yellowish brown = Y. b

Brownish pink = Globose = G

Rusty brown = R. b.

Globose to slightly elliptical = G.S.E

Long = + Columnar = Col.

Short = -

Rough = Ro.

Quietly = Q

Smooth = Sm.

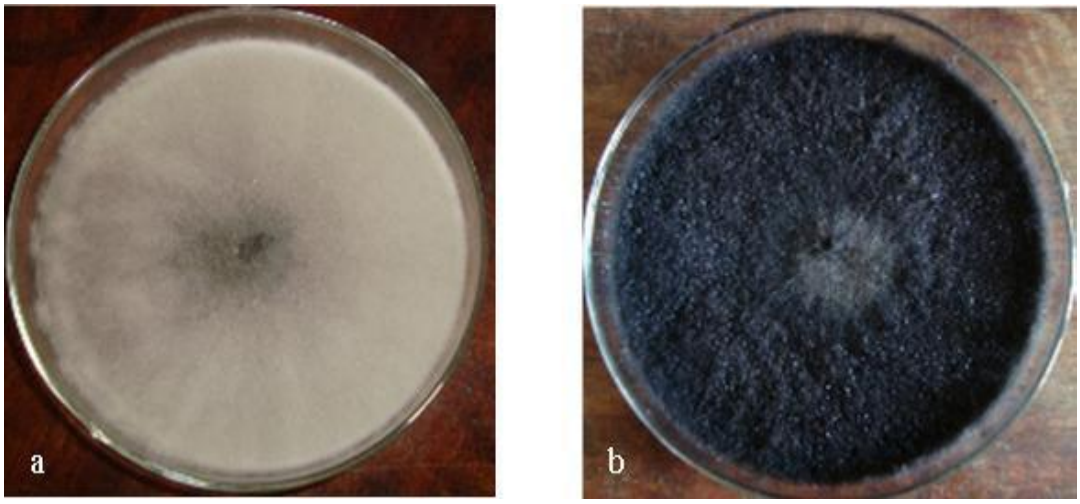

Fig 1: *Botryosphaeria sp. on malt extract agar (a) 10 day old culture and (b) one month old culture*

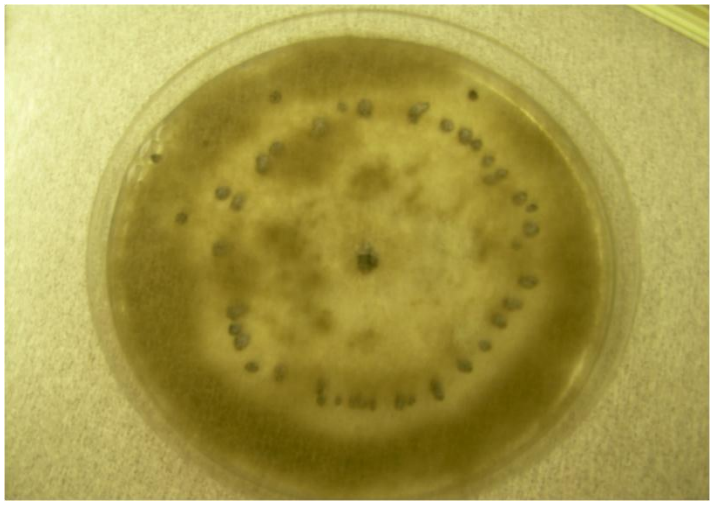

Fig2: *Botrytis sp. on malt extract agar 5 day old culture*

Biological control of pathogenic Fungi:

Botryosphaeria was the most prevalent fungal isolate among all isolated mycoflora with relative density of 35-45%, for that it was selected to be controlled biologically table 3. Extraction from neem leaves was obtained in hot and cold water separately and each was applied to the growth media in a concentration of 20%. Then the media were inoculated with Botryosphaeria sp. and incubated at 28^{o}C for 14 days, a control was prepared by inoculating the tested fungi to the untreated media. The diameters of Botryosphaeria sp. were measured on both treated and control media at interval of two days till 14 days. Table3 and Fig 4 showed the antagonistic effect of Azadirahta leaves extract (one in cold water and the other in hot water) on Botryosphaeria isolate. The neem cold extract suppressed the growth of the tested fungal isolate up to 65% at the day 14th while the hot extract was completely destroyed the fungi

Table 3: *Effect of Azadirachta indica leaves extract on the growth of Botryosphaeria sp.*

Azadirachta indica leaves extract	Days	*Botryosphaeria* colony diameter in untreated medium (control) mm.	*Botryosphaeria* colony diameter in treated medium (mm.)	Percentage decrease of fungal diameter %
	4	4.5	2.7	
	6	4.7	1.5	44.4

Leaves extract in hot water	8	6	1.1	59.3
	10	7.2	0.8	70.4
	12	8.2	0.4	85.2
	14	9.2	Zero	100
Leaves extract in cold water	4	4.5	2.3	
	6	4.7	2.1	9
	8	6	1.9	17.4
	10	7.2	1.5	34.8
	12	8.2	1.2	47.8
	14	9.2	0.8	65.2

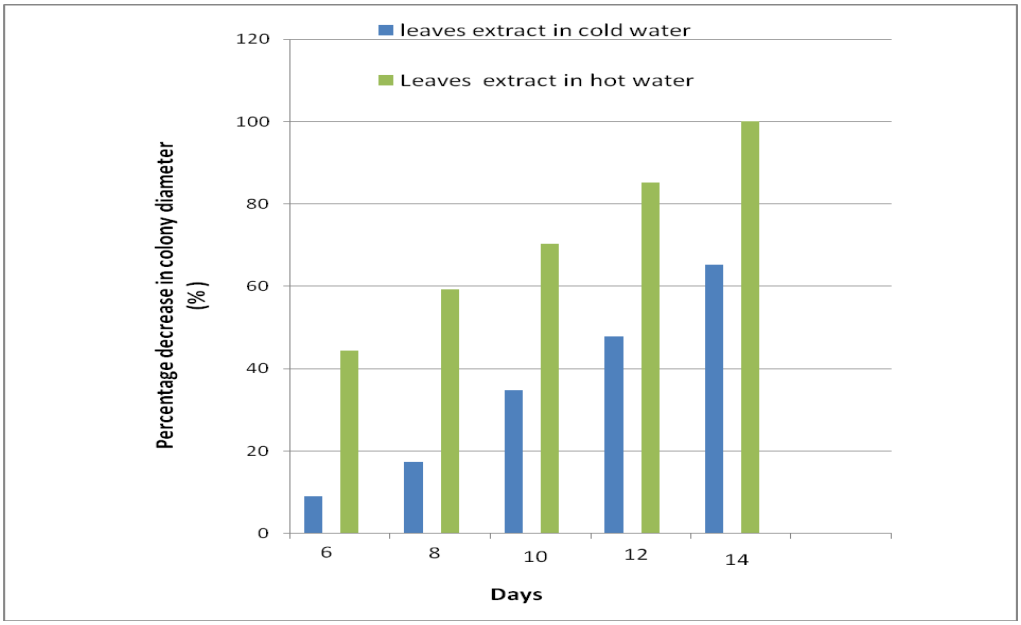

Fig 4: *Effect of Azadirachta indica Leaves Extract on the Growth of Botryosphaeria sp.*

Discussion:

Dieback and wilt diseases are considered as most severe and economically important diseases. The diseases have not been so epidemic but sporadic attacks have caused tremendous damage [13]. Different fungal genera were reported as causative agents of dieback diseases such as, Fusarium oxysporum, Fusarium semitectum, Rhizoctonia solani, Alternaria alternata, Curvalaria lunata, Botryosphaeria sp. Aspergillus niger and Aspergillus flavus [1]. In this study five mycoflora were isolated from stem canker of Ficus bengalensis and Ficus retusa and were designated as Bt, B, A1, A2 and A3 table 1. The incidence of infection of the two Ficus species by the above mentioned fungal isolates was determined by the percentage of relative density. Isolate Bt, was the most the prevalence among all mycoflora, the second dominant isolate was A1, B2,A3 and A2 recorded the lowest value of relative density. Similar results were found by Charles et.al. [14] who detected Phomopsis sp. and Botryosphaeria spp. in 89% and 40% of commercial orchards shoots respectively. Identification of the isolated mycoflora revealed that three fungal isolates out of five, were characterized by the presence of the typical Aspergillus heads having different colours. This suggests that the isolates A1, A2 and A3 belong to the genus Aspergillus[11]. Based on all these properties Isolate A1 was identified as Aspergillus niger and according to the colour and texture of the conidia this isolate was also identified as Aspergillus niger var. niger. The morphological characterization of the isolates A2 and A3 suggest that this organisms were Aspergillus terreus and Aspergillus flavus respectively as described by [15]. The fungus Bt was identified as

Botryosphaeria sp. as described by [16]. while B2 colony on malt extract agar were compact, cottony, radial or in concentric rings. According to these properties isolate B2 was identified as Botrytis sp. as described by [16]. The results of biological control showed the efficacy of hot aqueous extract of Azadirchta indica leaves against Botryosphaeria sp. This result is agreed with that obtained by [17] who found that the effect of 20% aqueous extracts from neem leaves inhibited the fungal growth up to 100 and 70.55% for F. oxysporum and Alternaria solani respectively. In Egypt Mahmoud et. al. [18] reported the efficiency of aqueous extracts from neem leaves on different fungal genera and they showed complete inhibition (100%) in the growth of A. niger obtained in assay with 20% concentration. Bohra and Purohit [19] also mentioned that the aqueous extracts of A. indica gave the highest inhibition of A. flavus growth.

Conclusion:

Dieback disease is caused by different fungal genera and this may depend on the plant genus and locations, for that isolation and identification of a causal agent of this disease is very important. To control pathogenic fungi isolated in this study aqueous Azadirachta indica leaves extract in both cold and hot water were tested. The results of the present studies showed that aqueous hot extract is very effective when compared to the cold extract and this would suggested that hot aqueous neem extracts holds promise control of Ficus spp. dieback disease.

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