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Evaluation of the Efficacy of Thiram and Benomyl on Radial Growth, Spore Germination and Sporulation Density of *Curvularia lunata* and *Fusarium semitectum*

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Abstract **– The effect of Thiram, benomyl and the mixture of the two fungicides was studied on *Curvularia lunata* and *Fusarium semitectum* which were isolated from the seeds of cowpea (*Vigna unguiculata* (L.) Walp). The radial growth of the two pathogens on potato dextrose agar (PDA) was significantly reduced at all concentrations of the tested fungicides. The fungi toxic effects of the fungicides were expressed at a concentration as low as 0.1 ppm. The two fungicides and their mixture became fully fungicidal causing 100% inhibition of spore germination in both fungi at concentrations of (1.000 and 10.00ppm). Sporogenesis was similarly 100% inhibited in *F. semitectum* at these concentrations but in the case of *C. lunata*, the sporulation inhibition was achieved at 10.000 ppm. A mixture of these fungicides was found to be more effective and potent than when they were used single under the same experimental conditions.**

Keywords **– Fungicide, Radial Growth, Sporulation Density, Spore Germination, Pathogens.**

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp is a major food crop grown in many African countries. The grains are well known for their protein content (20 – 30%) and a very good source of cheap plant protein to resource-poor who can hardly afford animal proteins from fish, meat, milk and eggs (IITA, 1984; Anderson, 1985). Cowpea is very rich in minerals, oils, fats and vitamins. The plant leaves, husk and other by-products are used to feed livestock and a good delicacy in Nigeria, where it may be consumed as moi-moi or akara (bean cake), "gbegiri soup" among the Egbas in Ogun State, Nigeria. The crop is grown in the tropical and sub-tropical region of the world on diverse soil types and conditions (Alghali, 1991). The yields are generally low (Olatunde *et al,* 1991) and total yield losses and crop failure may occur due to activities of some pathogenic fungal pathogens which decimates the crop in the field, storage and at various stages of growth.

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. In some cases death of individual plants particularly from legume in the tropics occur, when the seeds are not treated (Allen, 1995).

Fungi have become important experimental tools for studies of fundamental biological processes. (Taylor *et al,* 1993) and some of them have been reported to-be responsible for reduced agriculture production (Trutmann, *et al,* 1993). Benomyl and thiram have been widely used, as fungicides against varied species .of pathogenic fungi of various crop plants. (Ventural *et al;* 1970: Allison. *et al* 1975; El-Nagar *et al*, 1990; Subrahmanyani 1991, Kolte, 1994)

Cowpea fungal diseases of economic importance in the tropics include cowpea anthracnose induced by *Colletotrichum lindemutianum* (Sacc and Magu) with profuse sporulation in susceptible varieties but resistant varieties does not sporulate or lesions (Williams 1975; Emechebe and Shoyinka 1985), transmitted through seed (Emechebe and. MacDonald 1979; Qureshi *et al.,* 1985). Seed rot and seedling mortality caused by the pathogen was reported to reduce germination in India (Prasanna, 1985). Ascochyta blight was reported as one of the major diseases of cowpea-in African, Latin America and Zambia (Emechebe and Shoyinka 1985; Lin and Rios, 1985; Kannaiyam *et al.,* 1987). Black leaf spot and smut disease occurs widely in tropical Africa, Central America, Brazil, India and Nepal (Vakili, 1978; Rios, 1985). Brown blotch induced by *Colletotrichum* species is primarily seed transmitted and survives harsh dry season weather in seed (Alabi, and Emechebe 1992; Emechebe, 1981). This plant is reputed for having or susceptible to numerous fungal pathogen which may be seed borne, soil borne, air borne or transmit from one plant species to another. Among the widely and well distributed pathogens common encountered on cowpea include: *Rhizoctonia* spp, *Colletotrichum* spp. *Fusarium* spp, *Curvularia* spp, *Penicillium* spp, *Aspergillus* spp, *Cercospora* spp, *Phytophotra* spp which causes various diseases ranging from, rot, damping-off, seed decay, anthracnose, blight, seedling mortality, leaf spot, scab, brown blotch etc (Allen, *et al.,* 2004; Florini, 1997; Amadioha, 1999; Ajibade and Amusa, 2001; Adejumo and Ikotun, 2003; Bosah, 2013).

This study was carried out to evaluate the efficacy of thiram, a dithiocarbarmate, non-selective fungicide and benomyl, a benzimidazole systemic fungicide used either singly, or as a mixture against radial growth, spore germination and sporulation density of *F. semitectum* and *C. lunata* in culture.

II. MATERIALS AND METHODS

The seed borne fungi (*Curvularia lunata* and *Fusarium semitetcum*) used for this study were isolated, from Cowpea seeds. 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 General of Imperfect Fungi", (Barnette and Hunter, 1972).

Potato dextrose agar (PDA) was the substrate used to culture the fungi species. This was sterilized in the autoclave at a temperature of 12°C (1.1 kg/cm^2 pressure) for 15 minutes. Media which were not immediately used were stored in the refrigerator. Glasswares were wrapped with aluminum foil and sterilized in the oven at 160°C for 24 hours while inoculating needles were sterilized in the oven at 160°C for 24 hours while inoculating needles were sterilized in a flamed spirit lamp.

Benomyl, thiram solutions and chemical media

Stock solution of 10,000ppm formulated product (2g/100ml sterile distilled water) was used. Serial dilutions of 1,000ppm, l00ppm, l0ppm, 1 ppm and 0.1 ppm were prepared by adding 50ml of stock solution (10,000ppm) to 450ml sterile distilled water to obtain 1,000ppm; 50ml of 1,000ppm was added to another 450ml sterile water to get 100ppm. Similar transfers were made to obtain l0ppm, 1ppm and 0.1ppm.

For the fungicide PDA medium containing, 1,000ppm, l00ppm, l0ppm, 1ppm and 0.1ppm, 10,000ppm thiram (T) and Benomyl (B) dilution series (I.0ml PDA-T or PDA-benomyl mixture to 9ml agar blanks) were prepared. Plates for each chemical concentrations and those without the fungicides (controls) were replicated four times each and placed in a complete randomized block design manner in the inoculating chamber for two clays to ensure absence of contaminants in the plates.

Effect of thiram and benomyl on radial growth of C. lunata and F. semitectum

Investigating the effect of Thiram and benomyl on radial growth of *C. lunata* and *F. semitectum*, a flamed-sterilised 5mm diameter cork borer was used to cut mycelial discs from 2-day old culture of the two species of fungi and transferred to the center of PDA containing different concentrations of seed-dressing fungicides. Bottom of the Petri dishes were marked with two perpendicular lines along the center for easy measurement and control plates were similarly .inoculated with the test fungi containing no fungicides. Treatment consisted of four replicates which were incubated at 25±1°C for 3 days. Radial growth was measured .in two directions along the perpendicular lines and mean for each colony calculated.

Fungicides effect on spore germination of C. lunata and F. semitectum

To determine the effect of the fungicides on spore germination of *C. lunata* and *F. semitectum*, the PDA-sporulating culture of each fungus was washed and filtered in test tubes and centrifuged at l,000ppm for a minute. The sedimented spores after decanting the. supernatants were re-suspended in 2ml sterile water-to give a concentration of 2 x 10^3 spores/ml (*C. lunata*) and 2 x 10^1 spores ml (*F. semitectum*). A ml of each spore suspension was added to 9ml sterile distilled water (control) shaken thoroughly to obtain uniform suspension of thiram and benomyl. Drop of the concentration were placed separately in different glass slides and incubated in petri dish moist chambers at 25 ± 1°C. Each treatment had four replications and were incubated for 12 hours. To stop further germination of the spore, a drop of lactophenol in cotton blue was added; cover slides were placed on each drop and germination count taken under low power light microscope with a tally counter.

Thiram and benomyl effect on sporulation density of C. lunata and F. semitetcum

Investigation of the effect of Thiram and Benomyl on sporulation density of *C. lunata* and *F. semitetcum* was carried out with 5mm-diameter mycelial discs from a 2-day old non-sporulating cultures of the test fungi on PDA. Different concentration of the fungicides in PDA were obtained by serial dilution from the stock of 10,000pp. PDA plate that served as controls were pure PDA without the test fungicides and were inoculated with the test fungi. Each treatment had four replications arranged in a randomized complete block design at room temperature (25°C) and diffused sunlight. Sporulation density was determined 6 days after incubation with haemacytometer slide. Ten 5mm-diameter discs were randomly cut and transferred to 5ml distilled water in test tubes, manually shaken to dislodge the spores into the water. Agar-mycelium-spore suspension was filtered to obtain pure spore suspension and final volume made up to 5ml with sterile water. Three drops of each suspension were examined and counted in 10 large square/ml and (X) was calculated thus:

$$\frac{X - Nv}{V}$$

where

N = means no of spore in the large square counted,

v = 1 ml= 1000mm^3 and V = volume of suspension under cover slip (0.1 x l/25mm^3).

III. RESULTS

The effect of thiram and benomyl combination of the two fungicides on radial- growth of *F. senitectum* and *C. Iunata* (Table I) showed significant reduction in the radial growths of the two pathogenic fungi at all concentrations. The degree of inhibition increased significantly with successive increase in the concentrations of fungicides applied.

At 10,000ppm, the combination of thiram and benomyl gave a radial growth of 2.0mm which was significantly reduced than when they were used singly i.e. 6.5mm and 4.5mm for thiram and benomyl respectively for *F. semitectum*. The same trend was obtained with *C. lunata* which gave 2.3mm, 1.5mm and for thiram; benomyl and the combination respectively. There was no significant difference in the degree of inhibition by benomyl alone, and the mixture but this was significantly lower when thiram was singly used at (P = 0.05).

Results on the effect of thiram, benomyl and the mixture of the two on spore germination of *F. semitectum* and *C. lunata* is presented in Table 2). The percentage germination of the spore reduced significantly with successive increase in the concentrations of the chemicals

for, *F. semitectum.* At 1000ppm – 10,000ppm, there was 100% inhibition of spore germination in *C. lunata* followed the same trend and pattern of significant decrease in the germination of the spores of *F. semitectum* with 100% inhibition at 100ppm and 10,000ppm. It was however observed that benomyl had higher inhibitory properties than thiram alone on both fungi but their individual inhibitory effect was lower than, when the mixture of the two was applied.

On sporulation density of *F. semitectum and C. lunata* with the different chemical treatments (Table 3), there were significant decreases with each successive increase in the concentrations of the seed dressings when fungicides were used singly or in combination against the test fungi in culture. At 100ppm there was complete stoppage of sporulations when the combination of the fungicides was used. 100% inhibition of sporulation was achieved at 1000ppm for all the chemical treatments on the test fungi except C. *lunata* with 0.63 X 10^5 sporulation density when treated with 1000ppm of thiram alone which nonetheless was not significantly higher than results obtained with the same concentration of benomyl or in combination.

IV. Discussion

The fungicidal effects of thiram and benomyl applied singly or in combination were expressed at concentration as low as 1.0ppm. Radial growth of *F. semitectum* and *C. lunata* at this concentration were significantly reduced at (P = 0.05) compared to growth on fungicide free agar medium. Optimum temperature for radial growth in artificial culture of some species of fungus particularly *Collectotrichum* has been reported to be 25°C (Alabi and Emechebe, 1992) which was the temperature at which the test fungi were cultured, inhibition of Curvularia species was equally reported in culture when benomyl and thiram were applied (Fajemisin and Okunyemi 1976**,** Ogundana 1986**,** Emechebe *et al.,* 1994). Growth of the test fungi were significantly reduced with successive increase in the concentration of the fungicides.

At concentration of 0.1 ppm and 100ppm, the spore germination of *F. semitectum* and *C. lunata* were significantly reduced when compared with control experiment. At higher concentration (1000ppm and 10000ppm) these chemical either alone or in combination became fully fungicidal causing 100% inhibition of spore germination in the two test fungi in .culture. In the sporulation studies (Tables 3), all tested concentration of the fungicides significantly inhibited conidial production in culture. At 100ppm and 10000ppm sporegenesis was 100% inhibited in *F. semitectum* by either thiram or benomyl alone or in optimum temperature of 25°C for sporulation (Alabi and Emechebe, 1992). From this study, it is evident that the .combination of the seed dressing fungicides is more potent and effective on *F. semitectum* and *C. lunata* than when they were used separately under the same experimental condition. Combination of seed dressing fungicides, a viable option for peasant farmers in West Africa has been applauded than when used singly because of the wide spectrum of activity against fungal diseases (Emechebe *et al.,* 1994). Thiram and benomyl has been reported effective against the growth of many fungi species, such as *Phythium, Corticium, Macrophomina, Botrytis, Fusarium, Curvularia, Alternaria* etc. which may cause remarkable reduction in cowpea and other related legume production when not timely controlled, (Williams 1975a; Ventural *et al*, 1970; Allison *et al.,* 1975). Benomyl appeared more potent, effective and of wider spectrum than thiram when applied alone.

The study has established these fungicides as effective inhibitors to mycelial growth, spore germination and sporulation of the test fungi, the effects of which were concentration dependent. It is therefore recommended that farmers who wish to embark on cultivation and. production of cowpea are encouraged to combine thiram and benomyl as seed dressing there by potentiating the potency against activity of fungi pathogens.

References

[1] Adejumo, T. O. F. Florini and T. Ikotun (2003). Effect of planting date on incidence and severity of leaf smut of cowpea in Northern Nigeria. *Moor J. Agricultural Research*, 4:106 – 110.

[2] Ajibade, S. R. and N. A. Amusa (2001). Effects of fungal diseases on some cowpea in the humid environment of South Western, Nigeria. *J. Subst. Agric. Envirt*. 3:246 – 253.

[3] Alabi, O. and A. M. Emechebe (1992), Effect of Temperature on Growth and Sporulation of cowpea. Brown Blotch Pathogen (*Collectotrichum Capsici*) (Syd) Butler and -Bisby *Samaru Journal of Agricultural Resources* 9:09-192.

[4] Alghali, A. M. (1991). Studies on cowpea farming practices in Nigeria with emphasis on insect pest control. *Tropical Pest Management*. 37:71 – 74.

[5] Allen, D. F. (1995). An Annotated List of Diseases', Pathogen and Associated Fungi of the Common Beans. *(Phaseolus vulgaris)* in Eastern and Southern . Africa *Phytpathological Papers* 34. *International Phycological Institute*, Egham U.K.

[6] Allen, T. W. Enebak, S. A. and Carey, W. A. (2004). Evaluation of fungicides for control of species of Fusarium of long leaf pine seed. *Crop Protection*. 23: 979 – 982.

[7] Allison, D. A., Peak, R. and Tomalin, J. (1975). Control of Seed Borne Disease of Winter Wheat by Benomyl and Carbondazin-Dithiocarbonnle Mixture Proc. 8th Insecticide - *Fungicide Conference* 2, Brighton pp 177.

[8] Amadioha, A. C. (1999). Evaluation of some plant leaf extract against *Colletotrichum lindemuthianum* in cowpea. *Archives of Physiopathology and Plant Protection*. 32:141 – 149.

[9] Barnette. H. L. and Hunter, B. B. (1972). Seed-borne Pathogenic Fungi and Bacteria of Cowpea in North Nigeria. *PANS* 25 (4) :401-404.

[10] Bosah, B. O. (2013). Some fungal pathogens of cowpea (*Vigna unguiculata* (L.) Walp) and their control in Asaba area of Delta State. *Ph.D Thesis* submitted to Faculty of Agriculture, Delta State University, Abraka 122p.

[11] El-Nagar, M.A.A., EI-Said. S.I.A., Dia M. M and Makladi F. M. (1990). Effect of using some fungicides and Seed Inoculation with Rhizobium Lupini on Controlling Crown Rot Disease Incidence and Plant Growth of Peanut Crop. *African Journal of Agricultural Science* 17:199-207.

[12] Emechebe, A.M. and S.A. Shoyinka (1985). Fungal and Bacterial Diseases of Cowpeas in Africa Pages 173-192 in *Cowpea Research, Production and Utilization* edited by S.R. Singh and K. O. Rachie. Chichester; John Wiley and Son.

[13] Emechebe, A.M.(1981). Brown Blotch of Cowpea in Northern Nigeria. *Samaru Journal of Agricultural Research* 1(1): 20-26

[14] Fajemisin, J. M. and Okunyemi O. (1976). Fungicidal Control of Curvularia Leaf Spot of Maize. *PANS* 22:23-1-238.

[15] Florini, D. A. (1997). Nematodes and other soil borne pathogens of cowpea. In: Advances in cowpea research. Singh, B. B., D.R. Mohan Raj, K. E. Dashiell and LEN Jackai Co Publication of

IITA and Japan: *JIRCAS*. IITA, Ibadan, Nigeria. Pp. 193 – 206.
[16] Hawksworth, D. L. (1991). The Fungal Dimension-of Biodiversity: Magnitude, Significance and Conservation. *Mycol. Res.* 95: 641-655.
[17] Kannaiyam, J. D.C. Greenberg. H. C. Haciwa and M.N. Mbeewe (1987). Screening Cowpea for resistance to major diseases in Zambia. *Tropical Grain Legume Bulletin* 34:23-26
[18] Kolte, S. (1994). Disease Problems in Seed Crops of Oilseeds and Methods to Tackle *India Farming* (ICAR) 44(5) 7-12.
[19] Lin. M, T. and G.P. Rios (1985). Cowpea Diseases and their Prevalence in Latin America- Pg. I99-204 in *Cowpea Research Production and Utilization.* Edited by S.R. Singh and. K.O. Rachie, Chichester, U.K. John Wiley and Sons.
[20] Neergard, P. (1979). Seed Pathology Vols. 1 & 2 I87pp New York. John Wiley and Sons Inc.
[21] Ogundana, I. K. (1986.); Control-or-Pythium Wet Rot of Cowpea (*Vigna sinensis*) in Nigeria *India Phytopathology* 39(2): 245-248.
[22] Prasannn, K.P.R. (1985). Seed Health lasting of Cowpea with Special. Reference to Anthracnose Caused by *Colletotrichum Lindemutianum*. *Soc. Sc. Tech.* 13:821 -827. Qureshi, S. H., M, Bashir and S.S. Alam (1985) Anthrcnose of Cowpea - a New Disease Record in Pakistan. *Tropical drain Legume Bulletin* 50:26.
[23] Rios. G. P. (1988). Fungal and Bacterial Diseases of Cowpea in Brazil Pages 233-253 in *Cowpea Research in Brazil,* edited by E.E. Wait and J.I'P de Araujo. Translated by E., L. Walt IITA/EMBRAPA, Brasilia. Brazil.
[24] Subrahmanyam P. (1991). Control of Seedling Diseases of Groundnut in Niger. *Tropical Pest Management* 37:118-119.
[25] Taylor, J. W. Bowman, B. Berbee, M. L. and White, T. J. (1993). Fungal Model organisms: Phylogenetics of Saccharoinyees, *Aspergillus* and *Neurospora*. Sys. Biol. 42:440 – 457.
[26] Trutmann, P; Voss, J. and Fairhead, J. (1993). Management of common Bean Diseases by farmers in the Central African Highlands. *International Journal of Pest Management* 39:334-342.
[27] Vakili, N. G. (1978). Distribution of smut of Beans and Cowpeas in Tropical America and its Possible Centre of Origin. FAO Plant Protection Bulletin 2691): 19-24.
[28] Ventura, E; Bourdus, J. and Berthier, G. (1970). Etude Delefficacite De Quelques Fungicides Systemiques Nouveaux vis-à-vis Des Principaux Parasites Des Semences De Cereals Vill int. Congr. Pl. Prot. 1970 (Ab, tra).
[29] Williams R. J. (1975). Control of cowpea seedling mortality in Southern Nigeria. Plant Dis. Reptr. 59:245 – 248.
[30] Williams, R. J. (1975a). The Control of cowpea diseases in the IITA Grain Legume Impr. Prog. Pg 139 – 146 in *Tropical Disease of Legumes*. Edited by J. Blid and K. Maramorosch., New York: Academic Press.

Table 1: Thiram and Benomyl effects on radial growth of *Fusarium semitectum* and *Curvularia lunata* in culture

Fungicide concentration	Radial Growth (mm)**					
	F. semitectum			*C. lunata*		
	Thiram	Benomyl	T+Ben	Thiram	Benomyl	T + Ben
0.1	71.0a (a)	70.3a (a)	59.0b (b)	30.5b (c)	42.5b (b)	28.3a (b)
1.0	57.3b (a)	56.0b (a)	32.6c (b)	20.0c (c)	23.8c (c)	17.5c (d)
10	36.5c (a)	37.8c (a)	18.5d (b)	16.1c (b)	15.0d (b)	12.8cd (c)
100	21.0d (b)	29.8d (a)	13.3e(c)	11.3d (c)	11.0de (c)	7.5d (d)
1,000	13.6e (a)	11.8e (a)	5.5f (c)	9.3d (h)	6.3ef (c)	2.0e (d)
10,000	6.51f (a)	1.5f (b)	20.0f(c)	2.3e (c)	1.5f (d)	1.5e (d)
0.0 (ppm) (Control)	74.8a (a)	74.8a (a)	74.8a (a)	49.3a (b)	49.3a (b)	49.3a (b)

**Means of 4 replications/concentration after 72 hours. Column means followed by same letter are not significant at 5% while means in rows followed by the same letter in parenthesis are not equally significantly different at 5% separation by Duncan's New Multiple Range Test.

Table 2: Thiram and Benomyl effects on spore germination of *Fusarium semitectum* and *Curvularia lunata* in culure.

Fungicide concentration	Germination percentage (%**)					
	F. semitectum			*C. lunata*		
	Thiram	Benomyl	T+Ben	Thiram	Benomyl	T + Ben
0.1	49.0b (a)	44.0b (b)	40.0b (c)	43.0b (b)	39.5b (a)	34.0b (d)
1.0	31.5c (a)	28.5c (b)	27.0c (b)	30.0c (a)	25.0c (e)	20.5c (d)
10	21.0d (a)	17.5d (b)	13.5d (c)	17.0d (b)	13.5d (c)	8.5c (d)
100	2.0e (a)	6.0e (b)	2.0e(d)	9.0c (a)	4.3e (c)	0.25ef (e)
1,000	0.0f (a)	0.0f (a)	0.0f (a)	0.0f (a)	0.0f (c)	0.0f (a)
10,000	0.0f (a)	0.0f (b)	0.0f (a)	0.0f (a)	0.0f (c)	0.0f (a)
0.0 (ppm) (Control)	100a (a)	100a (a)	100a (a)	100a (a)	100a (a)	100a (a)

**Means of 4 replicates/concentration 12 hours after incubation. Column means followed by same letter are not significant at 5% probability while row means follow by same letter in parenthesis are not different.

Table 3: Thiram and Benomyl effects on sporulation density of *Fusarium semitectum* and *Curvularia lunata* in culture.

Fungicide concentration	Radial Growth (mm)*					
	F. semitectum			*C. lunata*		
	Thiram	Benomyl	T+Ben	Thiram	Benomyl	T + Ben
0.1	10.06b (a)	10.25b (a)	8.55b (b)	9.38b (a)	7.0b (c)	5.83b (d)
1.0	7.81c (a)	5.81c (b)	3.50 (f)	5.63c (b)	4.33c (d)	2.75c (c)
10	3.75d (a)	1.75e (c)	0.75d (d)	2.81d (b)	1.55d (f)	0.63dcc (d)
100	1.35e (a)	0.50ef (b)	0.0c (c)	1.25d (b)	0.23ef (d)	0.0e (e)
1,000	0.0f (a)	0.00ef (b)	0.0c (b)	0.63ef (a)	0.0f (b)	0.0e (e)
10,000	0.0f (a)	0.0f (b)	0.0c (a)	0.0f (d)	0.0ef (a)	0.0e (e)
0.0 (ppm) (Control)	20.75a (a)	20.75a (a)	20.75a (a)	15.69a (b)	15.69a (b)	15.69a (b)

*Means of 4 replicates/concentration 5 days after incubation. Column followed by same letter are significantly different from each other at 5% probability while row means followed by the same letter in parenthesis are not equally different from each other at 5% probability separated by Duncan's New Multiple Range Test.