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ANTIFUNGAL EFFECT OF *AZADIRACHTA INDICA* A. JUSS EXTRACTS ON *COLLETOTRICHUM LINDEMUTHIANUM*

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ABSTRACT

Amending substrates with neem extracts was directly inhibitory to *Colletotrichum lindemuthianum*. Percentage inhibition was distributed in a decreasing order of potency among the seed, fruit, leaf, root and bark of *A. indica*. As the antifungal efficacy of the extract increased with decreased concentration, fungal conidia were ruptured and completely prevented from germinating in 90 and 100gl^{-1} aqueous neem seed extracts. Water extract was superior to the organic solvent extracts in reducing fungal spore germination, mycelial growth and dry weight. Among the organic solvent extracts, methanolic extract rated best, followed by ethanolic extract while chloroform extract was the least effective. The efficacy of need seed extracts was significantly reduced when stored at 40 - 90°C before use.

KEYWORDS: *Azadirachta indica, Colletotrichum lindemuthianum* antifungal effect.

INTRODUCTION

The damage caused by *Colletotrichum lindemuthianum* (Sacc) Briosi is often a great impediment to cowpea production in Nigeria. The fungus is known to cause stem anthracnose disease of cowpea and if left unchecked, it may lead to total crop failure (Singh and Allen, 1979). Adoption of any plant disease control measure depends greatly on the level of expertise and socio-economic situation of the farmer. The use of synthetic pesticides has been most widely adopted (Gruzdyev *et al*, 1988). However, the prohibitive costs and toxic side effects associated with synthetic pesticides has necessitated the search for pesticides of plant origin which are not only cheap and readily available but also environmentally friendly.

Grainge and Ahmed (1988) reported that about four thousand two hundred (4,200) plant species possessed pesticidal properties. Among these flora, the neem (*Azadirachta indica* A Juss; Meliaceae) has been used to control a wide range of insect, bacterial, nematode and fungal species (Latum, 1985, National Research Council, 1992; Onifade, 1998).

The present study was designed to evaluate the antifungal efficacy of extracts of neem leaf, bark, fruit, seed and root on *C. lindemuthianum in vitro*.

MATERIALS AND METHODS

Preparation of Extract

Ripe neem fruits (with yellow pericarp), harvested from the local plant, were air-dried for two weeks. A portion of the dried fruits was cracked to remove the seed from the hard shell. Fresh leaves, bark and root were also collected, cut into small pieces and air-dried for two weeks. The tissue samples were pulverised separately using sterile mortar and pestle. A 100g portion of each powdered sample (from fruit, seed, leaf, bark or root) was extracted with 500ml of acetone, n-butanol, chloroform, ethanol, hexane, methanol or petroleum ether, for 5 days. The extract was concentrated *in vacuo* using rotary evaporator. Thereafter, dilutions of 1:10, 1:100 and 1:1000 were prepared from the stock using dimethyl sulphoxide (DMSO).

Aqueous extract was prepared by suspending 120g of each powdered sample in 100ml sterile distilled water for 20 hours. Thereafter, the suspension was filtered through sterile muslin cloth. Different concentrations; 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 gram sample per litre of water were then prepared and their suspension filtered as above. All the extracts were sterilized by passing through millipore membrane filter with pore size of 0.22μm (Singh *et al.*, 1980).

Isolation of Pathogen

C. lidemuthianum was isolated from anthracnose - infected cowpea plants as

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described by Alabi (1986). Four-day old pure culture of the fungus was scraped with sterile forceps into a 200ml conical flask containing sterile distilled water (150ml). The flask was gently rotated to dislodge the conidia after which spore concentration was determined with the aid of a Heamocy tometer slide.

Effect of Extracts on Spore Germination

A 1ml portion of the conidial suspension (1 x 10^4 spores per ml) was added to each tissue extract contained in a test tube. A test tube containing conidial suspension and sterile distilled water served as the control. The set up was incubated in microhumidity chambers at 24 - 29°C. The number of spores that germinated and those that did not germinate per treatment were determined every six hours for three days. Thereafter, percentage inhibition was calculated according to Obaleye *et al.* (1994).

Effect of Extracts on Mycelial growth

Fifteen millilitres aliquots of molten potato dextrose agar (PDA) were dispensed into 9cm diameter petri dishes. A 3ml portion of each extract was then transferred aseptically onto the cooled medium and the plate was gently rotated to ensure even distribution of extract on the medium. By means of a sterile 5mm diameter cork borer, inoculum plug (5mm diam.) obtained from the advancing edge of 4-day old pure cultures of the pathogen was transferred to the centre of each plate. The controls consisted of PDA plates without extracts, but inoculated as described. There were ten treatments, each replicated ten times. The plates were incubated at 24-29°C for 4 days. Thereafter, the colony diameter was measured using electronic calipars. Percentage reduction of fungal growth was determined according to Mishra *et al* (1995).

Effects of Extracts on mycelial dry Weight

Five ml of the respective extracts was added to 25ml of malt extract broth contained in each of 200ml conical flasks. Control flasks contained equal amounts of sterile distilled water instead of extract. The flasks were then inoculated with 1 x 10^4 spores of the fungal pathogen and incubated at 24 - 29°C for 4 days. Thereafter, the mycelium was filtered, washed twice with sterile distilled water, blotted well and the dry weight was determined at 80°C for 40 hours. Percentage reduction in mycelial dry weight was determined as described by Singh *et al* (1980).

Effect of temperature on the efficacy of 100gl^{-1} aqueous extract of neem seed

Each of the sterile McCartney bottles, containing 10ml of 100gl^{-1} aqueous extract, was placed in a water bath for 40 minutes at 20, 30, 40, 50, 60, 70, 80, or 90°C. The bottles were then exposed to room temperature for two hours before each extract was tested for its effect on fungal spore germination, mycelial growth, and dry weight as earlier described.

Each treatment, in ten replications, was randomly incubated in the laboratory. The data obtained were subjected to analysis of variance and means were separated using Duncan's New Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

A general trend of increased antifungal activity was observed with a corresponding increase in concentration of aqueous neem seed extract (Table 1). For instance, *C. lindemuthianum* spore germination, radial growth, and dry weight was completely reduced in 80, 90 or 100gl^{-1} aqueous neem seed extract. The antifungal potency of extract, however, declined as the concentration reduced down to 10gl^{-1}.

Table 1: Effect of the Different Concentrations of aqueous neem seed extract on spore germination, mycelial growth and dry weight of *Colletotrichum lindemuthianum*

Extract Conc. (gl-1)	Percentage inhibition in:		
	Spore germination	Mycelial growth	Mycelial dry weight
10	5.7d	7.2d	5.9d
20	10.3d	13.0d	11.1d
30	27.6c	29.9c	26.5c
40	43.6c	44.1bc	39.8c
50	62.9b	60.4b	64.9b
60	75.8b	80.0ab	79.4b
70	94.1a	90.7a	93.4a
80	100.0a	99.3a	100.0a
90	100.0a	100.0a	100.0a
100	100.0a	100.0a	100.0a

Values in a column with the same letter(s) are not significantly different at probability 5% ($P \leq 0.05$) level.

Table 2. Effect of different parts of *Azadirachta indica* (at 100gl^{-1}) on spore germination, mycelial growth and dry weight of *Colletotrichum lindumithianum*

Part of Neem	Percentage inhibition:		
	Spore germination	Mycelial growth	Mycelial dry weight
Seed	100.0a	100.0a	100.0a
Fruit	96.8a	100.0a	100.0a
Leaf	50.1b	57.2b	52.0b
Bark	32.2c	28.6c	31.7c
Root	19.8d	22.9c	20.4d

Values in a column with the same letter are not significantly different at probability 5% ($P \leq 0.05$) level.

Table 3. Effect of extraction solvent on the toxicity of neem seed extract (at 1:10) to *Colletotrichum lindemuthianum*

Extraction Solvent	Percentage inhibition in		
	Spore germination	Mycelial growth	Mycelial dry weight
Acetone	34.4c	34.0d	36.3c
Butanol	42.3c	37.5d	40.0c
Chloroform	19.0d	23.1a	20.9d
Ethanol	69.6b	72.0b	70.5b
Hexane	64.7b	60.9b	62.9b
Methanol	78.4b	79.9b	75.2b
Petrol ether	53.2c	49.8c	50.2c
Water	100.0a	100.0a	100.0a

Figures in a column with the same letter are not significantly different at probability 5% ($P \leq 0.05$) level.

Table 4: Effect of temperature on the efficacy of 100gl-1 aqueous neem seed extract against *C. lindemuthianum*

Temperature (0°C)	Percentage inhibition in		
	Spore germination	Mycelial growth	Mycelial dry weight
20	100.0a	100.0a	100.0a
30	100.0a	100.0a	100.0a
40	99.2a	93.4a	95.0a
50	61.4b	60.0b	62.0b
60	43.3c	40.9c	41.2c
70	21.0d	19.2d	20.9d
80	9.0de	0.0a	0.0e
90	0.0de	0.0de	0.0e

Figures in a column with the same letter(s) are not significantly different at probability 5% ($P \leq 0.05$) level.

Besides, the antifungal efficacy of neem extracts was found to be distributed in a decreasing order of seed, fruit, leaf, bark and root of neem (Table 2). The observed superiority of seed to other parts of neem is in conformity with a previous experiment (Ermel *et al.*, 1987), in which neem seed contained the highest concentrations of azadirachatin and other biocidal susbstances.

The direct effect of solvent employed in the extraction of plant material on the toxicity of neem extract is equally evident in this study (Table 3). For instance, water extract of neem seed was most effective in reducing the spore germination and growth of *C. colletotrichum* when compared with the organic solvent for extracting the fungitoxic principle in neem seed as earlier suggested by Feuerhake (1984) and Onifade and Alabi (1998). One possible explanation for the superiority of water extract for organic solvent extract is that the organic solvents may have reacted with the active ingredients in neem seed, forming inactive compounds. That is, a chemical change may have occurred during the exraction with organic solvents. Among the organic solvent extracts, methanolic extract was the most effective, followed by ethanolic, hexane, petroleum ether, and butanolic extracts respectively, while chloroform extract was the least potent.

Aqueous extract of neem seed (100gl^{-1}), when incubated at 20°C or 30°C before pesticidal assay, competely reduced fungal spore germination and mycelial growth. Furthermore, increase in temperature (between 40° and 90°C) progressively reduced the efficacy of the extract. The extract was completely inactivated at 80° or 90°c (Table 4). This suggests that high temperature degraded or inactivated the active principles inherent in the neem seed sample. But there is the need to subject each of the active ingredients to varying temperature regimes.

Also from this study, 5,036 and 8,254 conidia of *C. lindemuthianum* were ruptured after 28 hours in 90gl^{-1} and 100gl^{-1} respectively. This observation may give a clue to the possible mode of action of the exract against the test fungus.

Toxicity trials have shown that neem extracts were toxic to *C. lindemuthianum*, but fungitoxicity depends on the concentration of extracts, the solvent and part of neem plant used in extract preparation as well as the temperature at which extract was kept

before use. It is hereby established that neem extracts have desirable antifungal activity that can be harnessed for use in plant protection. With further studies, neem-based pesticides can be incorporated into a well-planned integrated pest management programme to reduce dependence on synthetic chemicals which are not only toxic but also expensive.

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