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EVALUATION OF THE EFFICACY OF FIVE FUNGICIDES ON SPORE GERMINATION AND MYCELIAL GROWTH OF *Curvularia lunata* and *Fusarium solani* ISOLATED FROM SEED ROT OF OKRA IN CALABAR, NIGERIA.

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Abstract

The efficacy of fungi toxicity of five fungicides was tested in the inhibition of spore germination and radial growth of *Curvularia lunata* and *Fusarium solani* isolated from seed rot of okra in the screen house. The concentrations of the fungicides used were LD_{50} at 10, 50, 100, 150, 200, 250 and 300µg/cm^3 on potato dextrose agar (PDA) for the vegetative growth. Kokotine recorded highest minimum inhibitory concentration (MIC), with *Curvularia lunata* (17.2MIC) and *Fusarium solani* (18.4MIC at LD_{50} of 9.2µg/cm^3. This was closely followed by Apron Plus at *C. lunata* (61.0MIC) and *F. solani* (65.0MIC) at LD_{50} (15.0µg/cm^3) concentration, while Dithane M-45 recorded the lowest inhibition with *C. lunata* (295MIC) and *F. solani* (300MIC) at same concentration. At 250 to 300 µg/cm^3 fungicide concentrations Kokotine and Apron Plus completely inhibited the mycelial growth of the fungal isolates (100%) inhibition. Dithane M-45 again recorded the lowest inhibition at 100µg/cm^{3}with *C.lunata* and *F. solani* recording 31.1% each. The fungicides in order of their efficacy in reducing spore germination and vegetative growth of the fungal isolates are Kokotine, Apron Plus, Benlate, Captan and Dithane M-45.

Key words: Evaluation, five fungicides, efficacy, spore germination, mycelial growth, *Curvularia lunata*, *Fusarium solani*, seed rot, okra.

1.0 Introduction

Okra (*Abelmoscus esculentus* (L.) moench is a widely cultivated vegetable crop in the tropics and sub-tropical regions of the world including Nigeria, primarily for its mucilaginous fruits. In Calabar, Nigeria, the short season and high yielding variety (velvet-35) is cultivated in both wet and dry seasons of the year on diverse soil types and conditions. The young tender fruits (a capsule) can be sliced and used in thickening soups, sauces and stews (Kochhar, 1986). The fruit is rich in vitamins A, B and C; and also minerals especially iodine (Akinsoyoye, 1979; Philips, 1974). It also contains water (86.1%), protein (22%), fat (0.2%), carbohydrate (9.7%) and fibre (1.0%) Purseglove (1969). The leaves and stems are sources of folder for goats and sheep. In traditional medicine, the mucilage in the fresh fruit is used in the treatment of ulcers and for the relief of hemorrhoids (Kochhar, 1986).

Okra, like other vegetable crops is susceptible to several pathogenic fungi both in the field and in storage; and this has become a major problem among farmers in the cultivation of the crop in Calabar and environs. Also, seeds of various crop plants have been implicated in the spread of diseases, with attendant yield losses if they are not treated to inhibit or destroy deep-seated pathogenic organisms in or on seeds; and to protect the seeds and emerging seedlings from both soil and seed-borne pathogens (Eboh and Okoh, 1980). In some cases death of individual plants particularly from legumes and capsules in the tropics occur, when the seeds are not treated. (Esuruoso, 2010). These fungal pathogens may be seed-borne soil-borne, air-borne or transit from one plant species to another (Atage and Akueshi, 1980 and Singh, 2003).Several species of pathogenic fungi like *Cercospora, Fusarium Curvularia, Aspergillus* and *Penicillium* have been reported to infect grains, okra and other vegetables crops in the field causing various diseases ranging from rots (Idowu and Osunlaja, 1999); and leaf spots (Onuegbu and Emiri, 2011; Arum and Chaudhary, 1980 and Okoi *et al.,* 2015). Others are moulds and damping off, (Kothari and Shekhawat, 1992; Vidyasekesan and Kaudaswary, 1980); as well as blights (Chand, 1980).

Chemical control measures have been tested and found effective in the control of several crop diseases (Ogundana and Denis, 1981; Plumbley, 1985). Different protectants and systemic fungicides have been reported to be used in vitro in the control of *Fusarium* sp. and other fungal diseases of crops (Tunwari *et al.,* 2014; Manej *et al.,* 2013). Also, kokotine and Apron plus have been reported to have successfully controlled leaf spot disease of okra caused by *Curvularia lunata* (Okoi *et al.,* 2015). Fungicides may act on or interrupt the metabolic system of the pathogen (Bilgrami and Dube, 1976). Also, the effectiveness of a pathogen depends on its innate toxicity and permeation. Certain protective fungicides although harzadous to environment are still used for the control of fungal crop diseases (Vaish and Sinha, 2003; Singh, 2006) especially if they are disease resistant – free varieties as is the case with velvet -35, cultivated here in Calabar and environs. Therefore, in the present investigation, inhibition of spore germination and mycelial growth of *C. lunata* and *F. solani* exposed to different concentrations of some fungicides were studied. The objectives of the study were to evaluate different fungicides under laboratory conditions to ascertain the most effective ones suitable for the control of seed rot diseases of okra caused by some pathogenic fungi in Calabar, Nigeria. The results of these studies will also be useful to the farmers who cultivate the crop.

2.0 Materials and methods

2.1 Isolation of *C. lunata* and *F. solani* from okra seeds

Seeds of locally important okra variety (cv. Velvet – 35) were obtained from Cross River State Agricultural Development Project (ADP), Calabar, Nigeria. Isolation of *C. lunata* and *F. solani* associated with the okra seeds was carried out in accordance with the recommendation of International Seed Health Testing Association (ISTA, 1966), using the blotter method. Okra seeds (500g) slightly

getting rot were surface sterilized by immersing in a 100% mercuric chloride solution for 1 minute and transferred into 95% ethanol for 10 seconds in the Biology Laboratory of the Cross River University of Technology, Calabar. The seeds were immediately rinsed in four changes of sterile distilled water and dried between layers of sterile filter papers. Ten(10) seeds of the sterilized okra were plated on a three-layer sterile Whatman filter paper, moistened with sterile distilled water in a 9cm sterile Petridish. The Petridish was incubated at a temperature of 27±2ºC for 7 days. The incubated seeds were later examined under a binocular light microscope for identification of *C. lunata* and *F. solani* fungi. Identification was made on the basis of their growth habits and characteristics. These were further confirmed by examining slide preparations of the spores/mycelia using binocular light microscope with the aid of "Illustrated Genera of imperfect fungi", (Barnette and Hunter, 1972).

2.2 Preparation and inoculation of fungal inoculum

Spore suspensions of *C. lunata* and *F. solani* were prepared by washing off conidia and mycelia (Propagules) of the test fungi with 10ml of sterile distilled water per petri-dish from 7 day old pure cultures of each fungi into two separate 250ml of Erlenmeyer flask. The suspension of the test fungi were filtered through a single layer of sterile muslin cloth and adjusted to a concentration of 5x104 spores/ml using a haemocytometer (Fajola and Nwufo, 1985).

2.3 Preparation of fungicides concentrations

A stock solution of 1000 ug/cm^3 of each of the five fungicides (Benlate, Dithane, M-45, Captan, Apron Plus and kokotine) was prepared in sterile water and three separate dilutions made with sterile distilled water (for comparison) to give concentrations of 2,4,6,8,10; 20, 40,80,100 and 150, 200, 250 and 300 ug/cm^3. Spore suspensions each of *C. lunata* and *F. solani* (5x104 spores/cm^3) were used for each investigation.

2.4 Effect of fungicides on the germination of *C. lunata* and *F. solani*

The relative efficacy of five fungicides were tested against *Curvuluria lunata* and *Fusarium solani* in vitro using potato dextrose agar (PDA) medium. The medium was sterilized in the autoclave at 1.1kg/cm3 at 121ºC for 15 minutes. Media plates and other glass wares were wrapped with aluminum foil and sterilized in the oven at 160ºC for 24 hours while inoculating needles were sterilized by flaming to red heat in a flamed spirit lamp. Media which were not immediately used were stored in the refrigerator at 4ºC. Germination tests of the fungal spores of the test fungi were carried out by inoculating drops of 0.01cm^3 suspension of each fungi to 0.01cm3 of each fungicide in separate glass slides and incubated in sterile petri dish moist-chambers at 26±1ºC for 3 days. Each treatment had four replications, and conidia germination was examined at one hour intervals for a period of 12 hours. In each case, a set of treatments without fungicides served as control. Sterile cover slides were placed on each drop and germination count taken under a low power light microscope with a tally counter. A conidium was assessed to have germinated when its germ tube was observed to be longer

than its width (Igbal *et al.*, 2010). The percentage inhibition of fungal spore germination was determined using the lethal dose 50 (LD_{50}), which is the minimum amount of concentration of each fungicide that can inhibit or kill 50% of the fungal pathogens under test. The minimum inhibitory concentration (MIC) and the fungicidal concentrations of each fungicide were determined against the two fungal pathganes under test, as follows: LD_{50} at 10, 50, 100, 150, 200 and 300µg/cm^3.

2.5 Assessment of mycelial growth of *C. lunata* and *F. solani* on fungicides.

Exactly 18ml of freshly prepared PDA medium was poured into each of the sterilized 9cm Petri dishes. Also, 2 ml of each fungicide solution was added into each Petri dish and agitated slightly to give a thorough mixing of the contents and left to solidify. The media plates were inoculated at the centres with a 4mm inoculum disc from a 7day old culture of *C. lunata* and *F. solani* using sterile inoculating needles. Each treatment was replicated three times. PDA plates inoculated with the test fungi but without fungicides served as control. All the media plates were incubated at 27±1°C for 7 days, after which the zones of inhibition on colony diameter were compared with controls using the formula of Whipp, 1987 and Percentage growth inhibition was expressed as follows:-

$$\mathrm{CP} = \frac{\mathrm{C-T}}{C} \; x \; \frac{100}{1}$$

Where CP = Percentage inhibition of colony growth

C = Colony growth in control plate

T = Colony growth in treatment plate.

The experiment was repeated thrice in a randomized complete block design with 5 treatments and 3 replications.

2.6 Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and treatment means separated according to Duncan's Multiple Range Test at 5% probability level.

3.0 Results

Results of the effect of fungicides on the germination of *C. lunata* and *F. solani* spores are shown in Table 1. All the fungicides showed significant differences ($P<0.05$) in the inhibition of germination of the fungal spores of both fungi. The lethal dose 50(LD_{50}) and the minimum inhibitiory concentration (MIC) of the fungicides for both *C. lunata* and *F. soluni* are recorded in Table 1. The results showed that the efficacy of the fungicides to inhibit spore germination of the two fungal pathogens increased with decrease in the LD_{50} concentration of the fungicides. Kokotine (lindane) gave the highest minimum inhibitory effect, and strongly reduced the spore germination of both fungi with *C. lunata* (17.2MIC) at LD50 (9.2ug/mt3), and *F. solani* (18.2 MIC) at same concentration. This was closely followed by Apron plus at LD_{50} (15 gu/cm^3) with *C. lunata* (61.0 MIC), and *F. solani* (65.0 MIC) at same concentration; while Dithaue M-45 at LD_{50} (145 ug/cm^3) recorded the least, with *C. lunata* (295 MIC) and *F. solani* (300 MIC) at same concentration

(Table 1). Generally, the fungicides in order of their efficacy in reducing spore germination of *C. lunata* and *F. Solani* are kokotine, Apron plus, Benlata Captan and Dithane M-45.

Table 1: In vitro effects of fungicides on conidial germination and minimum inhibitory concentration of *C. lunata* and *F. solani* treated with fungicides 24 hours after inoculation.

Fungicide	**Lethal Dose Concentration *LD_{50}(ug/cm^3)**	**Minimum Inhibitory Concentration (MIC)**	
		C. lunata	***F. solani***
Dithane M-45	145	295	300
Captan	60	120	125
Benlate	55	100	110
Apron plus	15	61	56
Kokotine	9.2	17.2	18.4

* LD_{50}:Is the amount of concentration of fungicide that can kill or inhibit 50% of fungal pathogens under test.

The results of the effect of fungicides on the vegetative growth of *C. lunata* and *F. solani* are recorded in Table 2. All the five fungicides tested under laboratory conditions significantly ($p<0.05$) inhibited the mycelial growth of *C. lunata* and *F. solani* at all concentrations. The result showed that there was a general reduition in the vegetative growth of the fungus with increase in concentration of the fungicides. The highest percentage inhibition of mycelia growth of *C. lunata*and *F. solani*were observed at 250 to 300 µg/cm^3 fungicides concentrations where Kokotine and Apron plus completely inhibited the mycelia growth of the fungus at 100% inhibition. Also, the lowest percentage inhibition of the fungus was recorded in Dithane M-45 (31.1%) closely followed by Captan (44.4%) as compared to 40mm in control with no (0%) inhibition (Table 2). The rest of the fungicides in order of their decreasing fungitoxic efficacy against the two pathogenic fungi were again Benate, Captan and Dithane M-45. Generally, the fungicides significantly ($p<0.05$) differed from one another in checking the growth of the two test fungi at different concentrations used in vitro.

Table 2: In vitro effects of mycelial growth and percentage inhibition of *C. lunata* and *F. solani* treated with fungicides, 7 days after inoculation

	Curvularia lunata					*Fusarium solani*				
Fungicide conc (ug/cm 3)	Captan inhibition (%)	Dithane M-45 inhibition (%)	Benlate inhibition (%)	Apron plus inhibition (%)	Kokotine inhibition (%)	Captain inhibition (%)	Dithane M-45 inhibition (%)	Benlate inhibition (%)	Apron plus inhibition (%)	Kokotine inhibition (%)
100	44.4^e	31.1^e	60.4^e	68.9^c	88.9^c	33.3^e	31.1^e	46.7^e	55.6^e	75.6^d
150	55.6^d	50.3^d	66.7^d	84.4^c	93.3^b	44.4^d	42.2^d	55.6d	71.1^d	82.2^c
200	68.9^c	66.7^c	80^c	93.3^b	100^a	57.8^c	55.6^c	68.9c	80^c	95.6^b

250	77.8[b]	80.6	90.1[b]	100[a]	100[a]	68.9[b]	66.7[b]	80b	88.9[b]	100[a]
300	90.6[a]	84.4[a]	94.8[a]	100[a]	100[a]	82.2	70.2[a]	86.7a	93.3[a]	100[a]

0.0 (µg/cm^3) control = 40mm, 0% inhibition.

Mean values followed by different letters in each fungicide (per colum) are significantly different ($p<0.05$) according to Duncan's Multiple Range Test.

4.0 Discussion

The results obtained showed that the five fungicides tested in vitro inhibited the mycelial growth and spore germination of both *Curvularia lunata* and *Fusariumsolani* at all concentrations. The results also showed that Kokotine and Apron Plus proved to be better fungicides than others in inhibition of mycelial growth and spore germination of *C. lunata* and *F. solani*. Kokotine recorded the highest inhibition of spore germination of *C. lunata* (17.2MIC) at a concentration of LD_{50} (9.2µg/cm^3) and *F. solani* (18.4 MIC) at same concentration. This was closely followed by Apron Plus at LD_{50} (15µg/cm^3) with *C. lunata* (61.0MIC) and *F. solani* (65.0MIC) at same concentration. However, Dithane M-45 at LD_{50} (14.5µg/cm^3) concentration recorded the lowest inhibition at *C. lunata* (29.5MIC) and F. solani (300MIC) at same concentration (Table 1). Kokotine and Apron Plus completely inhibited (100%) the vegetative growth of *C. lunata*and *F. solani*at 250 to 300µg/cm^{3}concentrations. (Table 2). Benlate, Captan and Dithane M-45 were also observed to be significantly effective when compared to controls in inhibiting the vegetative growth and spore germination of the test fungi in vitro. These observations corroborate the findings of previous researchers in the control of fungal diseases of similar vegetable crops (Tunwari, 2014; Chaudhary and Chaudhari, 2013 and Sauders and Langston, 2008).

Statistical analysis in this study also indicated that *C. lunata* was more sensitive than *F. solani* in the efficacy of the five fungicides to inhibit spore germination and mycelial growth (Tables 1 and 2). The study also showed that low concentrations of Kokotine, Apron Plus and Benlate effectively inhibited mycelial growth and spore germination in the two test fungi in vitro. This observation also agreed with previous reports that low concentrations of Benlate (Benomyl) and chlorothalonil inhibited the spore germination and mycelial growth of *Septoria apicola*, the causal agent of leaf spot disease of celery plant (*Apium graveolus* L.) (Bambridge *et al.,* 1985). In this study, Dithane M-45 exhibited least fungitoxicity in the inhibition of both fungi tested at lowest concentrations (Tables 1 and 2). This same fungicide at higher concentration (300µg/cm^3) was however reported to give 100% inhibition of vegetative growth of *Alternaria alternate* causal fungus of *Capsicum annum* (Manoj *et al.,* 2013). Also, Dithane M-45 (Mancozeb) was reported to have exhibited highest mycelial growth inhibition of 100.0% and spore germination of 80.0% at 500 ppm in color rot of Indian mustard (Rana and Tripathi (2003); and in dry root rot of chiken pea (Prajapati *et al.,* 2002) as well as sclerotia of *Macrophomina phaseolina*, causal fungus of charcoal rot of soybean (Dubey and Kumar, 2003). In previous works, Captan and Cabendazim in higher concentrations were reported to have

significantly arrested the mycelial growth and spore germination of *Fusarium mangiferae* (Gaur and Chakraberti, 2009).

The five fungicides assayed in this study were all found to inhibit *C. lunata* and *F. solani* at all the concentrations used. Low concentrations of Kokotine, Apron Plus and Benlate could however, be recommended for control of the two pathogenic fungi, to reduce residue in the controlled plant. Also, since in vitro results do not always reflect field experiments, this study was complemented by field trials which is ongoing to prove or disprove the efficacy of these fungicides on *C. lunata* and *F. solani* as inocula on the okra plant, and to compare them to new fungicides towards control of fungal rot diseases of some important vegetable crops grown in Calabar and environs.

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