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Evaluation of Fungicides on Growth and Conidial Germination of *Verticillium theobromae* Isolated from Plantain

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Abstract: An evaluation of three fungicides, namely, benomyl [methyl l-(butyl-carbamoyl)-2-benzimidazole carbamate], calixin (tridomorph) and dithane M-45 (mancozeb), was carried out for their inhibitory effects on *Verticillium theobromae*, the causal organism of cigar-end rot disease of plantain (*Musa paradisiaca)*. The LD_{50} of calixin, benomyl and dithane M-45 in inhibiting mycelial growth, were 0.14, 0.98 and 535.17 μg mL^{-1}, respectively. Calixin was also the most effective in conidial germination inhibition with an LD_{50} of 21.78 μg mL^{-1}, as compared with dithane M-45 86.39 μg mL^{-1} and benomyl 275.50 μg mL^{-1}. Germ tubes of the test organism were distorted by the fungicides at varying concentrations. Calixin had greater distortion effect at a lower concentration of 10 μg mL^{-1}, dithane M-45 (100 μg mL^{-1}) and benomyl (1000 μg mL^{-1}). The relevance of these results in relation to the control and management of cigar-end rot disease of plantain is discussed.

Key words: Fungicide, growth inhibition, *Verticillium theobromae*, plantain

INTRODUCTION

Since fungicides exhibit varying toxic effects on fungi, it is necessary to carry out screening trials on them to detect their effectiveness, method of application and possible phytotoxic effects (Ikhatua, 1997). Such screening trials should involve the use of *in-vitro* tests of mycelial growth or spore germination inhibition. They have also been used to explain the mode of action of some pesticides. Dijkhuizen *et al.* (1983) attributed the more sensitive reaction of *Monilia fructicola* to prochloraz than captan to the ability of prochloraz to suppress mycelial growth and germ tube elongation at a low concentration (1.0 μg mL^{-1}). Ogundero (1987) also reported that benomyl at 200 μg mL^{-1} completely inhibited the production of exo-enzymes of crown rot fungi of Nigerian bananas.

To preserve plantain and other fruits during long term storage or shipping, there is the need for fungicidal treatments. Of the artificially formulated chemicals, therapeutic action is characteristic of systemic fungicides, of which benomyl is the best known and most widely used against a wide range of fungi (Whitney, 1976; Alvarex *et al*., 1977; Ramsey *et al*., 1987). Other studies have documented the effectiveness of benomyl used solely or in combination with other chemicals as a fruit treatment pesticide for fruit rots of guava (Wills *et al*., 1982) and anthracnose of chilli (Mishra, 1988). In Nigeria, the results of Nwufo *et al.* (1995) showed that avocado fruits treated with benomyl, captan and potassium permanganate, remained fresh durably under refrigeration at 10°C. Ayodele and Ikotun (1996) also reported that for the control of black sigatoka disease of plantain at Ibadan, Nigeria, benomyl was most effective in eradicating *Mycosphaerella fijiensis*. Rawal *et al.* (1983) indicated that field sprays of bavistin, daconil and benomyl were effective in controlling anthracnose caused by *Colletotrichum gloeosporioides* during storage of papaya fruits.

The effectiveness of other protective and systemic fungicides for control of postharvest decay of fruits and for field applications have been reported. Ramarkrishnan and Kandaswamy (1978) found Dithane M-45 as the most effective and economical control of *Alternaria solani* on tomato. Enwezor *et al.* (1989) and Swennen (1990) reported that black sigatoka of plantain can be controlled with aerial applications of tridomorph and other fungicides. Eckert and Ogawa (1985) reviewed the postharvest application of chemical treatments and suggested pre-harvest fungicidal treatment (especially systemic fungicides) as a major step in the achievement of disease control strategies. Also Singh and Shukla (1985) indicated that of nine fungicides sprayed on inoculated brinjal plants in plot tests before harvest, brestan-60 (fentin acetate), followed by Dithane M-45, difolatan, cumin L. and zineb gave the best control of the disease.

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Postharvest cigar-end disease control of plantain has to begin in the field, since infection takes place at an early stage of bunch development (Pasberg-Gauhl and Gauhl, 1996). Slabaugh (1994) reported that effective control begins in the field with frequent removal of dead flowers followed by bagging bunches with perforated poly-ethylene sleeves. Field and packing station sanitation is helpful in reducing *Verticillium theobromae* inoculum pressure and subsequent cigar-end rot. Fungicides are necessary during peak cigar-end rot seasons as well as in ware houses. These operations call for critical selection and evaluation of effective fungicides for control of the disease. This research reports on the evaluation of three fungicides namely benomyl, calixin and dithane M-45, on growth and conidial germination of *V. theobromae*, causal organism of cigar-end rot disease of plantain (*Musa paradisiaca*).

MATERIALS AND METHODS

Verticillium theobromae (Turconi) E. Mason and S.J. Hughes was isolated from infected fingers of plantain (*Musa paradisiaca)* by the method described by Igeleke and Ayanru (2004). Pure cultures were kept as stock on Potato Dextrose Agar (PDA) slants in MaCartney bottles at room temperature (28±2°C). Sensitivity of the isolate to three test fungicides was based on its mycelial growth (culture diameter) and conidial germination. The test fungicides were benomyl (Benlate, 50% a.i.,) [methyl-1 butyl-carbanomyl -2-benzimidazole carbamate] manufactured by E.I. Du Pont de Nemours and Company Incorporated, Biochemicals Department, United Kingdom, calixin (Tridemorph 76% a.i.,) [2, 6- dimethyl-4-tridecyl morpholine] manufactured by BASF, Federal Republic of Germany and dithane M-45 (Mancozeb, 80% w.p.), a complex of zinc and maneb containing 20% manganese and 2.5% zinc, manufactured by Bentrus Chemicals, East Berlin, Germany.

The bioassay was carried out in two stages-an initial assessment of the fungicides aimed at selecting the most effective one and the determination of the range of effective concentrations. The concentrations were calculated as follows: a stock solution was made by dissolving 0.2 g a.i. in 10 ml of sterile distilled water. Serial dilutions of the stock solution gave lower dilutions ranging from 1.0×10^{-1} - 1.0×10^{3} μg mL^{-1} a.i.

For growth assessment, PDA cooled to ca. 45°C in a water bath was amended with each test fungicide to obtain initial concentrations ranging from 10-1,000 μg mL^{-1} a.i. and, subsequently, from 1×10^{-1} - 5.0 μg mL^{-1}. The fungicide-amended medium was shaken to ensure an even distribution of the fungicide, dispensed into 9 cm diameter petri-plates and allowed to stand overnight before its inoculation. Control plates were not amended with fungicides.

Plugs of inoculum were cut from the edge of 14-day-old PDA cultures of *V. theobromae* using a 5 mm diameter sterile cork borer. Plugs (discs) were transferred aseptically to the centre of test Petri-plates and incubated in the dark at room temperature for 14 days before assessing them for growth. Mean growth inhibition (reduction in colony radius as compared to untreated controls) for each fungicide concentration was determined. Data were subjected to analysis of variance in a completely randomized design and means separated by Duncan's multiple range tests (Finney, 1964).

Suspension of *V. theobromae* conidia was obtained by flooding the surfaces of 14-day-old cultures with 10 mL of sterile distilled water and dislodging the spores with a sterile bent glass rod. The spores were washed with sterile distilled water by repeated centrifugation at 5000 rpm. The suspension was made up with sterile distilled water to produce a concentration of ca. 10^{5} spores mL^{-1}, using a haemocytometer.

To test for the effect of the fungicides on conidial germination, water agar (1.2% w v^{-1}) was amended with benomyl, calixin and dithane M-45, to give concentrations of 1000, 500, 100, 50, 10 and 1.0 μg mL^{-1}. Using a sterile dropping pipette, a drop of the conidial suspension was placed on the surface of the amended water agar and spread over the surface with a sterile bent rod. The cultures were incubated in the dark as described. Spores were fixed and stained after 18 h of incubation, by placing a drop of lactophenol containing cotton blue (0.01%) on cut agar pieces, using a sterile 2 mm cork borer. The percentage number of germinated conidia was determined. A conidium was considered germinated if the germ tube length was greater than 50% of the conidium length. The data obtained were subjected to analysis of variance in a completely randomized design and means separated by Duncan's multiple range tests. Fungicide efficacy on growth and conidia germination were analysed by Probit analysis according to the method of Finney (1964, 1971), using the MSTAT-C software. It calculated the lethal (effective) dose or LD_{50} at 50% rate of growth reduction. The reduction values were computed as percentage reduction of the control values. Effect of varying concentrations of the fungicides on the distortion of conidial germ tubes were also noted.

RESULTS

Examination of the growth of *V. theobromae* cultures after 14 days of incubation showed inhibitory effects of the fungicides as compared to the control. Of the three fungicides assayed, the most effective at the concentrations tested was calixin. No growth occurred on PDA cultures of *V. theobromae* amended with concentrations greater than 10 and 50 μg mL^{-1} for calixin and benomyl, respectively (Table 1). However for dithane M-45, growth was observed at concentrations of 0 to 500 μg mL^{-1}. Complete inhibition was observed only at a concentration of 1000 μg mL^{-1}. A repeat experiment produced similar results (Fig. 1). Data on the percentage inhibition of colony growth, using lower concentrations of benomyl and calixin varying from 0.1-5.0 μg mL^{-1}, showed that calixin was more effective than benomyl (Fig. 2).

The three fungicides had varying degrees of inhibitory action on the germination of *V. theobromae* conidia as compared with unamended control cultures. Germination inhibition varied from 9.4 for benomyl at 100 μg mL^{-1} to 100% for calixin and dithane M-45 at 500 μg mL^{-1}, respectively (Table 2).

Results of Probit analysis on colony growth inhibition data produced LD_{50} values of 0.137, 0.975 and greater than 500 μg mL^{-1}, respectively for calixin, benomyl and dithane M-45 (Table 3). Similarly, Probit analysis on conidial germination data showed that calixin was the most effective fungicide with LD_{50} value of 21.78 μg mL^{-1} followed by dithane M-45 (86.39 μg mL^{-1}) and benomyl (275.50 μg mL^{-1}) (Table 3).

Table 1: Percentage inhibition of colony growth of *Verticillium theobromae* on PDA amended with benomyl and calixin

Fungicide	Concentration (μg mL^{-1})	Mean colony radius (mm) diameter	Colony growth as % of control	Inhibition of growth as % reduction control
Control	0.0	56.6	100.0	0.0
Benomyl	10	10.7	18.9	81.1
	50	0.0	0.0	100.0
Calixin	10	0.0	0.0	100.0
	50	0.0	0.0	100.0

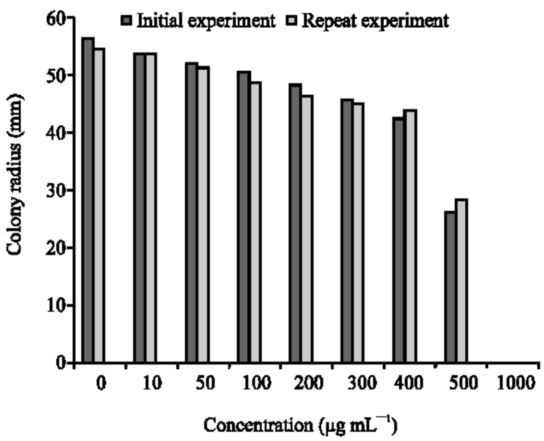

Fig. 1: Colony radius of cultures of *V. theobromae* grown on PDA amended with concentration of dithane M-45 varying from 0-1000 μg mL^{-1}

Table 2: Inhibition of germination of *Verticillium theobromae* on water agar amended with benomyl, calixin and dithane M-45 after 18 hours of incubation

Fungicide concentration (μg mL^{-1})	Germination (%)	Inhibition (%)
Control		
0.0	100.0⁺a	0.0a
Benomyl		
50.0	100.0a	0.0a
100.0	90.6b	9.4b
500.0	39.0b	61.0c
1000.0	13.3d	86.7d
Calixin		
1.0	100.0a	0.0a
10.0	80.0b	20.0b
50.0	44.3c	55.7c
100.0	12.39	87.7
500.0	0.0e	100.0e
Dithane M-45		
10.0	100.0a	0.0a
50.0	86.0b	14.0b
100.0	49.0c	51.0c
500.0	0.0d	100.0d

+Mean of three replications; means in a column not followed by the same letter are significantly different at ($p \leq 0.01$)

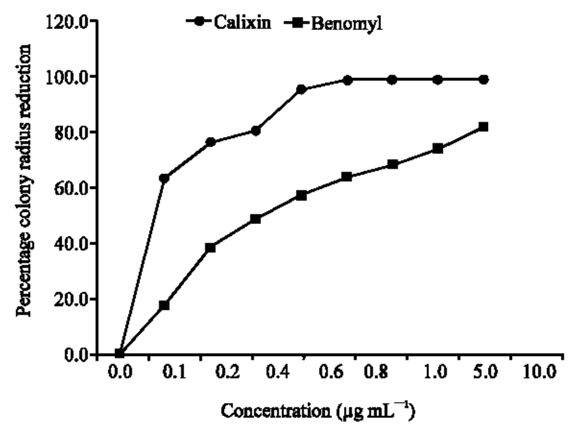

Fig. 2: Mean Percentage reduction in colony radius of *V. theobromae* on PDA media amended with varied low concentrations of benomyl and calixin

Table 3: Probit analysis Table of the effect of benomyl, calixin and dithane M-45 on colony growth reduction (%) of Verticillium theobromae after 14 days of incubation

Fungicide⁺	Degree of freedom	Slope (b)	Intercept (a)	Calculated chi-square	Tabular chi-square	Log ED_{50}	Variance of log	LD_{50} (μg mL^{-1})
Growth (Colony Diameter) Reduction								
Benomyl	7	1.7095	4.4948	100.1623**	18.475	0.2955	6.7022-0.004ᵉ	0.975
Calixin	7	14.2296	4.2043	66.7040**	18.475	0.0559	2.3338-0.005ᵉ	0.137
Dithane M-45	6	0.7315	2.6693	26.6256**	16.812	3.1862	2.4454-0.002ᵉ	535.171
Conidial germination reduction								
Benomyl	5	2.5706	-1.6186	2.2460 ns	11.070	2.5747	1.0962-0.003ᵉ	275.5
Calixin	5	2.0204	1.9649	7.5877 ns	11.070	1.5022	1.6606-0.003ᵉ	21.78
Dithane M-45	5	3.9575	-2.8520	0.4226 ns	11.070	1.9841	7.1524-0.004ᵉ	86.39

**- Significant at p<0.01; ns - Not significant, + Concentrations used were 1.0, 10, 50, 500 and 1000, Source: Finney (1964, 1971)

Germ tubes of conidia of the test fungus were distorted by the fungicides at varying concentrations. Distorted germ tubes were swollen (or enlarged) and bent as compared to the control with slim, long germ tubes. Calixin induced greater distortion on the germ tubes at a lower concentration of 10 μg mL^{-1} as compared to benomyl which effected distortion of germ tubes at 1000 μg mL^{-1}. Dithane M-45 effected distortion on germ tubes at a concentration of 100 μg mL^{-1}.

DISCUSSION

Calixin was the most effective of the test fungicides in its inhibitory action on mycelial growth at a low concentration (LD_{50} 0.14 μg mL^{-1}). A low LD_{50} is one of the ideal characteristics of a good fungicide (Whitney, 1976) and should make calixin a better choice for the control of *V. theobromae*. There is no report on the use of calixin to control the disease caused by *V. theobromae* on plantain fingers *in vitro*. However, Enwezor *et al.* (1989), Swennen (1990) and Ayodele and Ikotun (1996), reported on the use of calixin as a foliar spray for the control of black sigatoka disease of plantain caused by *Mycosphaerella fijiensis*.

Benomyl was next to calixin in inhibiting mycelial growth with an LD_{50} of 0.98 μg mL^{-1}. A number of reports (McMillan, 1973; Alvarex *et al.*, 1977; Wills *et al.*, 1982; Ullasa and Rawal, 1988) have indicated that benomyl is an effective fungicide for the control of postharvest disease of fruits. It may, therefore, be a promising candidate fungicide for the control of cigar-end rot disease of plantain.

Calixin was also the most effective test fungicide for conidial germination inhibition with an LD_{50} of 21.78 μg mL^{-1} as compared with dithane M-45 (86.39 μg mL^{-1}) and benomyl 275.50 μg mL^{-1}). Van Gestal (1991) recognized the existence of differences in the ability of a fungicide to act differently on radial growth in comparison with spore germination. Of the three fungicides tested, calixin was the most effective in its inhibitory action on mycelial growth as well as conidial germination. However, a recommendation for the use of a fungicide for disease control can be reliably made only after testing it in the field for several years (Ridings and Clayton, 1970). The results of this study suggest that calixin has a promise for testing under field conditions.

Conidia of the test fungus were also observed to be distorted by the fungicides at varying concentrations. Calixin has greater distortion effect at a lower concentration of 10 μg mL^{-1} as compared to dithane M-45 (100 μg mL^{-1} and benomyl (1000 μg mL^{-1}). This finding is similar to the report of Solel (1970) on the sublethal effect of TBZ, CITBZ and benomyl on *Cercospora beticola*, resulting in numerous initials and distorted germ tubes.

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