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Growth, Morphological Alterations and Adaptation of Some Plant Pathogenic Fungi to Benlate and Zineb. A New Look

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Abstract: The fungicides benlate and zineb were tested *in vitro* on growth, sporulation and morphological alterations of *Alternaria solani*, *Fusarium oxysporum* and *Sclerotium cepivorum*. Reduction in growth criteria of the tested pathogenic fungi was more correlated with elevation of benlate dose than with zineb. The target fungi showed a limited resistance to zineb training transfers, but could tolerate up to 5- folds of sublethal benlate dose specially *Sclerotium cepivorum*. The pigmentation and the dark colour of colonies are the most morphological alterations occurring in the growth media, in response to toxic stress of both fungicides. Fifty and about 100% sectoring were incident just before LD_{50} and lethal dose respectively of both chemicals. Percentage increase of sectoring was associated with progressive reduction in sporulation. Tolerance of these fungi towards zineb, is correlated with increased ability to synthesize extracellular melanin under fungicidal stress. A new look to the mode of action of each fungicide was discussed in the light of current oxygen toxicology theory.

Key words: Morphology, growth, fungi, plant pathogenic, *in vitro*

Introduction

Benlate is a potent fungicide used to decrease the aggressiveness of several phytopathogens (Minuto *et al.*, 1997; Kummuang, 1997; Mackinaite, 1997; Mercer and Ruddock, 1997; Amadioha, 1998; Casa *et al.*, 1998). Zineb has also been studied as a fungicide on a number of fungal pathogens on some worldwide crops (Cristopher and Nair, 1993; Kummuang, 1997; Jamjanya, 1997).

It is important to know the mode of action of the fungicides used, specially before they are introduced into the field in order to evade or minimize the appearance of resistant strains of the pathogen. Benzimidazoles (include benlate) are widely used as anthelmintic agents and systemic fungicides. In susceptible organisms, benzimidazoles bind to beta tubulin and block microtubule polymerization (Li *et al.*, 1996; Tikhomirova and Inge-Vechtomov, 1996). Despite the isolation of numerous resistant isolates (Molnar *et al.*, 1985; Souza Silva and Melo, 1997; Yamaguchi *et al.*, 1998) during the past few years, information on their mode of resistance to the fungicides is scanty. These isolates show cross resistance to all toxic benzimidazole derivatives which put limitations on their use in disease control. Therefore, the dicarboximide fungicides were largely introduced in attempt to combat resistance to the benzimidazole group of fungicides. Recently the precise mode of action of dicarboximide fungicides has been suggested that they may cause

the formation of reactive free radicals (Christopher and Nair, 1993, Elskens and Penninckx, 1997; Radice ***et al.***, 1998) which may be the ultimate cause of cell death in dicarboximide treated fungi.

This recent classification of dicarboximides (include zineb) as oxidants urged us to choose benlate and zineb, in this experiment to study their comparative effectiveness on growth, sporulation and adaptation in relation to morphological deformations of the phytopathogens ***Alternaria solani***, ***Fusarium oxysporum*** and ***Sclerotium sepivorum***. Such a study may be a new look to the angle of disease control in light of a recent mode of action.

Materials and Methods

Fungal cultures

Three phytopathogens were used in this study: ***Alternaria solani*** the causative of tomato and potato early blight, ***Fusarium oxysporum*** f. sp. ***Lycopersicii*** the causative of tomato wilt and ***Sclerotium cepivorum*** the causative of onion and garlic rot. These pathogens were isolated from the target host and were maintained on Czapek-Dox's agar medium for the first two pathogens and potato dextrose agar medium for the third one.

Fungicides

The fungicides used were benlate (Methyl 1-(butyl-carbamoyl) benzimidazol-2-carbamate] and zineb [zinc ethylenebis (dithiocarbamate)];(Ciba Geigy). The fungicides were used at concentrations: 0.01, 0.1, 1.0, 1.5, 2.0, 2.5, 2.75, 3.0, 3.2 and 3.3 mg ml^{-1}.

Rate of growth (Kr)

Petri dishes supplied with 20 ml Dox's agar medium and amended with the desired concentrations of either benlate or zineb were used. After solidification, each plate was inoculated with 5mm diameter agar disc from the periphery of a 7-day old culture plate. The plates were then incubated at the suitable temperature (28°C for *A. solani* & *F. oxysporum* and 20°C for *S. cepivorum*) and the diameter of developing colonies was recorded daily over a period of 7 days. The radial growth rate (Kr) was then estimated and compared with that of untreated plates (control).

Mycelial deformation (Sectoring)

This was carried out according to Sharma and Brown (1983). Plates of CDA, either treated with the tested concentrations of the fungicides or untreated (control) were inoculated with an agar plug (5mm) cut from the leading edge of normal colonies of each fungus. The inoculated plates were incubated at 25°C under 12 h nuv/12 h dark for 13 days prior to examination for sectoring. The morphological characters of the sectored colonies were examined. The mean number of the sectored colonies was also calculated and percentage of sectoring related to colonies developed on the control plates was calculated.

Sporulation

Plates of Czapek-Dox's agar supplemented with the tested concentrations of the fungicides

used were inoculated each with a 5 mm diameter agar disc of the used fungus. The plates were then incubated for 7 days. A 1 cm^2 block was cut from margin of the colony and transferred to a vial containing 10 ml sterile distilled water. The suspension was continuously shaken for 5 min and the number of spores (or sclerotia) per ml was counted in a haemocytometer. Three plates were used for each treatment.

Training of fungi on solid medium

The tested fungi were trained to progressively increasing levels of each fungicide by serial transfers on petri dishes each containing 20 ml of Czapek-Dox's agar medium amended firstly with the sublethal concentration of either fungicide for each fungus. The plates were inoculated with 5 mm diameter disc taken from the periphery of 7 days old culture plates and incubated at the suitable temperature. Diameter of the developing colonies was measured daily for 7 days and the radial growth rate was then calculated. After that, fungal discs were transferred two successive times, 7 days each, to plates containing the same sublethal concentration. Serial transfers to progressively increasing concentrations of the fungicides then followed and the rate of growth for each transfer was calculated. The training passages continued till the fungus failed completely to tolerate any transfer.

Statistical analysis

The arithmetic means of the replicate estimations were tabulated and the least significant difference (LSD) at 1% confidence limits was calculated.

Results

Reduction in the radial growth rate (Kr) of the tested pathogenic fungi was linear with the elevation in benlate and zineb concentrations. The lethal dose varied according to species and fungicide. ***Alternaria solani*** continued to grow on benlate or zineb amended media up to the sublethal concentrations which were 3.2 and 3.0 mg ml^{-1}, respectively. (Table 1) ***Fusarium oxysporum*** f.sp. ***lycopersicii*** and ***Sclerotium cepivorum*** behaved similarly towards zineb, but they were very sensitive to benlate as their growth was almost completely inhibited at the lower concentrations; the recorded growth rate of *F. **oxysporum*** was only 0.1 mm^{-1} day at 0.01 mg ml^{-1} benlate and that of *S. **cepivorum*** was 0.2 mm^{-1} day at 0.1 mg ml^{-1}. Fifty percent inhibition of growth rate (LD_{50}) was reached at concentration 1.5 mg ml^{-1} of either fungicides for the three tested pathogens in case of zineb and for ***Alternaria solani*** only in case of benlate.

A gradual increase was evident in percentage of sectoring of *A. **solani*** colonies corresponding to increase in the fungicides doses (Table 2). About 100% sectoring was reached at the sublethal dose of either fungicides, whereas 50% sectoring was attained at a concentration of 1 mg ml^{-1}. It was obvious that the sectored colonies of *A. **solani*** became darker with purple pigmentation diffused in the agar medium. Such change in color matched with increase in the fungicide concentration. The produced colonies of *F. **oxysporum*** and *S. **cepivorum***, whenever grew on benlate, showed 100% sectoring from the beginning. On zineb, the sectoring of two species was gradually common as the fungicide concentration was increased.

Table 1: Radial growth rate (mm^{-1} day) of the tested plant pathogens grown on Dox's agar medium supplemented with different concentrations (mg ml^{-1}) of benlate or zineb

	Benlate concentrations (mg ml^{-1})												Zineb concentrations (mg ml^{-1})									
Fungal species	Control (0.0)	0.01	0.1	1.0	1.5	2.0	2.50	2.75	3.0	3.2	3.3	LSD P≤0.01	0.01	0.1	1.0	1.5	2.0	2.50	2.75	3.0	3.2	LSD P≤0.01
Alternaria solani	6.4	6.3	5.5	4.6	3.4	3.0	2.5	2.0	1.5	0.8	-	0.4	6.9	5.9	4.0	3.5	2.8	1.7	1.2	0.9	-	0.5
Fusarium oxysporum	7.3	0.1	-	-	-	-	-	-	-	-	-	1.8	7.0	6.3	4.4	3.6	2.9	1.8	1.1	0.4	-	0.7
Sclerotium cepivorum	7.6	0.6	0.1	-	-	-	-	-	-	-	-	2.1	8.3	6.2	5.1	3.9	3.9	2.5	1.8	1.0	-	0

(6) No growth

Table 2: Percentage of sectored colonies of the tested plant pathogens grown on CDA medium supplied with different concentrations of benlate or zineb and incubated for 13 days under 12 h nuv/12 h in dark

	Benlate concentrations (mg ml^{-1})											Zineb concentrations (mg ml^{-1})								
Fungal species	Control (0.0)	0.01	0.1	1.0	1.5	2.0	2.50	2.75	3.0	3.2	3.3	0.01	0.1	1.0	1.5	2.0	2.50	2.75	3.0	3.2
Alternaria solani	0.0	18.3	41.3	52.6	76.6	81.6	90.6	97.3	98.3	100	-	0.0	25.0	51.7	59.3	68.6	80.0	88.0	95.6	-
Fusarium oxysporum	0.0	100	-	-	-	-	-	-	-	-	-	3.3	23.3	48.0	60.6	75.1	89.3	92.3	96.6	-
Sclerotium cepivorum	0.0	10.0	100	-	-	-	-	-	-	-	-	8.3	31.3	49.6	62.8	71.9	83.6	91.2	100	-

(-) No growth

Table 3: Effect of different concentrations (mg ml^{-1}) of either benlate or zineb on the sporulation of *Alternaria solani* and *Fusarium oxysporum* (spores x 10^5/ml) or sclerotial production of *Sclerotium cepivorum* (sclerotia/19.6 mm^2)

	Benlate concentrations (mg ml^{-1})												Zineb concentrations (mg ml^{-1})									
Fungal species	Control (0.0)	0.01	0.1	1.0	1.5	2.0	2.50	2.75	3.0	3.2	3.3	LSD P≤0.01	0.01	0.1	1.0	1.5	2.0	2.50	2.75	3.0	3.2	LSD P≤0.01
Alternaria solani	50.2	39.1	30.3	24.8	19.2	15.1	13.8	10.7	6.2	0.0	-	4.1	33.5	29.2	25.5	20.7	18.9	11.7	5.9	0.0	-	5.1
Fusarium oxysporum	78.3	3.2	-	-	-	-	-	-	-	-	-	30.4	60.1	49.7	36.1	29.3	20.8	11.3	7.6	0.0	-	4.3
Sclerotium cepivorum	21.7	6.3	0.0	-	-	-	-	-	-	-	-	9.1	18.2	15.3	10.6	4.1	0.0	0.0	0.0	0.0	-	3.1

(-) No growth

Table 4: Radial growth rate (Kr mm^{-1} day) of the tested plant pathogen grown on Dox's agar medium supplemented with progressively increasing concentrations (mg ml^{-1}) of either benlate or zineb starting with the sublethal concentration

Fungal species	Benlate		Zineb	
	Treatments (mg ml^{-1})	Kr	Treatments (mg/ml)	Kr
Alternaria solani				
	3.2	0.9	3	1.1
	3.2	1.4	3	1.5
	3.2	2.2	3	1.8
	3.3	0.9	3.1	1.3
	3.4	0.5	3.2	0.6
	3.5	0.2	3.3	0.4
	3.6	0.1	3.4	0.2
	3.7	-	3.5	0.0
LSD ($P \leq 0.01$)	0.29		0.20	
Fusarium oxysporum				
	0.01	0.1	3.0	0.4
	0.01	0.2	3.0	0.6
	0.01	0.4	3.0	1.0
	0.02	0.1	3.1	0.8
	0.03	-	3.2	0.5
			3.3	0.1
			3.4	-
LSD ($P \leq 0.01$)	0.18		0.24	
Sclerotium cepivorum				
	0.1	0.2	3.0	1.1
	0.1	0.3	3.0	1.3
	0.1	0.3	3.0	1.9
	0.2	0.5	3.1	2.8
	0.3	0.3	3.2	2.1
	0.4	0.2	3.3	1.4
	0.5	0.1	3.4	0.7
	0.6	-	3.5	0.3
			3.6	-
LSD ($P \leq 0.001$)	0.14		0.4	

(-) No growth.

The highest sectoring percent (about 100% for *F. **oxysporum*** and *S. **cepivorum***,) was attained at the sublethal concentration of the fungicide. Fifty percent sectoring was detected at 1 mg ml^{-1} of zineb. The altered colonies of *F. **oxysporum*** had stout hyphae, rare chlamydospores and

macroconidia with indistinct septation of the latter. The extracellular blue exudations, accompanying *F. oxysporum* growth, became more darker and the intensity of darkness increased with the increase in fungicide concentration. The colonies of *S. cepivorum* grown on fungicide-amended medium turned brownish with thick walled hyphae at the margine but flaccid at the center. The sectored colonies became darker than the normal unsectored ones; this darkness became more intensive with increasing both fungicide concentration and sectoring percent.

Results reveal a progressive reduction in sporulation matched with percentage sectoring of *A. solani* and *F. oxysporum* grown on media amended with increasing fungicidal dose; however, at the sublethal dose of benlate and zineb, the mycelia of both fungi were sterile and lost its capability to produce any spores. The number of sclerotia of *S. cepivorum* was greatly reduced on benlate-amended medium to reach 6.3/19.6 mm^2 at 0.01 mg ml^{-1} compared with 21.7/19.6 mm^2 obtained under control condition(Table 3). Zineb was less effective and sclerotia continued to grow up to 1.5 mg ml^{-1}. Whenever formed, the produced sclerotia appeared small, immature and poorly developed. The effective concentration which possessed antisporulant activity and exerted about 50% inhibition for conidial formation by *A. solani* and *F. oxysporum* or sclerotial formation by *S. cepivorum* was 1 mg ml^{-1} of either benlate or zineb.

The process of training transfers raised the sublethal dose to 3.6 and 3.4 mg ml^{-1} in case of *A. solani* grown on benlate and zineb, respectively. *F. oxysporum* and *S. cepivorum* could tolerate the training passages on elevated concentrations of zineb up to 3.3 and 3.5 mg ml^{-1} respectively (Table 4). With benlate, *F. oxysporum* was very sensitive as the sublethal dose was raised up to 0.02 mg ml^{-1} only compared with its starting sublethal dose (0.01 mg ml^{-1}) whereas *S. cepivorum* succeeded to tolerate up to 0.5 mg ml^{-1} compared to 0.1 mg ml^{-1}.

Discussion

The present results indicate that the reduction in the radial growth rate (Kr) of the tested pathogenic fungi was linear with the elevation of benlate and zineb concentrations. The toxicity of benlate and zineb to the growth criteria has been implicated in laboratory experiments by Ohazurike (1996); Abdel Malek *et al.* (1997); El Moughith (1998) and Lim TaeHeon *et al.* (1998).

From these results, the target fungi reacted against the toxic effect of fungicides by inducing morphological distortions (sectoring) to its characteristic features. The morphological alterations were induced in the texture, colour of the colonies, pigmentation and sporulation. It has been reported that many systemic fungicides are known to induce alterations in fungi directly and indirectly depending on their mode of action (Yasuda and Noguchi, 1972; Richmond and Phillips, 1975). Incorporation of fungicides to the growth medium of *Trichoderma* sp. (Peterbauer *et al.*, 1992; El Moughith, 1998) and *Collitotricum trancatum* (Bankole and Adebanjo, 1996) led to the formation of very restricted and abnormal mycelial morphogenesis.

Interestingly, 50 and about 100% sectoring were incident just before LD_{50} and lethal doses of both fungicides for the target fungi, respectively. A progressive reduction in sporulation matched with percentage increase of sectoring. Reduction in sporulation was consequently followed by inhibited rate of growth. The antisporulation activity of benlate and zineb was investigated by measuring the concentration of each fungicide required to inhibit 50% of the

conidial formation. The results clearly indicated that those concentrations for most fungi were less than those required to inhibit the rate of growth. The antisporulation activity of fungicides was previously reported in some fungi (Echerl, 1977).

The relatively high lethal dose of zineb and its LD_{50} (3.2 and 1.5 mg ml^{-1} respectively) for the tested pathogens indicate their tolerance towards dicarboximides. Razak ***et al.*** (1991) claimed that ***Alternaria tenuis*** and ***Fusarium*** sp. isolated from rotted tomato fruit tolerated relatively high levels of the fungicide, zineb, up to 2560 ppm on solid media. Dicarboximide resistance has since been recorded in a number of fungal pathogens on a variety of worldwide crops e.g. ***Botrytis cinerea*** (Christopher and Nair, 1993) ***Drechslera*** sp. (Jamjanya, 1997) and ***Rhizoctonia*** spp. (Kummuang, 1997).

The differences in susceptibility and resistance of fungi, towards a definite fungicide, may be due to its detoxification before the site of action has been reached, lack of conversion of a compound into the fungi-toxic principal or compensation for toxic effect by an increased production of inhibitory factors (Parry, 1990). The third possibility of the above-mentioned directions is most probably the reason for such high resistance against carboximides revealed in this study. Melanin may be the inhibitory factor playing the role. All tested fungi already contain melanin pigments in its cell wall or have the ability to excrete such pigment through the medium. The natural occurrence or the induction of pigment secretion may be a mode of pathogen defense against the toxic effect of the fungicide. Melanized cells posses increased resistance to environmental stress (Fogarty and Tobin, 1996).

Nevertheless, it is important to know the mode of action of any fungicide before being used. Recently, the net fungitoxic effect of dicarboximides (zineb included) is free radicals formation leading to the eventual destruction of membrane lipid (Radice ***et al.***, 1998; Elskens and Penninckx, 1997; Christopher and Nair, 1993), which may be the ultimate cause of cell death in dicarboximide-treated fungi.

Therefore it can suggest that resistance or tolerance of these fungi, towards dicarboximides, is correlated with increased ability to synthesize extracellular melanin, as fungal metabolite, under fungicide stress. Melanine in turn may be a respective cause of reduced fitness of the fungicide. Various mechanisms have been proposed to explain the protective role of melanin which invoke the radical scavenging properties of the polymer (Lukiewicz ***et al.***, 1981; Tadeuz ***et al.***, 1986; Eric and Herschell, 1991). The present report thus, provides the link between melanization and resistance to dicarboximides. The relatively high resistance of melanized fungal pathogens to carboximidies can be explained in the light of current oxygen toxicology theory. The free radical species generated from carboximides become opposed by melanin synthesized by the pathogen. Such opposition and resistance is based on the scavenging properties of melanin which may be by binding, reducing and/or inactivating such lethal free radicals.

In this study, pigmentation was obviously the most morphological alteration induced in response to fungicide treatment. The pigmentation was illustrated by darking in the colour of the colony and/or by increasing the dark extracellular secretion. Such response insure that the tested fungal pathogens are suffering from oxidative stress, in the growth media, in response to carboximides acting as oxidants, thus leading to extra synthesis of melanin by tolerant fungi. Eric

and Herschell (1991) and Abo Ellil (1999) linked the oxidative stress with the extra melanin synthesized.

It is important to note here, that the three pathogens succeeded to pass the training transfers up to a limited dose of zineb (elevation by = 1/9th of sublethal dose only). Dickinson and Wallace (1976) reported that repeated sprays of zineb inhibited the development of many yeasts and filamentous fungi.

With respect to the fungicide benlate, ***Alternaria solani*** seemed to be very tolerant to it, while ***Fusarium oxysporum*** and ***Sclerotium cepivorum*** were supersensitive and sensitive towards benlate, respectively. Suza Silva and Melo (1997) found that ***Alternaria alternata*** strain, isolated from benlate-treated soil, could degrade carbenadazim, a product of benlate hydrolysis, while Monistrol ***et al.*** (1988) reported that a very low concentration of benlate completely prevented ***Cladosporium cucumerinum*** growth. Frequent use of benomyl resulted in production of tolerant isolates of moulds (Hopkins, 1984). This was revealed and insured by our results where ***A. solani*** exhibited a higher level of adaptation to benlate than to zineb.

Although ***S. cepivorum*** seemed to be very sensitive to benlate, it was able to adapt itself and could tolerate about 5 folds of sublethal concentration of benlate. A similar trend was evident in ***Saccharomyces cerevisea*** (Kelly ***et al.***, 1994) as well as many isolates of ***Monilinia fructicola*** which showed double tolerance to benzimidazole (Lim TaeHeon ***et al.***, 1998). This behaviour may be due to its ability to produce a larger number and size of more dark sclerotia at high concentration of benlate reached in this study work. Natural occurrence of melanin in cell wall of ***A. solani*** and sclerotia of ***S. cepivorum*** acted as antipenetrants (Sisler, 1985) which reduced the uptake of that fungicide leading to loss of its efficiency.

Interestingly, ***Fusarium oxysporum*** f.s.p. ***lycoperscii*** revealed very limited adaptation towards benlate where it passed only the first transfer (1-fold of the sublethal dose). This might explain why benzimidazoles are still used widely more than 30 years, as a proper fungicide for this phytopathogen in Egypt.

The precise mode of action of dicarboximide fungicides reflects no inhibitory effects on cell organelles or cell function, at concentration capable of inhibiting growth (Pappas and Fisher, 1979). Raj Kumar ***et al.*** (1998) recorded that zineb improved growth of apple tree, increased the height and girth of treated cultivars, ***Fusarium oxysporum*** and others were either controlled, or made ineffective by fungicide treatments. Fontem and Bouda (1998) claimed that dicarboximide significantly increase leaf area and pod and green fodder of ***phaseolus vulgaris***.

Although a high tolerance was illustrated by the 3 pathogens under test from the starting point of contact with the high doses of zineb, yet they showed a limited resistance to the chemical training transfers (elevation by 1/9th of the sublethal dose only). This behaviour (limited adaption) can be considered in favour of zineb application.

In contrast, benlate (benzimidazoles) functions by interfering with a number of cellular processes such as mitosis, meiosis, intracellular transport of molecules and maintenance of cell shape (Tikhomirova and Inge-Vechtomov, 1996; Orbach ***et al.***, 1986; Peterbauer ***et al.***, 1992); such toxic effects may be extended to include the host cells (Davidse 1973, Hammerschlag and Sisler, 1972). Moreover, the usefulness of benzimidazole has been reduced in the light of the present

data, by the frequent appearance of resistant strains which can tolerate up to 5 fold concentration of benlate. Therefore, it is suggested that carboximides (included zineb) are preferred than benzimidazoles (included benlate) as fungicides used for the control of many plant fungal diseases.

In conclusion, efficient inhibitors of melanin synthesis are nowadays needed and recommended in the field of phytopathology. Such inhibitors are necessary to enhance drug efficiency, by causing selective toxicity to melanized cells. This mechanism is highly desirable for fungicides used against soil-borne pathogens that survive as melanized propagules.

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