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Effect of some botanicals on *Colletotrichum destructivum* O`Gara of cowpea

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Colletotrichum destructivum **is the causal pathogen of cowpea anthracnose; botanical extracts and benlate fungicide were evaluated as Biopesticides/chemical control strategies in cowpea (*Vigna unguiculata* L). Botanicals of four plants: *Azadiractha indica*, *Cymbopogon citratus*, *Ocimum gratissimum*, and *Xylopia aethiopica*, proved effective in reducing spore germination and colony growth *in vitro* and the growth of the pathogen *in vivo*. The extracts of *X. aethiopica* and *A. indica* more effectively reduced both the growth of the pathogen *in vitro* and the spread of the disease *in vivo*. Extracts and benlate applied both before and after pathogen inoculation of cowpea significantly reduced the size of pathogen induced lesion.**

Key words: Anthracnose, diseases, biopesticides, botanical, benlate, *Colletotrichum destructivum.*

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp) is an important source of protein and other essential nutrients for human and livestock consumption, particularly among the low income segment of the populace in the semi-arid regions of the tropics and subtropics (Adebanjo and Bankole, 2004; Sun and Zhang, 2009). The optimum production of the crop is hindered by several factors including the plant pathogenic organism like *Colletotrichum destructivum* O`Gara (Allen et al., 1998; Akinbode and Ikotun, 2008).

The pathogen *C. destructivum* is seed borne and causes anthracnose disease in cowpea (Fokung et al., 1997; Allen et al*.,* 1998) where the inoculum sporulates readily at localized infection foci and produce symptoms within 96 h of susceptible crop inoculation (Latunde-Dada et al*.*, 1996).

In a susceptible crop, all its parts are attacked by the fungus including: seedlings, hypocotyl, stems, peduncles, flowers, leaves and pods and can lead to yield loss of up to 50% in cowpea (Emechebe and Lagoke, 2002; Adebanjo and Bankole, 2004).

Several control methods have been adopted including the application of chemical (fungicides) and integrated pest management (Emechebe and Lagoke, 2002), biocontrol (Adebanjo and Bankole, 2004**;** Akinbode and Ikotun, 2008), host resistance (Amusa et al., 1994; Latunde-Dada et al., 1999)**,** use of phosphorus fertilizers (Adebitan, 1996; Owolade et al*.,* 2006) and cultural practices like spacing and plant pattern **(**Adebitan and Ikotun, 1996).

Resistance development in plant pathogens (Emechebe and Lagoke, 2002), and chemical build up both of which are hazardous to man and his environment (Airold and Critter, 1996; Jansch and Frische, 2009) have led to search for bioactive molecules in plants and plant parts as alternative and/ or complements in the control methods of crop health issues (Alkhail, 2005; Win et al., 2007; Necha and Barrera, 2008). And just recently Mogle and Maske (2012) reported the leaf extracts of *Argemone*

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mexicana L., *Semecarpus anacardium* L., *Cassia fistul*a L., and *Tephrosia purpurea* (L.) pers to control *C. destructivum pathogen* on seeds of cowpea.

The enhancement of Biopesticides therefore remains a viable pest control strategy for efficient and an effective cowpea crop protection. The United States Environmental Protection Agency (EPA-USA) defines a Biopesticides as a pesticide derived from natural materials such as animals, plants, bacteria and certain minerals (http://www.polyversumla.com).

The objective of the present study was to evaluate biofungicidal effectiveness (*in vitro*) of *Azadiractha indica*, *Cymbopogon citratus*, *Ocimum gratissimum*, and *Xylopia aethiopica* on the anthracnose pathogen*, C. destructivum* and in comparism (*in vivo*) with a conventional fungicide.

MATERIALS AND METHODS

Botanical materials

Mature fruits from *A. indica* A. Juss (neem) were harvested, dehisced and then oven-dried for two days at 60ºC. The seed coats were then split for the cotyledons which were subsequently washed in sterile distilled water and oven dried together with the sterile fruits of *X. aethiopica* (Dunal) A. Rich at 60^{o}C for 24 h. The fruits and the seeds were separately ground in a sterile mortar to obtain 0.1 kg of dry powder from each material. Fresh leaves of *C. citratus* (DC.) Stapf. and *O. gratissimum* L., were washed thoroughly in sterile distilled water, air-dried at 26ºC, weighed (0.1 kg) and ground separately in sterile mortar.

Botanical extraction

Oils were extracted from 80 g of each of the oven dried mortar-grounded seeds of *A. indica* and fruit of *X. aethiopica* in 500 mL (diethyl ether (TKM Pharma, Andhra Pradesh, 500020, India) in a Soxhlet extractor (Bionics Scientific Technolgies Pvt.Ltd, India) for six hours. The solvent was evaporated initially using a water bath and then left overnight at 26ºC for evaporation of the remaining solvent.

Hot water extracts (HWE) were obtained by infusing 100 g each of the four ground test materials separately with 100 mL sterile distilled water in a water bath at 80ºC for 1.5 h, then filtering the extract through 4 layers of sterile cheese cloth. Cold water extracts (CWE) from *C. citratus and O gratissimum* were obtained by adding 100 g to 100 mL sterile distilled water. The mixture was stirred vigorously and allowed to stand for one hour, then filtered through 4 layers of sterile cheese cloth.

Evaluation of botanical extracts in vitro on pathological activities of Colletotrichum destructivum

The bioassay was on the pathological activities of spore germination, colony extention and sporulation density. The effect of botanicals on fungal growth was determined by growing *C. destructivum* on a PDA (potato dextrose agar) media containing extract in Petri plates. A 50% concentration of crude extract in PDA was prepared by adding 50 mL of the oil or hot water extract to 50 mL molten PDA (primed by dissolving 3.70 g PDA in 50 mL sterile distilled water). One hundred percent botanicals in PDA were primed by smearing one milliliter of each botanical (full strength) on the surface of the solidified PDA-botanical medium contained in Petri dishes and PDA without botanicals served as standard.

With a cork borer within a lamina flow chamber, a disc (3 mm in diameter) of ten day old *C. destructivum* culture was aseptically transferred to the center of the solidified PDA-extract medium in the Petri plates. Treated cultures were incubated at 26ºC for seven days. Colony growth of the *C. destructivum* was measured, with a line gauge on each Petri dish. The experiment was replicated five times per treatment.

Suspension of the virgin cultures of *C. destructivum*, were prepared for evaluation of spore germination using a disc (3 mm diameter) in 1 mL each of the undiluted (100% concentration) and diluted (50% concentration) extracts in test tubes. Similar spore suspensions were prepared in sterile distilled water as control. The contents of the tubes were subsequently centrifuged at one hundred revolutions per minutes for ten minutes and then filtered through four folds of cheese cloth.

With a Pasteur Pipette (Ningbo Mflab Medical Instruments Co., Ltd., Zhejiang, China Mainland), a drop (0.05 mL) of each spore suspension (10 × 10^4 spores/mL) was placed on triplicate sterile slides inside Petri dish-moistured chambers and incubated at 26ºC for 24 h. Further spore germination was then stopped by adding a drop of a biological stain, the Lactophenol Cotton Blue (LPCB) (Thomas Baker (Chemicals) Pvt Ltd, Mumbai India) to each spore suspension on the slide and 100 spores observed at random with a microscope (x10) and values used to determine the percentage extract inhibition of spore germination.

The culture plates used for the study on colony extension were employed in the study on the effect of Botanical extracts on sporulation density. Five millimeters of sterile distilled water was added to each of four replicate plates per treatment. Spores from each Petri plate were washed into suspension with the aid of a sterile scalpel and filtered through three layers of muslin cloth into a test tube. Spores counts per replicate spore suspension were made using Hemacytometer slide (Ningbo Mflab Medical Instruments Co., Ltd., Zhejiang, China Mainland) for each treatment.

Number of spores per treatment was calculated using the formular: (A+B+C+D+E) x50 equal numbers per cubic millimeter, multiplied by 1000 to get value in millimeter, where letters A, B, C, D and E, represent spore counts in 5, 1 mm^2 rulings of the Hemacytometer. The ruled surface is 0.1 mm below the cover glass on the Hemacytometer grid and volume of liquid over a square millimeter is 0.1 $mm^{3.}$ The sporulation densities data were divided by related colony areas, for differences in colony compensation, using the formular: πr^2 where r = radial growth and π = 3.142.

Evaluation in vivo of botanical extracts and benlate effectivity on disease development

With a susceptible *V. unguiculata* the inhibitory effect of botanicals on disease development *in vivo* were evaluated. The seeds were disinfected using a solution of sodium hypochlorite (The Stutz Company 4450W Carroll Ave. Chicago, IL 60624, USA) at 0.5% concentration for a minute and rinsed in sterile distilled water. The sterile seeds were sown in 4 kg of Metham Sodium (SPE Chemicals Co., Ltd. Shanghai, China Mainland) sterilized humus soil in 22.5 cm diameter clay pots at three seeds/pot randomly arranged into sets in a green-house.

The crops in the first set were inoculated with a spore load of 1 × 10^5 spores /mL distilled water of the pathogen two days before treatment application of botanicals and or benlate (3 g/L). Crops in the second set were inoculated two days after application of botanicals or benlate. Crops in the third collection were treated with the botanical extracts or benlate when anthracnose symptoms became evident (21 days after inoculation). Control crops were treated with sterile water.

Twenty five (25) days after crop treatment with botanical extracts and benlate four lesions per pot were selected at random and cut out, using a surgical blade, into test tubes containing 5 mL distilled water.

The tubes were subjected to centrifugation at 100 rpm for 5 min to release conidia from lesions into suspension. Three replicate drops of each spore suspension were placed on a Hemacytometer slide and sporulation density evaluated as earlier described under evaluation *in vitro*. The mean sporulation density of three replicate tubes was then divided by four to obtain the mean sporulation density per lesion for per extract treatment.

RESULTS

Treatments effect of botanical extracts *in vitro* on three pathological activities of *Colletotrichum destructivum*

The *in vitro* inhibitory potentials of the extracts of *A. indica*, *C. citratus*, *O. gratissimum* and *X. aethiopica* on three pathological activities of spore germination, colony growth and sporulation density is as indicated in Table 1. The comparative effect of treatment as indicated in Figure 1 showed the oil extract of *A. indica* (Aoe) and *X. aethiopica* (Xoe) to inhibit spore germination of *C. destructivum* by up to 100% at full strength fat extract concentration. At half dose concentration *C. citratus* water extract (Ccwe) had 9.3% inhibitory effect contra spore germination. This is also the least control value among the screened plants on *C. destructivum.*

Though there was inhibitory effect of over 60% from *A. indica* oil (Aoe), *O. gratissimum* cold water (Ocwe) and *X. athiopica* hot water (Xhwe), the extracts of *A. indica* and *C. citratus* at different forms and concentration induced or supported sporulation of the test pathogen. While *A. indica* extracts (hot water and oil at half dose) aided pathogen sporulation with well over 150% intensity, all forms of *C. citratus* extracts assisted pathogen sporulation, between 86 and 222%, at all levels of treatment forms and concentration (Figure 1).

Extracts and benlate effect on Vigna unguiculata diseases development

The *in vivo* effect of extracts and benlate on *C. destructivum* is shown in Table 2. Disease development was inhibited at various degrees in all cowpea crops treated with either extracts or benlate in all three levels of evaluation. Comparatively, treatment at two days after crop infection (2dai) indicated *O. gratissimum* hot water (Ohwe) treatment to be the most effective with 70.4% disease inhibition.

The highest disease inhibition, of 22.2% on 21 days treatment after crop inoculation (21dai) was by *X. aethiopica* oil (Xoe). Disease inhibitory effect of 37.8% was exhibited by both *A. azadirachta* oil (Aoe) and *X. aethiopica* oil (Xoe) on crops treated two days before pathogen inoculation (2dbi) as shown in Figure 2.

DISCUSSION

The advocacy for the use of plant extracts with antifungal property in agriculture, particularly in organic farming system where synthetic pesticides are restricted, is documented (Wang et al*.,* 2004; Albiter et al*.*, 2007). This work adds to the list of plants screened for antifungal activity significant to crop protection. The four plants` extract indicated inhibitory effects on *C. destrcutivum.* The extracts of *A. indica and C. citratus* however stimulated sporulation in the *C. destructivum* at some different levels of concentration. For example in the *in vitro* evaluation the water and oil extracts of *A. indica* stimulated up to 140 and 184% sporulation in *C. destructivum* respectively. This suggests the suitability of *A. indica* and *C. citratus* for substrate base/components in the culture of phytopathogens as *C. destructivum.*

The observed variation in the inhibitory effect of the test plants may be due to qualitative and quantitative differences in antifungal principles. For example the leaf extracts of *Ricinus cummunis* (Linn), *Tephrosia vogelii* (Hooks) and *Psidium guajava* (L) were reported in the work of Nduagu et al. (2008) to stimulate instead of inhibit the growth of *Colletotrichum capsici* (Synd.) Butler and Bisby, pathogen of Pepper anthracnose. However, the same *Ricinus cummunis* effectively inhibited growth of *C. destructivum* (Akinbode and Ikotun, 2008).

Anthracnose lesions were observed on both the leaves and stems of the inoculated cowpea crops. This tends to contradict the work of Frayssiinet (2008) who, though on different crop types, reported that lesions of *C. destructivum* were observed only on the leaves and petioles but non on the stems of Medicago sativa (Lucern) test plant.

It is possible that the antifungal property exhibited by the *C. citratus* in the study was due to the Citral content which corroborates the work of Palhano et al. (2004), who successfully inactivated spores of *C. gloeospoioides* using high hydrostatic pressure separate and combined with Citral essential oil.

The presence of Xylopic acid in *X. aethiopica* fruit is documented (Woode et al*.,* 2012). This diterpenes might be responsible for the antifungal property exhibited on the test pathogen in this work.

In the induction of plant defense responses by *O. gratissimum* leaf extracts, Colpas et al*.* (2009) reported the effective defense response in sorghum and soybean against the pathological activities of *Colletotrichum lagenarium* and attributed such feat to the several polar compounds and or essential oils, such as geraniol and nerol. These components could as well be responsible in the effect exhibited against *C. destructivum* in this evaluation.

The plant tissue extracts and the standard fungicide ex-

Table 1. Treatments effect (WE and OE)[1] *in vitro* on three pathological activities of *Colletotrichum destructivum.*

Treatment	Percentage inhibition[2]											
	Spore germination				Colony growth				Sporulation density			
	Water extract		Oil extract		Water extract		Oil extract		Water extract		Oil extract	
	A[3]	B[4]	C[3]	D[4]	E[3]	F[4]	G[3]	H[4]	I[3]	J[4]	K[3]	L[4]
Azadiractha indica	56.0	45.7	100	69	51.5	50.8	56.2	56.0	-26	-140	80	-184
Cymbopogon citratus	20.3	9.3	31.3	15	53.9	53.4	55.2	54.5	-86	-232	-72	-222
Ocimum gratissimum	39.3	19.7	48.7	41	51.7	49.4	52.4	50.0	64	12.0	76	18
Xylopia aethiopica	78.7	59	100	72	53.9	53.4	57.3	52.2	86	52	18	58

[1]Extracts were water (WE) or oil (OE) with data means of 5replicates. [2]Inhibition measured as a reduction on number of spore germination, extent of colony spread and quantity of inocula available. [3]Treatment at full extract concentration. [4]Treatments at diluted (50%) extracts concentration.

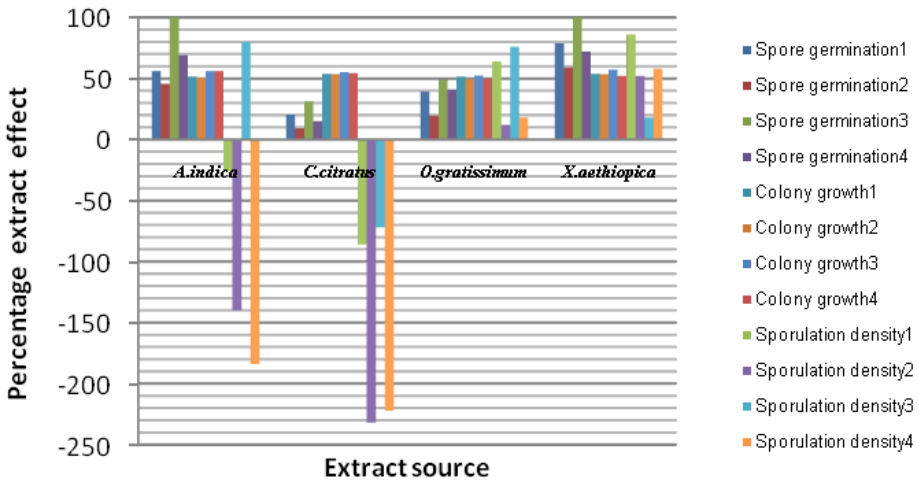

Figure 1. Comparative inhibitory effect of treatments, *in vivo*, on pathological activities of *C. destructivum.* 1 = Water extract at full concentration, 2 = water extract at diluted concentration, 3 = fat extract at full concentration, 4 = fat extract at diluted concentration.

Table 2. Effect of four botanical extracts and Benlate treatments on cowpea anthracnose disease development.

Treatment	Treatment form[2]	Percentage reduction in lesion spread[1]		
		2 days before inoculation (2dbi)	2 days after inoculation (2dai)	21 days after inoculation (21dai)
Azadiractha indica	Ahwe	29.6	23	13.3
	Aoe	37.8	30.4	20
Xylopia aethiopica	Xhwe	32.6	16.3	15.6
	Xoe	37.8	25.2	22.2
Ocimum gratissimum	Ohwe	8.9	70.4	8.9
	Ocwe	21.5	11.9	11.9
Cymbopogon citratus	Chwe	10.4	10.4	8.9
	Ccwe	25.9	11.9	8
Benlate	Bn	25.9	12.6	12.6

[1]Inhibition measured as a reduction in lesion spread as compared with those on control crops, means of 10 lesions of 5 replicates. [2]Extracts in the form of *Azadiractha* hot water (**Ahwe**) ,*Azadiractha* oil (**Aoe**), *Xylopia* hot water **(Xhwe**), *Xylopia* oil (**Xoe**), *Ocimum* hot water (**Ohwe**) *Ocimum* cold water (**Ocwe**), *Cymbopogon* hot water (**Chwe**) and *Cymbopogon* cold water (**Ccwe**) treatments at full concentration.

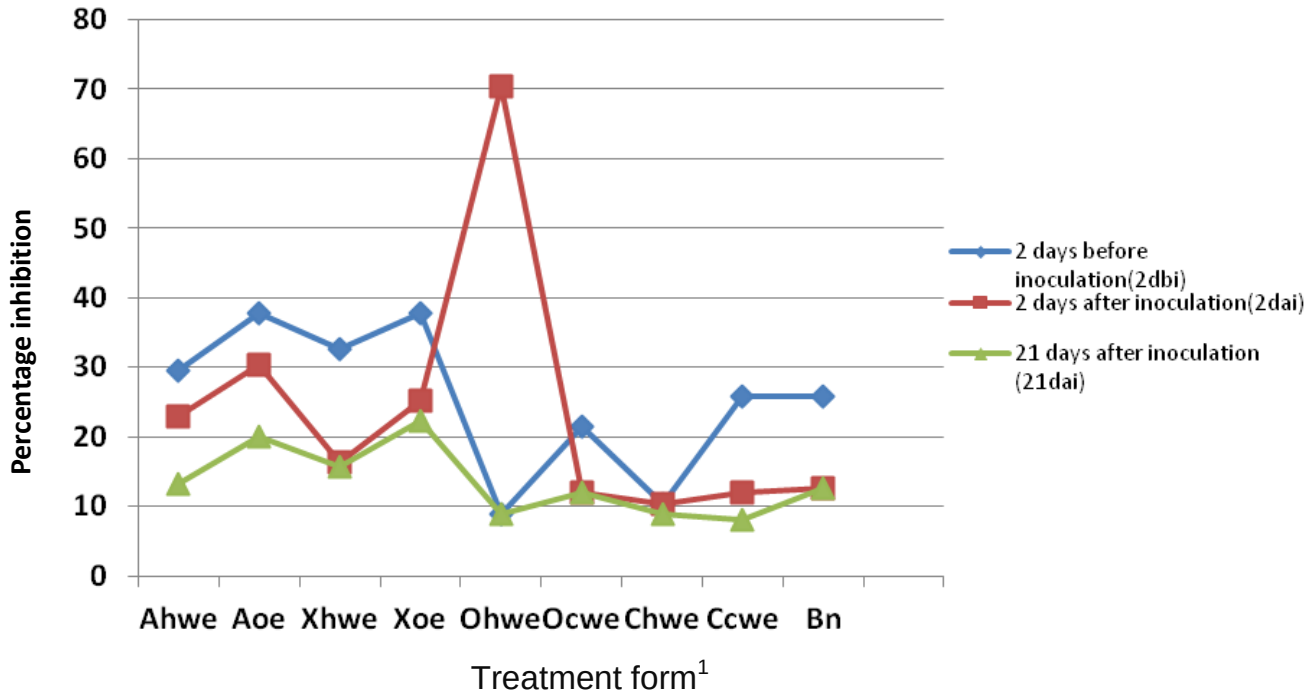

Figure 2. Comparative effect of treatment *in vivo* on disease spread based on periods of pathogen inoculation on *Vigna unguiculata.* [1]Ahwe = Azadiractha hot water extract; Aoe = Azadiractha oil extract; Xhwe = Xylopia hot water extract; Xoe = Xylopia oil extract; Ohwe = Ocimum hot water extract; Ocwe = Ocimum Cold water extract; Chwe = Cymbopogon hot water extract; Ccwe = Cymbopogon cold water extract; Bn = Benlate.

hibited more prophylactic than chemotherapeutic effects on cowpea anthracnose disease.

This tendency was further expressed in the physiological condition of the crop where plants treated 2 days before artificial inoculation looked healthier than the ones treated two days after pathogen inoculation which in turn were healthier than those treated after macroscopic symptom expression.

The systemic activity of these plant extracts corroborates the work of Ogwulumba et al. (2008) who achieved significant reduction in disease incidence of *Arachis hypogaea* L., in pre-germination treatment than in post-germination plant extract application.

The synthetic fungicides did not reduce the spread of the disease as effectively as the test plant extracts. The unimpressive control from the synthetic benlate fungicide could be due to tolerance of the pathogen to the standard formulation. For instance, in the survey of fungicide sensitivity in *Colletotrichum* spp., Sanders et al. (2000) reported the resistance of *C. gloeosporioides* to benlate.

Results from the present study, established the fact that the four botanicals: *A. indica, X. aethiopica, C. citratus, and O. gratissimum* possess antifungal substances significantly toxic to *C. destructivum* and a concentric comparism with the conventional fungicide (benlate); hence the potential of these botanicals as source of alternatives or complemental to synthetic fungicides in crop protection.

Non-standard abbreviations: HWE, Hot water extracts; **CWE,** cold water extracts; **LPCB,** lactophenol cotton blue; **2dai,** two days after crop inoculation; **2dbi,** two days before pathogen inoculation; **21dai**, twenty one days after crop inoculation; **Ahwe**, *Azadiractha* hot water extract; **Aoe**, *Azadiractha* oil extract; **Xhwe**, *Xylopia* hot water extract; **Xoe**, *Xylopia* oil extract; **Ohwe**, *Ocimum* hot water extract; **Ocwe**, *Ocimum* cold water extract; **Chwe**, *Cymbopogon* hot water extract; **Ccwe**, *Cymbopogon* cold water extract; **WE**, water extracts; **OE,** oil extracts; **Bn**, Benlate.

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