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Full Length Research

Timing and sequence of application of inoculum and plant extracts on disease incidence, severity and yield of three cowpea cultivars inoculated with *Colletotrichum lindemuthianum*.

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Field experiment was conducted to determine the effect of time and sequence of application of infective conidia and plant extracts on disease incidence, severity and yield of three cowpea varieties, namely; Ife brown, Ife bimpe and Tvx3236. The experiment was laid in a split plot design, cowpea plants in the first sub-plots were sprayed with the different concentrations of extracts and inoculated after forty eight hours. The second sub-plots were inoculated followed by application of extracts after forty eight hours while cowpea plants in the third sub-plots were inoculated followed by simultaneous application of extracts. Results from the study shows that disease incidence was significantly lower when plants extracts were applied two days before inoculation compared to application of the extract two days after inoculation or concurrent application of extract to the cowpea plants followed by inoculation. Similarly, disease incidence was significantly lower (20.4%) where extracts of *D. stramonium* were applied two days before inoculation. On the other hand, disease incidence was significantly higher (26.7%) when treatment with plant extracts preceded application of inoculum concurrently. Variations were also observed among the varieties of cowpea used in this study. There was no significant difference in disease incidence (28.1%) when *J. gossypifolia* extracts were applied two days after inoculation compared to when it was inoculated concurrently (30.4%) with extracts. Similarly, severity of the disease was significantly lower at higher concentrations of the extracts used in this study with corresponding increase in the percentage of normal seeds.

Keywords: *Colletotrichum lindemuthianum*, disease incidence, disease Severity, Cowpea varieties.

INTRODUCTION

Cowpea (*Vigna unguiculata* L.Walp),a member of the family Fabaceae (Leguminosae), is widely grown in the tropical and subtropical regions where it provides a major source of dietary proteins for man and livestock (Ademe *et al.,* 2013). Over 600 million people in Africa and Asia depend on it for food security, poverty reduction and income generation (FAOSTAT, 2012). The estimated world cowpea production in 2000 was 3.32 million metric tons (MT) with Africa accounting for 75% of the output (FAOSTAT, 2013). West Africa and the sub- humid to semi-arid zones, especially Nigeria, Niger, Senegal, Ghana, Mali and Burkina-Faso are the key cowpea producing areas. (FAOSTAT 2000).

The annual output in Nigeria of 2.1 million MT fall short of domestic demand by about 0.52 million MT that is partly met by importation from neighboring countries, mainly Niger and Burkina-Faso. This deficit is attributed to myriads of production constraints such as drought, low yield of local unselected cultivars, lack of good planting materials and pests and diseases (Abubakar and Olukosi, 2008).

Cowpea anthracnose is caused by the fungus, *Colletotrichum lindemuthianum.* It is the most important fungal disease of field grown cowpea capable of 75% yield reduction in Nigeria (Enyiukwu *et al.,* 2014). The disease affects all stages of the plant but more often in

the reproductive stages. Its symptoms include round brownish or purple specks which become darker and enlarge into lesions (about 2 cm in diameter). Individual lesions are usually lenticular to circular, tan to brown coloration and the size and distribution depend on the degree of severity (Sharon and Douglas, 2011). The symptoms are most visible on leaves and ripe fruits but the disease also produces cankers on petioles and stems thereby causing defoliation and rottening of fruits. Infected fruits have water-soaked and sunken circular spots (Davis *et al*., 1991). Severity of infection is affected by water availability to the plant (Dodd *et al*., 1991).

MATERIALS AND METHODS

Location of experiments

The field experiments were conducted at Ekiti State University Teaching and Research farm, Ado Ekiti.7.7129° N, 5.2523° E, Nigeria.

Collection and preparation of plant leaves

Leaves of the three plants namely; *D. stramonium* , *J. gossypifolia* and *R. communis* were collected at Ekiti State University. (Latitude 7 7212°N and longitude 5.2575°E) in south western Nigeria and air-dried at 28°C for 6-8 weeks until each plant had a constant weight. The dried leaves were milled using a blender (Okapi®, Mixer-Grinder), packaged into sealable nylon and refrigerated at 4°C for about 2 weeks until they were required for bioassay.

Preparation of plant extracts

Extracts were prepared by mixing equivalent grams of prepared plant powder (65, 50 and 30) with 100 ml of distilled water at 70°C in 500 ml bottles and kept in hot water bath-shaker for 30 minutes. Thereafter, the liquid extract was separated by vacuum filtration and poured inside standard bottles which were refrigerated at 4°C. These extracts were used as the stock solution from which 65%, 50% and 30% of each extract were prepared.

Isolation and identification of *C. lindemuthianum*

Cowpea plants showing distinct symptoms of the pathogen were collected from the cowpea fields at Ekiti State University. The leaves were cut into small pieces of about 1-2 cm^2 and surface sterilized by immersion in 0.2 % NaOCl for two minutes and followed by two rinses in sterile distilled water in a laminar flow cabinet. Three leaf cuttings per plate were placed on PDA. The plates were sealed with parafilm and incubated at 28°C for 5-6 days. Single spore of developing colonies was isolated and sub-cultured to obtain pure cultures. Samples from single spore cultures were used for identification on Malt Extract Agar (MEA) at x400 magnification of a compound microscope (OLYMPUS Binocular) (Zivkovic *et al*, 2010).

Effect of crude extracts on cowpea anthracnose disease

Three varieties of cowpea susceptible to anthracnose namely: Ife brown, Ife Bimpe and TVx3236 were used in the study. Three plant extracts (a) *D. Stramonium* (b) *J. Gossypifolia* and (c) *R. Communis* were used as foliar sprays at three different concentrations (30%, 50% and 65%). The experiment was a factorial experiment in a split plot design in which variety represented the main plot, concentrations were the subplots and the botanicals represented the sub-sub plot factor.

The plot size was 2 x 2 m^2 and separated by a boarder row of 1 m for a total farm area of 730 m^2. Three seeds of each variety of cowpea susceptible to anthracnose were sown in the first week of September at spacing of 60 cm x 30 cm. Seedlings were thinned to two per stand after one week. The planted seeds were previously sterilized by rinsing in 0.5% sodium hypochlorite solution for 30 seconds and rinsed in sterile distilled water three times before sowing. The total farm area was divided into three sub-plots and the cowpea was sprayed with either the *C lindemuthianum* ccurred suspension (10^4 conidia ml^{-1}) or the plant extracts were sprayed followed by ccurred .

The cowpea in the first sub-plot were sprayed first with the spore suspension and thereafter with different concentrations of the plant extracts at approximately 48 hours. The second sub-plot was sprayed with the different concentrations of plant extracts followed by *C. lindemuthianum* spore suspensions after 48 hours, while the third sub-plot was sprayed with the ccurred suspension and followed by immediate application of the plant extracts. Hand operated Knapsack sprayer (Presto) was used for the application of the ccurred and plant extracts.

Percentage disease incidence was determined using the formula:

$$\% \text{ disease incidence} = \frac{Number\ of\ plants\ infected\ X\ 100}{Total\ number\ of\ plants}$$

Severity of disease was recorded on a scale of 0 – 6 as shown in Table 1.

Table 1: Disease severity index

Scale	Rating	Symptoms of *Anthracnose* on cowpea
0	No disease	No trace of infection
1	Hypersensitivity	Hypersensitive spot on lower leaves only
2	Trace Infection	Small lesions on lower and upper leaves and stem
3	Slight Infection	Small lesions on lower and upper leaves and stem
4	Moderate infection	Advanced lesions on upper and lower leaves, with or without new infections on stems and petiole
5	Severe infection	Advanced lesions on upper and lower leaves, flowers, buds, stems and petiole and slight infection of pod.
6	Very severe infection	All features of five above with severe infection of pod

Source: Enikuomehin & Peters (2002)

RESULTS

Effect of time and sequence of application of ccurred and plant extracts on disease incidence in three cowpea cultivars.

Table 2: Effect of time and sequence of application of ccurred and plant extracts on incidence of cowpea anthracnose**.**

Leaf extract	2DB1	2DAI	CAEI
	Percentage Incidence cowpea anthracnose.		
Tvx 3236			
D. stramonium	20.4^{c}	24.3^{d}	26.7^{d}
R. communis	23.1^{c}	27.2^{c}	28.4^{b}
J. gossypifolia	24.8^{c}	28.1^{b}	30.4^{b}
Ife Brown			
D. stramonium	22.3^{d}	25.8^{d}	27.7^{c}
R. communis	23.9^{c}	27.4^{c}	28.6^{c}
J. gossypifolia	25.2^{b}	29.4^{b}	31.1^{b}
Ife Bimpe			
D. stramonium	23.4^{c}	26.1^{c}	28.2^{c}
R. communis	24.3^{b}	28.4^{b}	31.9^{b}
J. gossypifolia	24.0^{b}	29.1b	32.8^{b}
Control (water)	63.5^{a}	64.7^{a}	64.1^{a}

DBI, Days before Inoculation, DAI, Days after Inoculation CAEI, Concurrent Application of Extract and Inoculum
Values in the same column followed by similar letters are not significantly (P<0.05) (Tukey'

Table 2 shows the effect of time lag and sequence of application of plant extracts and infective Conidia of *C. lindemuthianum* on disease incidence where extracts were applied two days before inoculation of the plants with the conidia, disease incidence was significantly lower compared to application of the extract two days after inoculation or concurrent application of extract to the cowpea plants followed by inoculation. Similarly, disease incidence was significantly lower (20.4%) where extracts of *D. stramonium* were applied two days before inoculation. Disease incidence was significantly higher (26.7%) when treatment with plant extracts preceded application of ccurred concurrently. Variations were also observed among the varieties of cowpea used. There was no significant difference in disease incidence (28.1%) when *J. gossypifolia* extracts were applied two days after inoculation compared to when it was concurrently inoculated (30.4%). With extracts Similar results were observed with extracts of *R. communis* in this experiment.

Effect of *D. stramonium, R. communis* and *J. gossypifolia* leaf extracts on anthracnose disease severity on three varieties of cowpea

Table 3 shows the effect of foliar spray of three plant extracts; *D. stramonium, R. communis* and *J. gossypifolia* on severity of anthracnose disease. All the extracts at the three concentrations significantly (P≤0.05)

Table 3: Effect of *D*. s*tramonium, R. communis* and *J. gossypifolia* leaf extracts on anthracnose disease severity on three varieties of cowpea

Leaf extract	Conc.	Severity score Tvx 3236 2012 Season	2013 Season	Pooled Mean	Symptom Rating
D. stramonium	65	2	2	2	Trace infection
	50	3	3	3	Slight infection
	30	4	4	4	Moderate infection
R. communis	65	2	2	2	Trace infection
	50	3	3	3	Slight infection
	30	4	4	4	Moderate infection
J. gossypifolia	65	3	3	3	Slight infection
	50	4	4	4	Moderate infection
	30	4	4	4	Moderate infection
Control		6	6	6	Very severe infection
Ife Brown					
D. stramonium	65	3	3	3	Slight infection
	50	3	3	3	Slight infection
	30	4	4	4	Moderate infection
R. communis	65	3	3	3	Slight infection
	50	3	3	3	Slight infection
	30	4	4	4	Moderate infection
J. gossypifolia	65	3	3	3	Slight infection
	50	4	4	4	Moderate infection
	30	5	5	5	Severe infection
Control		6	6	6	Very severe infection
Ife Bimpe					
D. stramonium	65	3	3	3	Slight infection
	50	3	3	3	Slight infection
	30	4	4	4	Moderate infection
R. communis	65	3	3	3	Slight infection
	50	3	3	3	Slight infection
	30	4	4	4	Moderate infection
J. gossypifolia	65	3	3	3	Slight infection
	50	4	4	4	Moderate infection
	30	4	4	4	Moderate infection
Control		6	6	6	Very severe infection

Severity score was obtained at 8 WAP

reduced severity of the disease. Where relatively high concentrations of the extracts were applied (65%), trace infections of the symptoms were observed, compared to low concentration treatments where a moderate infection ccurred. Variations were also observed among the three cowpea varieties, such that at 65% concentration of *D. stramonium*, symptoms of anthracnose noticeable on Ife Bimpe and Ife Brown were trace infections compared to that of Tvx3236 that had slight infections.

Effect of *D. stramonium, R. communis* and *J. gossypifolia* leaf extracts on percentage of normal and abnormal seeds in three varieties of cowpea

The percentage incidence of normal and abnormal seeds of cowpea varieties which were treated with three plant extracts at three different concentrations (65, 50, and 30%) is shown in Table 4. Percentage of normal seeds (Tvx3236) in 2012 season at 65, 50, and 30% concentrations of *D. stramonium* were (86.5 ,82.3 and 76.5%) respectively. In 2013, the percentage of normal seeds also varied with concentration of the extracts. For example, the percentages of normal seed on Tvx3236 that was treated with *D. stramonium* at 65, 50 and 30% concentrations were 83.2, 78.9 and 74.2% respectively. The percentage of normal seeds were higher in 2012 than 2013 planting seasons in the three cowpea varieties. The treated plants at the two seasons produced significantly ($P \leq 0.05$) higher percentage of normal seeds, compare with the control. Generally, lower concentrations of the extracts gave higher abnormal seeds of the three varieties of cowpea used in the study.

Table 4: Effect of *D. stramonium, R. communis* and *J. gossypifolia* leaf extracts on percentage of normal and abnormal seeds in three varieties of cowpea

Incidence (%)							
		2012 Season		2013 season		Pooled Mean	
Leaf extract	Conc.	Normal seed	Abnormal Seed	Normal seed	Abnormal Seed	Normal seed	Abnormal seed
Tvx 3236							
D. stramonium	65	86.50[a]	13.50[c]	83.20[a]	16.80[c]	84.85[a]	15.15[c]
	50	82.30[a]	17.70[c]	78.90[a]	21.10[c]	80.60[a]	19.40[c]
	30	76.50[b]	23.50[b]	74.20[b]	25.80[b]	75.35[b]	24.65[b]
R. communis	65	83.80[a]	16.20[c]	81.30[a]	18.70[c]	82.55[a]	17.45[c]
	50	80.45[a]	19.55[c]	78.55[a]	21.45[c]	79.50[a]	20.50[c]
	30	75.77[b]	24.23[b]	74.10[b]	25.90[b]	74.94[b]	25.06[b]
J. gossypifolia	65	84.50[a]	15.50[c]	82.10[a]	17.90[c]	83.30[a]	16.70[c]
	50	79.40[a]	20.60[c]	77.45[a]	22.55[c]	78.43[a]	21.57[c]
	30	76.50[b]	23.50[b]	73.98[b]	26.02[b]	75.24[b]	24.76[b]
Control		52.90[c]	47.10[a]	54.20[c]	45.80[a]	53.55[c]	46.45[a]
Ife Brown							
D. stramonium	65	84.20[a]	15.80[c]	82.70[a]	17.30[c]	83.45[a]	16.55[c]
	50	80.10[a]	19.90[c]	79.10[a]	20.90[c]	79.60[a]	20.40[c]
	30	77.30[b]	22.70[b]	76.45[b]	23.55[b]	76.88[b]	23.12[b]
R. communis	65	81.40[a]	18.60[c]	79.30[a]	20.75[c]	80.35[a]	19.65[c]
	50	79.32[a]	20.68[c]	77.25[a]	22.75[c]	72.28[a]	21.72[c]
	30	73.50[b]	26.50[b]	71.48[b]	28.52[b]	72.49[b]	27.51[b]
J. gossypifolia	65	81.20[a]	18.80[c]	79.80[a]	20.20[c]	80.50[a]	19.50[c]
	50	78.50[a]	21.50[c]	76.18[a]	23.82[c]	77.34[a]	22.66[c]
	30	73.40[b]	26.60[b]	72.10[b]	27.90[b]	72.75[b]	27.25[b]
Control		53.20[c]	46.80[a]	56.58[c]	43.42[a]	54.89[c]	45.11[a]
Ife Bimpe							
D. stramonium	65	85.33[a]	14.67[c]	81.60[a]	18.40[c]	83.47[a]	19.53[c]
	50	82.49[a]	17.51[c]	80.40[a]	19.60[c]	81.45[a]	18.55[c]
	30	79.87[b]	20.13[b]	77.50[b]	22.50[b]	78.69[b]	21.31[b]
R. communis	65	83.76[a]	16.84[c]	82.17[a]	17.83[c]	82.67[a]	17.33[c]
	50	81.38[a]	18.62[c]	79.68[a]	20.32[c]	80.53[a]	19.47[c]
	30	78.40[b]	21.60[b]	76.75[b]	23.25[b]	77.58[b]	22.42[b]
J. gossypifolia	65	84.37[a]	15.63[c]	82.18[a]	17.82[c]	83.28[a]	16.72[c]
	50	81.50[a]	18.50[c]	78.76[a]	21.24[c]	80.13[a]	19.87[c]
	30	80.77[b]	19.23[b]	77.29[b]	22.71[b]	79.03[b]	20.97[b]
Control		52.37[c]	47.63[a]	51.67[c]	48.33[a]	52.02[c]	47.98[a]

Means with the same letter in each column are not significantly different (Tukeys HDS)

Effects of plant extracts and concentrations on seed weight of cowpea

The effect of the three plant extracts on seed weight of three varieties of cowpea is shown in Table 5. All the extracts at the three concentrations (65, 50 and 30%) significantly $P \leq 0.05$ increased seed weight of cowpea compared to the control. The trend showed that higher concentrations of the extracts caused significant yield increase. At 65%, concentration of *D. stramonium* cowpea seed yield of variety Tvx 3236 (539 kg/ha) was significantly ($P \leq 0.05$) higher than that of Ife Brown (502 kg/ha) and Ife Bimpe (452 kg/ha).

At 50% concentration of same extracts the yield of Ife Bimpe (391 kg/ha) was significantly higher than Ife Brown (353 kg/ha). The same trend was observed at 30% concentrations in all the treated plots. Overall, Tvx 3236 gave the best yield. Yields of cowpea from plots treated with *D. stramonium* were higher and this was closely followed by yield from the plots sprayed *R. communis* and *J. gossypifolia* respectively.

Table 5: Effect of plant extracts on seed weight of three cowpea varieties

Extracts Conc. (%)	Cowpea variety	Seed weight of cowpea (kg ha^{-1})		
		D. Stramonium	*R. communis*	*J. gossypifolia*
65	Ife Brown	502.33^{a}	439.33^{a}	430.33^{b}
	Ife Bimpe	451.67^{a}	424.67^{a}	402.33^{b}
	TVX3236	539.00^{a}	510.00^{a}	511.00^{a}
50	Ife Brown	353.00^{b}	319.33^{b}	355.33^{c}
	Ife Bimpe	391.00^{b}	311.33^{b}	327.67^{c}
	TVX3236	495.00^{a}	479.33^{a}	494.00^{a}
30	Ife Brown	176.33^{c}	143.00^{c}	184.00d
	Ife Bimpe	207.00^{c}	179.00^{c}	149.67^{d}
	TVX3236	288.33^{c}	285.00^{c}	272.33^{d}
0	Ife Brown	95.67^{d}	95.67^{d}	95.67^{e}
	Ife Bimpe	88.33^{d}	88.33^{d}	88.33^{e}
	TVX3236	93.67^{d}	93.67^{d}	93.67^{e}

Means with the same letter in each column are not significantly different (P<0.05) (Tukey's HSD)
Values are averages obtained for 2012 and 2013 planting seasons.

DISCUSSION

In this study, hot water extracts of the three plants: *D. stramonium, R. communis, and J. gossypifolia* were tested against *C*. *lindemuthianum* using field bio-efficacy trials. The leaves were air dried and powdered to increase the surface area between samples and extraction solvents. It has been reported that air dried plant materials are less fragile and do not tend to deteriorate an advantage which it has over fresh leaf samples (Azwanida, 2015). Bioactive constituents are endogenously synthesized by plants and they are present at varied concentrations in the tissues of different plant species (Eno, 2011: Gideon and Anita, 2013) as natural protectants against diseases (Ghost *et al.,* 2002). The use of hot water is considered one of the best methods of extraction of bioactive components in plants and it is capable of preserving the chemistry of constituents. This method was employed because it is relatively simple to use compared to other methods of extraction, it is less costly and can be recommended for small and medium enterprises (Vongsak *et al*., 2013). Variations in concentrations of bioactive components in plant extracts are known to be influenced by the method of extraction, age of plants at harvest, time of harvesting and mode of exposure of plant to air (Enyiukwu and Awurum, 2013).

In this study field trials were conducted to evaluate disease incidence where some of the plant extracts were applied before inoculation, shortly after inoculation and inoculation of the plant followed by application of the extracts. Disease incidence was significantly lower ($P \leq 0.05$), when extracts were applied two days before inoculation of the cowpea. The trend common to all the extracts at the three concentrations when compared to application of extracts two days after inoculation or inoculation followed by application of extract within the shortest possible time of about 20-30 minutes. The results of the field trial suggested that the extracts were more effective when applied as a preventive measure rather that for curative means. Amadioha (1999) reported the control of rice blast caused by the pathogen *Pyricularia oryzae invitro* and *invivo* with extracts of *A. indica*. The study also showed that the incidence and severity of infection were lower with extracts applied two days before inoculation.

In this study, incidence of the disease was concentration dependent where higher concentrations of the extracts were used (65%) disease incidence was lower. Severity of infection in extract sprayed plots was characterized by the presence of small lesions only on lower leaves of the cowpea plants why the control plots was characterized with advanced nations on leaves, pods and petiole. This suggest the reasons why lower incidence of abnormal seeds were recorded on the treated plots than the control. This is important if the seeds are to be used for planting in subsequent years since it will improve their germination percentage. This study is in line with the work of Jimoh et *al* (2016) who reported that incidence of *Cercospora* Species and *Fusarium* Specie was significantly $p \leq 0.05$ reduced on seeds sprayed with the extracts of *Chromolaena odoratum* and *Titonia diversifolia*, the study shows that lower number of abnormal seeds were recorded in treated plots than in the control.

Treatments with extracts of *D. stramonium, R. communis* and *J. gossypifolia* at the various concentrations increased the pod and seed yields of all the three cowpea varieties: Ife Brown, Ife Bimpe and Tvx 3236 in the two planting seasons. Tvx 3236 performed better than the other varieties in terms of higher pod and seed yield. In addition, the incidence and severity of the disease were lower compared to the other varieties probably because it has some level of natural resistance to the strain of *C. lindemuthianum*. The effect of the botanicals combined with this inherent resistance probably enhanced its overall performance and relatively higher yield. Emechebe and Florini (2003) reported that

TVx3236 showed resistance to anthracnose disease caused by *C lindemuthianum.* However, the yields of the three cowpea varieties were higher in 2012 than 2013 which could be attributed to relatively lower rainfall during the cropping season of 2012. The spread of disease was minimal when the rainfall was relatively lower.

D. stramonium was the best plant extract in terms of the ability to suppress anthracnose disease and its spread. In this study, the extracts of the plants used on the field for the control of C*. lindemuthianum* were effective. Pretorius *et al.* (2002) reported that extracts of some indigenous plants were effective in-vitro but failed to control the spread of the pathogen on the field, which contrasts the results of the current study. In the field, abiotic interactions such as temperature and solar radiation may negatively interfere with the performance of active constituents in botanical extracts. The stability of bioactive substances to temperature and ultraviolet radiation may vary with their chemical structure and this may be responsible for the failure reported by Pretorius *et al.* (2002). However, the results of this study are similar to the report of Muthukumar *et al*. ((2010) where the fungus, *Pythium aphanidermatum,* the causative organism of chilli disease of pepper, was controlled in-vitro and on the field with extracts of twenty-three medicinal plants. The result showed that *A. sativum, A. cepa* and *T. procumbens* extracts effectively controlled the disease. Similarly, Amadioha and Obi (1999) reported that alcohol and water extracts of *Piper betle, Ocimum sanctum* and *Citrus limon* significantly suppressed the mycelial growth of *C. lindemuthianum* in vitro and reduced the spread of the disease in the field. The current study therefore contributes to the list of research work that can be used for the control of plant diseases.

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