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Evaluation of *Allium sativum* (Linn) Crude Extracts and *Trichoderma asperellum* (Samuels. Lieckf) for Antifungal Properties against Cowpea Anthracnose Pathogen

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ABSTRACT

This study was carried out to evaluate the efficacy of Allium sativum (garlic) crude extract and Trichoderma asperellum against cowpea anthracnose pathogen (Colletotrichum lindemuthianum). Three concentrations of garlic crude extracts were evaluated in-vitro using the food poison method in a Potato Dextrose Agar growth medium, while three-time interval of T. asperellum inoculation was undertaken before the introduction of the cowpea pathogen and evaluated using the dual culture technique. The in-vivo experiment involved prophylactic foliar spray of the most promising concentrations of garlic crude extracts and conidia suspensions of Trichoderma from the in-vitro study on healthy cowpea plants. A total of seven treatments (35% and 45% garlic crude extracts, 10^3 conidia/ml at 48 and 72 hrs. inoculation time, 10^6 conidia/ml at 48 and 72 hrs. inoculation time and the control, pathogen only) were evaluated in 21 plastic pots. The experiment was laid out in a Completely Randomized Design. The result shows that garlic crude extract at 45% concentration gave 100% inhibition of mycelia growth of C. lindemuthianum in-vitro. The lowest values for disease incidence (4.17 at three weeks after treatment) and the highest value for pod yield (10.33 pods) were recorded for T. asperellum at 10^6 conidia/ml at 72 hrs inoculation time. Garlic extract at 45% concentration gave 9.33 pods. Garlic extract and T. asperellum compared favourably in protecting cowpea against the attack of C. lindemuthianum. Consequently, both Garlic crude extract and T. asperellum are recommended as control agents for anthracnose disease of cowpea.

Keywords: *Allium sativum*, *Trichoderma asperellum*, *Colletotrichum lindemuthianum*, Biological control, Pathogen.

INTRODUCTION

Opinion differs among scholars as regards the actual origin of cowpea. A school of thought is of the view that it originated from the Transvaal region of South Africa, while another believed it originated from West Africa. Cowpea is a warm season annual crop that may be erect, semi erect, prostate or climbing. Cowpea is well adapted to the dry savannahs of Africa because its cultivation requires minimal amount of water (Mortimore *et al.*, 1999). The crop thrives on well drained sandy loam soils with pH range of 5.5-6.5. It has been reported that close to 2.0 million tonnes is produced annually in Nigeria (Singh, *et al.*, 2002). Cowpea is an important source of protein for millions of Africans, especially the low income earners, who cannot afford other protein sources like milk, meat or fish (SADAFF, 2009). It is also important in the control of erosion and replenishing of soil nutrient through the addition of Nitrogen in form of Nitrate. This it does in association with certain Nitrogen fixing bacteria that lives symbiotically in its roots. Residue from cowpea plant is also important as fodder crop for livestock. (Enyiukwu and Awurum, 2013).

The cultivation of cowpea in Nigeria is faced with a number of challenges. Chief among these is the problem of pests and diseases. One of the most serious disease of cowpea is anthracnose caused by *Colletotrichum lindemuthianum.* It has been reported to cause yield loss as high as 70 -90% in some cases. (Emechebe and Lagoke, 2002, Barriuso-vagas, 2014; Enyiukwu *et al*., 2014). The disease manifest as tan to brown spots that appear sunken and form small circles on leaves, stems and branches. In severe cases the flowers are also not spared, while the pods are covered with black spots which contains spores of the pathogen (Adegbite and Amusan, 2008). The disease is usually seed borne, and the seeds therefore serves as reservoir of inoculum during the dry season.

Attempts at controlling the disease has been through the use of synthetic fungicides like Benomyl, Mancozeb and Carbendazin. Chemical disease control agents have associated problems, such as toxicity to both plants and animals, including humans, and are carcinogenic (Rueegg and Siegfried, 1996).

The development of resistance by the pathogen is also a reality and this often requires the development of supposedly more potent but ultimately more hazardous fungicides (Jansch *et al*., 2009). These problems have necessitated the search for environment friendly, save and economical alternatives. Plant extracts and biological control agents are two examples of these possible alternatives (Adebanjo and Bankole, 2004; Nduagu *et al*., 2008; Colpas *et al*., 2009). It is in the light of this that this study was designed to evaluate the efficacy of crude extract from *Allium sativum* (plant extract) and *Trichoderma asperellum* (a biological control agent) in the control of cowpea anthracnose disease caused by *Colletotrichum lindemuthianum.*

METHODOLOGY

Study area

The study was conducted in the screen house of the Department of Crop Soil and Pest management, the Federal University of Technology, Akure (FUTA) during the cowpea growing season.

Laboratory (in-vitro) experiment

(i). Preparation of Garlic extract: About 100 g of garlic bulb was crushed in a small mortar with a pestle. The paste was then soaked in 100 ml hot water (100^{o} C) and allowed to stand for 24 hrs. Thereafter, filtration was done with the aid of a sterile double layered muslin clothe. The filtrate was reconstituted to give 25%, 35% and 45% concentrations of garlic crude extract by adding sterile distilled water as appropriate. The different concentrations were used immediately.

(ii). Evaluation of garlic extract and *T. asperellum* against *C. lindemuthianum*: The food poison method was used to evaluate garlic extract. Exactly 2 ml each of the three different concentrations (25%, 35%, and 45%) of garlic crude extract were incorporated into 10 ml of Potato Dextrose Agar (PDA) in Petri-dishes. The PAD was prepared by dissolving 3.9 g powdered PDA in 100 ml distilled water in a conical flask. The mouth of the flask was plugged with non-absorbent cotton wool and sterilized in an autoclave at 121^{0} C, 15 mins. Garlic crude extract was incorporated into the PDA while it was still hot. The PDA/Garlic crude extract medium was then allowed to cool and gel. The control experiment had 2 ml distilled water incorporated into the PDA. Agar discs measuring 4 mm each were punched from a 7 days old culture of *C. lindemuthianum*, isolated from infected cowpea seeds, and placed upside down at the centre of each PDA/ Garlic extract medium. Each extract concentration was a treatment and each treatment was replicated thrice.

The evaluation of *T. asperellum* against *C. lindemuthianum* was done using the dual culture technique (Plate 1. Three inoculation time intervals of 24 hrs, 48 hrs and 72 hrs were evaluated. The control experiment had *C. lindemuthianum* only in a culture plate. *T. asperellum* was inoculated first in each treatment. Exactly 4 mm agar disc of *T. asperellum*, obtained from the International Institute of Tropical Agriculture (IITA) was placed at one end of a Petri-dish containing PDA, while the same size of agar disc from *C. lindemuthianum* was placed at the opposite end, such that the distance between the two discs was 8.4 cm. Fifteen Petri-dishes were used for each of garlic crude extract and *T. asperellum* treatment methods, making a total of 30 Petri-dishes for both study. The two disease control agents were evaluated independently and each experiment was laid out in a Completely Randomized Design (CRD) and incubation was done at 27^{0} C ± 2^{0} C for 7 days.

Screen house study

Collection and sterilization of soil

Sandy loam soil rich in humus was collected from the Teaching and Research farm of FUTA. The soil was sterilised using the steam heat method. Thereafter, about 4 kg of soil was dispensed into 5 litres plastic buckets after cooling

Evaluation of disease control agents: Two levels of garlic crude extract that gave the best result in the laboratory study, namely 35% and 45% as well as two concentrations of *T. asperellum* (10^{3} conidia/ml and 10^{6} conidia /ml) at 48 hrs and 72 hrs inoculation interval before the introduction of *C. lindemuthianum* were evaluated. The control experiment had *C. lindemuthianum* inoculated into healthy cowpea seedlings with no disease control agent applied. Thus a total of seven treatments were evaluated. The treatments are as follows:

i. Control experiment (no disease control agent applied after inoculation with *C. lindemuthianum*)
ii. 35% Garlic crude extract (G 35%) only
iii. 45% Garlic crude extract (G 45%) only
iv. *T. asperellum* at 10^{3} conidia/ml at 48 hrs interval before the inoculation of the pathogen (TA)
v. *T. asperellum* at 10^{3} conidia/ml at 72 hrs interval before inoculation of the pathogen (TB)
vi. *T. asperellum* at 10^{6} conidia/ml at 48 hrs interval before inoculation of the pathogen (TC)
vii. *T. asperellum* at 10^{6} conidia/ml at 72 hrs interval before inoculation of the pathogen (TD)

Each treatment was replicated thrice, giving a total of 21 pots for this phase of the experiment. The experiment was laid out in Completely Randomized Design (CRD). Exactly 20 ml garlic extracts was sprayed prophylactically by foliar application on healthy cowpea plants per pot for 24 hrs before inoculation with 10^7 spores /ml suspension of the *C. lindemuthianum.* The two levels of *T. asperellum* were also applied prophylactically on healthy cowpea plants at 48 hrs and 72 hrs time interval before infection with spore suspension of the pathogen. Spore suspensions were applied as foliar spray till run off and each cowpea plant was covered with a transparent polythene bag for 24 hrs to create sufficient warmth and moisture required for spore germination and penetration into the tissue of the host crops.

Data Collection and Statistical Analysis.

In the in-vivo study, data collected were converted to percentage, such that the values for percentage inhibition of mycelia growth by each treatment was obtained using equation 1. Count data were square root transformed while data in percentages were arc sin transformed before subjection to statistical analysis using Minitab version 17 software package. Means were separated using Tukey test.

$$\frac{Mycelia\ growth\ control - Mycelia\ growth\ treatment}{Mycecelia\ control} \ x\ 100 \quad equation \quad 1$$

Data were collected weekly (for 5 weeks) in the *in-vivo* study on the following parameters:

i. Number of leaves
ii. Number of pods produced
iii. Plant height
iv. Number of filled and unfilled pods
v Disease incidence was computed using equation 2

$$\frac{Number\ of\ infected\ leaves}{Total\ nuber\ of\ leaves} \ x \quad 100 \qquad equation \quad 2$$

Disease severity: Disease severity was determined with a visual rating scale of 0-5 as follows;

0 = no symptoms on leaves
1 = 1-10% of leaf infected with the pathogen
2 = 11-25% of leaf infected with the pathogen
3 = 26-50% of leaf infected with the pathogen
4 = 51- 75% of leaf infected with the pathogen
5 = 76% and above infected with the pathogen

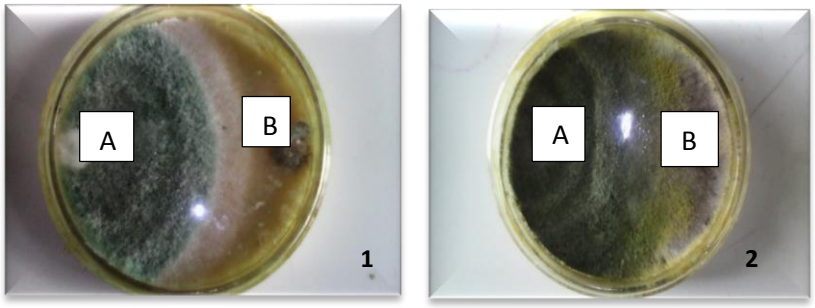

Plate 1: Dual culture (72 hrs interval) of *T. asperellum* (A) and *C. lindemuthianum* (B) at inoculation of *C. lindemuthianum* (1) and 8 days old culture (2).

RESULTS

Inhibition of mycelia growth (%)

The best value of 100.00% inhibition of mycelia growth was recorded for G 45. This was however not significantly different at (0 .05% significant level) from 96.31% which was obtained for G 35 (Table 1). At 72 hrs inoculation time interval, *Trichoderma asperellum* inhibited *C. lindemuthianum* mycelia growth by 94.06%. This was significantly higher than the value recorded for 24 hrs time interval which was 66.58% (Table 2.)

Table 1: Inhibition of mycelia growth of C. *lindemuthianum* by garlic crude extract.

Concentration (%)	IMG
25	35.66b
35	96.31a
45	100.00a

Mean values in the same column followed by different letters are significantly different ($p<0.05$)

Table 2: Inhibition of mycelia growth of *C lindemuthianum* by *T. asperellum*

Time (hrs)	IMG
24	66.58c
48	71.92b
72	94.06a

Mean values in the same column followed by different letters are significantly different ($p<0.05$)

Leaf production

The effect of treatments on leaf production is presented on Table 3. The highest number of leaves was 19.83 and 19.67 for G 45% and TD at the fourth week after application respectively. These were however not significantly different from the other. Also, the values obtained for the 2nd and 3rd weeks after treatment were not significantly different from one another.

Table 3: Effect of disease control agents on leaf production

Treatment	2WAT	3WAT	4WAT
Control	11.33a	13.00a	16.17a
G 35%	11.17a	13.63a	18.67a
G 45%	14.67a	16.50a	19.83a
TA	9.33b	11.33a	15.18a
TB	11.00a	13.27a	18.17a
TC	12.17a	15.83a	18.60a
TD	14.00a	15.33a	19. 67a

WAT = Week After Treatment. Mean values in the same column followed by different letters are significantly different ($p<0.05$)

Incidence (%)

Disease incidence was highest in the control at 2 and 3 weeks after treatment with the values 20.88% and 13.62% respectively (Table 4). TD had the lowest values of 5.90% and 4.17% at 2 and 3 weeks after treatment, respectively. The values recorded for the control and TD differed significantly at 0.05% significance level (Table 4).

Table 4: effect of disease control agents on *C. lindemuthianum* disease incidence of cowpea (%)

Treatment	2WAT	3WAT
Control	20.88a	13.62a
G 35%	12.50bc	10.23ab
G 45%	10.08bc	5.98ab
TA	17.36ab	11.21ab
TB	17.40ab	8.78ab
TC	12.27b	5.59ab
TD	5.90c	4.17b

Mean values in the same column followed by different letters are significantly different ($p<0.05$)

Disease severity

Infection was most severe in the control treatment at both 2 and 3 weeks after treatment. The lowest severity values of 1.67 and 1.33 were recorded for TD at 2 and 3 weeks after treatment respectively. Disease severity was significantly higher (at 0.05% significance level) in the control than all the other treatments, except TA at 2 weeks after treatment (Table 5).

Table 5: Effect of disease control agents on *C lindemuthianum* disease severity on cowpea (%)

Treatment	2WAT	3WAT
Control	3.83a	3.83a
G 35%	1.83b	2.33ab
G 45%	1.67b	1.33b
TA	3.33a	2.67ab
TB	2.83ab	2.17ab
TC	2.33ab	1.67b
TD	2.33ab	1.67b

Mean values in the same column followed by different letters are significantly different ($p<0.05$)

Plant height

Significantly highest value for plant height was recorded for TD at 3 weeks after treatment, while the lowest value of 9.85 was recorded for the control. At 4 weeks after treatment, TD gave 15.27 value for plant height. This value differed significantly from all the others (Table 6).

Table 6: Effect of treatments on plant height (cm)

Treatment	2WAT	3WAT	4WAT
Control	26.60a	29.57d	33.77c
G 35%	28.73a	33.33bcd	36.77bc
G 45%	30.93a	37.73ab	39.20b
TA	28.47a	31.47cd	37.70bc
TB	25.53a	29.60d	34.50bc
TC	29.93a	35.03bc	37.99bc
TD	32.70a	41.33a	45.83a

Mean values in the same column followed by different letters are significantly different ($p<0.05$)

Yield

Table 7 shows the effect of the various treatments on cowpea yield and yield quality (filled and unfilled pods). The highest number of pods, 10.33, was recorded for cowpea plants under TD, treatment while the lowest value, 4.67 was recorded for the control treatment. The number of filled pods for cowpea plants under TD treatment were significantly ($p\leq0.05\%$) higher than those obtained for plants under all the other treatments (Table 7).

Table 7: Effects of treatments on yield and yield quality

Treatment	No. of Pods	Filed Pods	Unfilled pods
Control	4.67c	3.00c	1.67a
G 35%	5.33b	4.00c	1.33a
G 45%	9.33ab	8.33ab	1.00a
TA	5.33b	4.33c	1.00a
TB	6.00ab	4.33c	1.67a
TC	8.00ab	6.33ab	1.67a
TD	10.33a	9.00a	1.33a

Mean values in the same column followed by different letters are significantly different ($p<0.05$)

DISCUSSION

Results from the *in-vitro* study showed that both garlic extract at 45% concentration (w/v) and TD were the most effective in the inhibition of mycelia growth of *C. lindemuthianum*. *T. asperellum* grew towards and overlapped the mycelium of *C. lindemuthianum*. The longer the period of pre-inoculation of *T. asperellum*, the greater the degree of inhibition of *C. lindemuthianum* mycelia growth. The control plate (consisting of *C. lindemuthianum* only) grew rapidly and sporulated massively, filling the Petri-dish in just five days after inoculation. Talukder, (2007) had reported a similar finding on the effect of biological control agent on pineapple disease of sugarcane.

Garlic at 45% gave 100% inhibition on the mycelial growth of *C. lindemuthianum in-vitro*. This is due to a number of bio-active compounds it contains. The concentration of these compounds increases with the increasing concentration of the crude extract of garlic, thus accounting for the 100 % inhibition exhibited by G 45%. These compounds are collectively referred to as allicin with antifungal, antibacterial and antiviral properties (Lawal *et al.,* 2010, Davies *et al.,* 2012). The conversion of alliin present in garlic to allicin by an enzyme called allinase is responsible for the characteristic aroma associated with garlic. This conversion occurs whenever garlic is chopped or cut. The efficacy of these compounds *in-vitro* has been confirmed by previous workers (Akinmusire *et al.,* 2014, Alan and Anant, 2008). This study has gone further to show that improvement in the growth and yield of cowpea can be achieved through foliar application of garlic extract as disease control agent. Worthy of note is also the fact that the effectiveness of garlic as disease control agent is concentration dependant. At very low concentration, the level of efficacy was low. The mode of action of garlic on fungal pathogens is through interference with the amino acid constituent of the cell, alteration of the cell wall through the oxidation of certain sulphur compounds within it and production of lipid hydro-peroxide in the plasma membrane which brings about increased permeability and eventual death of such cells (Horev-Azaria, *et al.,* 2009 and Gruhlke *et al.,* 2010). At harvest, TD gave the best value in terms of pod yield with 10.33 pods. The effectiveness of *Trichoderma* spp.as biocontrol agents' *in-vitro* have been reported by previous workers with varying degree of successes. Elad, *et al.* (1979) reported on the inhibitory activities of *T. harzianum* on *Sclerotium rolfsii*. Talukder, *et al.* (2007) found that *T. hazianum* exhibited strong antagonism against *Ceratocystis paradoxa*, the sugarcane sett rot pathogen, while Idowu, *et al.* (2016) reported on the significant effect *T. asperellum* had on disease incidence and severity of *Pythium aphanidermatum* of okra.

The concentration of conidia of *T. asperellum* and the duration of prophylactic application were two very important factors in the efficacy of *T. asperellum* as a disease control agent based on the results of this study. This is probably due to the fact that a higher concentration gives a higher quantity of inoculum required to initiate the colonization process, while the time duration gives room for establishment of the biological control agent within the tissue of the host plant (Sharma, 2012). *Trichoderma* spp. occurs in large number in soils, rotten log and in the tissues of some living plants like yam, jatropha and Azadirachtha. In such plants, they act as endophytes, protecting them from the attack of pathogen (Howell, 2002). They colonize root surfaces, bringing about increased surface area, enhancing the absorption of nutrients and consequently bringing about an improvement in the number of foliage, increased plant height and a general improvement in plant growth. They also attack plant parasitic organisms, using some as food, especially some fungal pathogen (Sharma, 2012). Trichoderma species have also been found to inhabit the cortex of some plants. Their mode of action is through antibiosis, mycoparasitism and inactivation of the enzymes of pathogens (Idowu *et al.,* 2016).

CONCLUSION AND RECOMMENDATION

Garlic extract and *Trichoderma asperellum* both showed promise in the control of *C. lindemuthianum* of cowpea *in-vitro* and *in-vivo*. Cowpea plants treated with TD gave the best yield value. This value was however not significantly different from G 45 %. Similar non-significant differences of the two treatments were also observed in most of the growth parameters evaluated. It is therefore suggested that both methods of disease control can be adopted by cowpea farmers in the control of anthracnose disease. Further study can be carried on the two disease control agents with the aim of producing handy and ready to use packs that will be made readily available to the local farmers. Extension agents can step in and with demonstration farms to further convince the farmers about the efficacy of the two disease control agents.

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