DETECTION OF BENOMYL RESISTANCE IN THE ANTHRACNOSE PATHOGEN, *COLLETOTRICHUM CAPSICI*

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SUMMARY: Conidial isolates of Collectotrichum capsici were obtained from four field sites in Malaysia. Benomyl-resistant isolates were detected by their mycelial growth on agar containing 100 µg/ml benomyl. Resistant isolates were found at three of the four sites examined where benomyl sprays were frequently used. Large differences in the effects of benomyl on mycelial growth of sensitive and resistant isolates were demonstrated; growth of sensitive isolates were completely inhibited at 2.5 µg/ml benomyl while the resistant isolates examined, all grew on agar containing 1000 µg/ml fungicide. The benomyl-resistant isolates were cross - resistant to thiophanate-methyl and carbendazim. Benomyl-resistant isolates were just as pathogenic to chillies as sensitive isolates. The implication of these results and other reports of benomyl resistance in Collectotrichum are discussed in relation to disease control.

Key Words: Colletotrichum capsici, anthracnose, resistance, benomyl.

INTRODUCTION

The benzimidazole fungicide benomyl, have been used for the control of many diseases caused by Deuteromycetous pathogens (6), owing to its systemic properties and their great efficacy in controlling plant diseases. It has also been recommended for a long period for the control of riperot disease of chillies (*Capsicum annum L.*) caused by *Colletotrichum capsici* (Syd.). Butler and Bisby in Malaysia. However, recently little or no control of this disease was observed in some major chilli growing areas (personnel communications with growers). The possible explanation offered for the reduced effectiveness of benomyl was insensitivity of *C.capsici* to benomyl, but this was not favoured because benomyl continues to give good control of ripe rot in other areas.

Development and occurrence in fungal populations of isolates resistant to this particular group of fungicides is now well known, both in the laboratory and in the field. Until 1979, resistant isolates had been reported in 16 different genera (7) and in many instances their frequency in the field has led to failure of disease control following the use of benzimidozole fungicides (3,11,14).

Thus this paper reports on the detection of benomyl-resistant isolates of *C. capsici* from chillies in Malaysia. The pathogenicity and response to benomyl and other fungicides of these and benomyl sensitive isolates are described.

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MATERIALS AND METHODS

Fungicides and Media

Potato dextrose agar (PDA) was used for culturing the fungus. The fungicide used were benomy (50% a.i.), thiophanate-methyl (80% a.i.) and carbendazim (50% a.i.).

Fungicide - amended agar was prepared at the required concentration by the addition of 1 ml. of fungicide solution in 2-ethoxyethanol to 99 ml. PDA cooled to 45°C. Control were prepared by the addition of 1 ml solvent or sterile distilled water to 99 ml of media. For both treatment and control five plates were poured from each 100 ml media. All fungicide concentrations were determined according to the amount of active ingredient in each material.

Agar plates were inoculated with mycelial discs, 5 mm in diameter, taken from the margin of a colony of the fungus grown on PDA, for 7 days. Mycelial growth was estimated by measuring colony diameters after 6 days 30°C in the dark.

In all experiments, unless stated otherwise, five replicates were used.

Isolates

Isolates were obtained from diseased plant material, collected from field sites as described below and were stored under mineral oil at room temperature and unless stated otherwise, were derived from single spores.

Isolation and Identification of Resistant Isolates in Field Crop

Isolates of *C. capsici* were obtained from diseased plant material collected from four different field sites in Selangor, Malaysia. Three of these sites had received application of benzimidazole fungicides

in previous seasons. Site 3 has never been treated with benzimidazole fungicide.

Active lesions caused by *C. capsici* on chilli fruits were washed in running tap water, immersed in 10% sodium hypochloride for 6-10 minutes and blotted dry on sterile filter papers. Diseased tissues were cut and thin slices containing acervulus were plated across the surface of PDA agar plate. After 4-6 days, these were subcultured onto fresh PDA plates which support blackish growth consisting of abundance of conidia.

Benomyl resistance was detected by the ability of mycelial isolates to grow on agar containing 100 μg/ml fungicide. After 5 days at 30°C isolates could be identified as benomyl-resistant or sensitive, and all isolates required for further work were than subcultered from unamended agar.

Effects of Benomyl on Mycelial Growth of Benomyl-Resistant and Sensitive Isolates

Preliminary work demonstrated that mycelial growth of sensitive isolates of *C. capsici* were inhibited at benomyl concentrations of 2.5 μg/ml and above. The effect of benomyl on mycelial growth of four such isolates (from field 3) was determined by inoculating plates containing 0, 0.01, 0.05, 0.075, 0.1, 0.25 and 0.5 μg/ml benomyl with mycelial discs of the fungus. Similar experiments were conducted for four benomyl resistant isolates (from field 1) by inoculating plates containing 0, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 μg/ml benomyl.

Cross-resistance Experiments

The effects of two benzimidazole fungicides (carbendazim and thiophanate-methyl) on mycelial growth of four benomyl-resistant isolates were evaluated at 0, 1.2, 5, 10, 25, 50, 100 μg/ml.

Production of Benomyl-Resistant Mycelial Colonies From Spores of Field Isolates

The number of mycelial colonies produced from candia in the presence and absence of benomyl was determined for benomyl-resistant and sensitive primary cultures. For each isolate, suspensions containing 200-500 candia/ml were pipetted onto PDA plates containing 0, 2.5, 5, 10, 25, 50, 100 μg/ml benomyl. After incubations for 3 days at 30°C in the dark the colonies present on each plate were counted.

Pathogenicity Experiments

Experiments were conducted on mature chilli fruits obtained from the local market to determine the pathogenicity of benomyl resistant and sensitive isolates.

RESULTS

Isolation and Identification of Resistant Isolates

From the four sites a total of 340 isolates of *C. capsici* were obtained from diseased fruits. The presence of other fast-growing fungi presented the isolation of *C. capsici* from some fruits and led to variation between sites in the number of isolates obtained.

Resistant isolates were found at rather high requencies in 3 of the four field sites. No resistant isolates were found among the 73 isolates from field 3 (Table 1).

Table 1: Occurence of benomyl-resistant isolates of *C. capsici* at four field sites.

Site location	Crop	Previous application of benzim fungicides	Number of isolates examined	Number of benomyl resistant isolates
Field 1	Chilli	Yes	110	60
Field 2	Chilli	Yes	93	40
Field 3	Chilli	No	73	0
Field 4	Chilli	Yes	64	22

Effect of Benomyl on the Mycelial Growth of Benomyl-Sensitive and Resistant Isolates

Benomyl was very inhibitory to mycelial growth of all four benomyl-sensitive isolates, none growing of 2.5 μg/ml fungicide. Inoculum discs from which no mycelial growth developed became darkened but mycelial growth occurred when these were transferred to medium lacking the fungicide.

Mycelial growth of four of the benomyl-resistant isolates tested showed no apparent reduction in colony diameter over the complete range of fungicide concentration tested (Table 2).

Table 2: The effect of various concentrations of benomyl on the mycelial growth of four benomyl-resistant isolates: mean colony diameter (mm) as percentage of control.

	Fungicide Concentration (μg/ml)									
Isolate No	0	2.5	5	10	25	50	100	250	500	1000
R_1	-	97.1	95.0	100.2	87.5	81.3	86.2	78.2	63.5	61.6
R_2	-	102.5	96.2	94.6	94.2	81.8	97.4	86.3	77.0	75.1
R_3	-	100.3	90.3	90.3	77.9	73.2	90.7	68.5	66.4	66.0
R_4	-	98.1	95.0	94.8	87.3	87.3	77.6	75.1	73.5	73.5

Table 3: Concentration of fungicide (μg/ml) which caused a 50% reduction in radial growth rate compared with control colonies. (benomyl-resistant isolates only).

	Fungicide (μg/ml)		
Isolate No	Benomyl	Carbendazime	Thiophanate methyl
R_1	>100	>100	>100
R_2	>100	>100	>100
R_3	>100	>100	>100
R_4	>100	>100	>100

Cross-Resistance of Benomyl-Resistant Isolates to Other Fungicides

All four benomyl-resistant isolates were also resistant to the other two benzimidazole fungicide, with mycelial growth unaffected or reduced by less than 50% at 100 μg/ml fungicides (Table 3).

Production of Benomyl-Resistant Mycelial Colonies by Spores of Field Isolates

For the four benomyl-sensitive isolates no conidia developed into colonies on any of the fungicide-amended plates. With four of the benomyl-resistant isolates there were only slight differences in the number of colonies on unamended agar and on the concentrations of fungicide amended agar (Table 4).

Pathogenicity of Benomyl-Sensitive and Benomyl-Resistant Isolates to Chilli Fruits

All the isolates tested, both benomyl-sensitive and benomyl-resistant, were pathogenic to chilli fruits (Table 5). The difference in the pathogenicity of resistant and sensitive groups of isolates in each experiments were however not significant.

Table 4: Mean number of colonies per plate produced on benomyl-amended agar from spores derived from field isolates after 5 days at 30°C.

Isolate No	Benomyl Concentration μg/ml					
	0	2.5	5	10	25	100
Sensitive isolates						
S_1	120	-	-	-	-	-
S_2	235	-	-	-	-	-
S_3	217	-	-	-	-	-
S_4	230	-	-	-	-	-
Resistant isolates						
R_1	230	230	253	288	196	235
R_2	302	233	332	292	308	298
R_3	205	223	219	119	236	213
R_4	152	238	251	154	201	154

DISCUSSION

The detection of isolates of *C. capsici* resistant to benzimidazole fungicides at these sites should not be regarded as unexpected. The development of resistance to benomyl previously has been described in a large number of other fungi, including *C. musae* (Berk and Curt) Arx (12) and *C. gloeosporioides* Penz. (16). This acquired resistance to systemic fungicides has been discussed by Dekker (8). The development of resistant to benomyl by chilli pathogen in Malaysia is probably the result of repeated use of benomyl on chillies to control anthracnose during production.

Table 5: Pathogenicity to chilli fruits of benomyl-sensitive and benomyl-resistant isolates of *C. capsici.*

Isolates	Degree of infection
Sensitive isolates	
S_1	xx
S_2	xxx
S_3	xxx
S_4	xx
Resistant isolates	
R_1	xx
R_2	xxx
R_3	xx
R_4	xxx

x = slight infection, xx = moderate infection, xxx = severe infection

Even though small numbers of conidia were examined in this work, and as expected, conidia of benomyl-resistant isolates produced resistant colonies and those of sensitive isolates produced sensitive colonies. This suggests that the choice of the conidial or mycelial method to estimate the extent of resistance in *C. capsici* field populations would have little influence on the results.

When the effects of benomyl on mycelial growth of sensitive and resistant isolates were compared, large differences, typical of bensimidazole fungicide resistance, were obtained. Minimum inhibitory concentration for benomyl-sensitive isolates was 2.5 μg/ml and for menomyl-resistant isolate was >1000 μg/ml. Benomyl-resistant isolates of *C.beticola* have been found to be 100 times more resistant than wild types (11) and mycelial growth of a benomyl-resistant isolate of *Botrytis cinerea* was less inhibited on PDA containing 1000 μg/ml fungicides than was growth of a wild type on agar containing 0.5 μg/ml (2).

Research with other fungi has also shown that resistance to benomyl is generally accompanied by resistance to related benzimidazole compounds (1,4,15). This relation held true in our research, although *C. capsici* isolates showed resistance in varying degrees. As mutation conferring resistance are considered to influence the binding of the benzimidazole nucleus to fungal tubelin (5), and because such mutations generally lead to resistance to all the benzimidazole compounds (17), this differential toxicity was unexpected. Because isolates were also resistant to carbendozim and thiophanate-methyl, use of these fungicides as combination/substitute for benomyl could be a problem, and would put us into the same position we now have with the development of pathogens resistant to benomyl. The answer is probably to develop a sufficient number of effective fungicides, such that their use can be alternated to avoid selection of resistant chilli pathogens for as long as possible.

Although differences did exist between the pathogenicity of isolates these could not be associated with benomyl sensitivity. While resistance to the ergosterol biosynthesis inhibiting fungicides may be associated with a decrease in pathogenicity (10), this does not generally occur for fungal isolates resistant to the benzimadazole fungicides.

Relatively high proportions of benomyl-resistant isolates were found at three sites described here. Such frequencies cannot be considered insignificant if they represent similar proportions of diseased plants which would no longer respond to fungicide treatment. Current available information suggests that the fitness of resistant isolates is not in any way inferior to sensitive isolates. It has been both predicted on theoretical grounds (13) and shown experimentally (9) that once fungicide resistance in a population reaches a frequency of 1%, only a few more sprays are required for almost total resistance to be obtained. If this is true for *C.capsici* then certain sites, such as three of those examined here must present a greater risk for the development of economically significant benomyl resistance.

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