STUDIES ON THE BIOCHEMICAL BASIS OF RESISTANCE AGAINST DICARBOXIMIDE FUNGICIDES

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ABSTRACT

On the basis of present knowledge of the mode of vinclozoline action in Botrytis cinerea, the specific activities of some flavin and oxygen detoxifying enzymes were compared in both wild-type and dicarboximide resistant strains. Only in the case of cytochrome c reductase were significant differences in enzyme activities correlated with the degree of resistance. Furthermore, in resistant strains the catalase activity was remarkably higher than in the sensitive strain. Both these effects are discussed in relation to the biochemical basis of dicarboximide resistance.

INTRODUCTION

A few years after the introduction of dicarboximide fungicides (DCOF), the first evidence of resistant populations was observed, at first in closed locations (greenhouses, store-rooms etc.) (Gullino et al. 1982, Spotts & Cervantes 1986). A loss of fungicide efficiency was also reported (Lacroix & Gouot 1981, Panayotaku & Malathrakis 1983). A short time later resistant isolates were also detected under field conditions, and these led to yield losses (Lorenz et al. 1981). Efficacy of DCOF's decreased especially in vineyards after repeated applications (Dieter 1983), but the loss of efficacy was never a total one (Pommer & Lorenz 1982).

DCOF resistant laboratory strains have been isolated from more than 15 different fungal species. However, the biochemical basis of resistance against DCOF's remains obscure. Many field isolates, as well as laboratory strains have been isolated which vary in resistance degree, vitality and virulence, but no strong correlation could be found between these parameters. Interpretation of these phenomena was more or less speculative, and only the general cross resistance with fungicides of the group of chlorinated hydrocarbons seems consistent (Lyr & Casperson 1982). No cross resistance could be detected against metalaxyl, chlorothalonil, folpet, captafol or thiram (Panagiotaku & Malathrakis 1981). On the basis of our earlier results regarding the mode of action of DCOF (Edlich & Lyr 1986) we studied some possible biochemical causes of the resistance to these fungicides.

In a previous report we demonstrated the interaction of DCOF with some flavin enzymes of B. cinerea (Edlich & Lyr 1987). It was suggested that a peroxidative destruction of membrane lipids was induced in sensitive fungal species via a free radical mechanism. The inner mitochondrial membrane as well as the nuclear envelope seem most sensitive. A number of side effects exist, such as the activation of

proteolysis, DNA single strain breaks etc. In this investigation we studied whether a target muation or specific defence mechanisms play any role in DCOF resistance.

MATERIALS AND METHODS

The following strains of B. cinerea were used in our experiments: S - DCOF sensitive wild-type strain; R_1 - DCOF highly resistant laboratory strain; R_2 - DCOF resistant field isolate from grapes. Isolates were cultivated as stock cultures and in fungicide sensitivity tests on 2% malt agar. For biochemical investigations the fungi were cultivated in a nutrient solution for 24 h. The degree of resistance is shown in Table 1. Resistant strains were stable in culture.

TABLE 1

Effect of vinclozoline on the mycelial growth of different Botrytis strains on 2% malt agar medium.

Strain	Concentration of vinclozoline	
	10 mg/l	100 mg/l
	% growth inhibition*	
S	100	100
R_1	2	37
R_2	50	58

* Growth inhibition is indicated as % of untreated controls from measurements of the diameter of the colonies.
Data represent averages from 3 independent experiments each with 5 repetitons.

Catalase activity was measured by iodometric titration according to Bellamy & Ralph (1968). A superoxide generating system containing xanthine oxidase from milk and xanthine was used for measurement of superoxide dismutase as described by Fukuzawa et al. (1981). Phenol peroxidase activity was determined by oxidation of p-phenylenediamine. Cytochrome c reductases (CytcR) were measured according to Beneviste et al. (1986). The change in extinction at 550 nm was recorded for up to 3 min in an incubation mixture containg 0.2 ml 0.1 M cytochrome c from horse heart, 0.1 ml 1 mM KCN, 0.2 ml 0.1 mM NADPH (or NADH or succinate), 0.2 ml enzyme suspension and 0.5 ml 0.05 M phosphate buffer, pH 7.6. Oxidation rates of NAD(P)H were determined by measuring the extinction at 340 nm for 5 min. Activity of glucose-1-oxidase was determined directly by determination of hydrogen peroxide via peroxidase/p-phenylenediamine.

RESULTS

The specific activities of glucose-1-oxidase, NAD(P)H CytcR, succinate CytcR as well as NAD(P)H oxidases were determined in 3000 x g supernatants of crude homogenates from both sensitive and DCOF resistant mycelia. At first we examined the effect of vinclozoline in

the culture medium on the activities of flavin enzymes. Results are shown in Table 2.

TABLE 2

Relative specific activities of some flavin enzymes in 3000 x g supernatants of crude homogenates from strains S and R_1 cultivated with or without vinclozoline in the media.

Strain	Concentration of vinclozoline (mg/ml)	Relative specific enzyme activities							
		GOD		NADPH CytcR		NADH CytcR		Succ. CytcR	
		*	**	*	**	*	**	*	**
S	0	100		100		100		100	
S	0.3	88		62		21		17	
R_1	0	75	100	80	100	55	100	87	100
R_1	250	56	75	93	116	58	105	86	99

* relative to the value of S-mycelium cultivated without vinclozoline.
** relative to the value of R-mycelium cultivated without vinclozoline.
GOD - Glucose-1-oxidase (100% enzyme activity is equivalent to an increase of extinction at 485 nm of 0.245 per mg protein per min).
NADPH CytcR - NADPH:cytochrome c reductase (100% enzyme activity is equivalent to an increase of extinction at 550 nm of 0.0836 per mg protein per min).
NADH CytcR - NADH:cytochrome c reductase (100% enzyme activity is equivalent to an increase of extinction at 550 nm of 0.0661 per mg protein per min).
Succ. CytcR - Succinate:cytochrome c reductase (100% enzyme activity is equivalent to an increase of extinction at 550 nm of 0.0125 per mg protein per min).

All enzyme activities measured in the R_1 strain were significantly lower in comparison to the wild-type strain. But these differences cannot explain a tolerance of 250 mg/l vinclozoline by the highly resistant R_1 strain. With the exception of GOD which was influenced only slightly, all other flavin enzyme activities in the S strain showed a significant inhibition by vinclozoline. Application of vinclozoline (0.3 mg/ml) caused approximately 50% inhibition of hyphal growth as well as some cytotoxic effects in the sensitive strain. In the resistant strain, the CytcRs were not influenced by vinclozoline in the culture medium, even in 1000 times higher fungicide concentration than was present in the culture of the wild-type strain.

Here, a biochemical difference exists which could play a role in the resistance mechanism because of a decreased target-fungicide interaction. In further experiments under the same conditions the activities of NAD(P)H oxidases were determined in S as well as in the R_1 strain (Table 3).

TABLE 3

Specific activities of NAD(P)H oxidases in 3000 x g supernatants of crude homogenates form both S and R_1 strains cultured with or without vinclozoline in the medium.

Strain	Concentration of vinclozoline	Relative specific activity			
		NADPH oxidase		NADH oxidase	
		*	**	*	**
S	–	100			100
S	0.3	43			35
R_1	–	69	100	67	100
R_1	250	39	57	47	70

* Relative to the value of S-mycelium cultivated without vinclozoline.
** Relative to the value of R-mycelium cultivated without vinclozoline.
NADPH oxidase (100% enzyme activity is equivalent to a decrease of extinction at 340 nm of 0.0882 per mg protein per min).
NADH oxidase (100% enzyme activity is equivalent to a decrease of extinction at 340 nm of 0.0775 per mg protein per min).

The effects of vinclozoline were not so clear as in the case of CytcR, but losses in activity could not be detected in cultures grown in the presence of vinclozoline. Compared to the results with the reductase activities, 250 mg/ml vinclozoline in the culture medium caused less effect on oxidase activities of the R_1 strain than did 0.3 mg/ml vinclozoline in the culture medium of the wild-type strain. We obtained similar results for the effects of vinclozoline under in vitro conditions (Table 4). 0.25 mM vinclozoline inhibited strongly all CytcRs studied. Therefore, a drastic mutation of reductases resulting in a total loss of affinity towards vinclozoline could be excluded.

TABLE 4

Effect of vinclozoline (0.25 mM) on cytochrome reductase (CytcR) from both S and R_1 strains in vitro.

Strain	Relative specific enzyme activities	
	NADPH CytcR	NADH CytcR
S	49	25
S*	31	0
R_1	72	63
R_1*	35	23

All data are expressed in % of untreated controls.
S* – cultured in 0.3 mg/l vinclozoline.
R_1* – cultured in 250 mg/l vinclozoline.

Besides changes in target affinity, activation of oxygen detoxifying enzymes could provide an additional mechanism for DCOF resistance. Therefore, the specific activities of catalase (CAT), superoxide dismutase (SOD) and phenol peroxidase (POD) were compared in both wild strain and the resistant strains ($R_{1,2}$). Results are summarised in Table 5.

TABLE 5

Comparison of the relative specific activities of catalase (CAT), superoxide dismutase (SOD) and phenol peroxidase (POD) in extracts from sensitive and DCOF resistant strains of Botrytis cinerea.

Strain	Concentation of vinclozoline (mg/l)	Relative specific enzyme activity		
		CAT	SOD	POD
S	0	100	100	100
S	0.3	909	26*	n.d.
R_1	0	266	53	104
R_1	250	357	41	n.d.
R_2	0	175	76	188

All data represent averages from 5 independent experiments and are related to the value of the untreated sensitive mycelium.
* Reaction was disturbed by vinclozoline from homogenate.
n.d. - not detected.

Comparison of activities of oxygen detoxifying enzymes in sensitive and DCOF resistant strains revealed a 9-fold increase in CAT activity in the sensitive strain after treatment with 0.3 mg vinclozoline per litre culture medium. By contrast, in the resistant strains vinclozoline resulted in only a slight increase in CAT activity, but the level of both CAT and POD activity was significantly higher in untreated resistant mycelia without addition of vinclozoline, than in untreated sensitive mycelia.

DISCUSSION

In all experiments with B. cinerea the presence of flavin enzymes, which possibly act as a target for dicarboximides could be detected in all strains. Consequently a mutation in one of these enzymes could be excluded experimentally as a resistance mechanism. Such enzymes are obviously essential for fungi. Especially in the case of cytochrome c reductases these were less sensitive both in vivo and in vitro to vinclozoline but this depended on the degree of resistance in each strain. Homogenates from vinclozoline-treated mycelia generally showed a significantly lower level of enzyme activities in comparison to the untreated controls. We suggest that cytochrome c reductases were inactivated by the fungicide, possibly via the generation of active oxygen. DCOF resistance is apparently characterised partly by reduced affinity of these reductases for vinclozoline, even at very high fungicide concentrations.

In previous studies (Edlich & Lyr 1987) on the mode of action of dicaboximides, it was suggested that vinclozoline induced an oxidative stress in sensitive fungi by generation of active oxygen species. Therefore, DCOF resistance could be caused by an activation of oxygen detoxifying enzymes. Comparing the specific activities of CAT, SOD and POD of both sensitive and resistant strains, we found that the CAT level in resistant strains is much higher than in the wild-type strain. CAT activity rises rapidly in the sensitive strain after vinclozoline treatment due to an increased level of reduced oxygen. This, however, seems insufficient to prevent oxidative damages in cells. In contrast, high CAT activity in resistant mycelium, in combination with other factors, provided enough protection against active oxygen. Possibly this is the reason for the cross resistance to the chlorinated hydrocarbons which are also known to induce oxidative stress (Edlich & Lyr 1986).

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MODES OF ACTION OF CARBENDAZIM AND ETHYL N-(3,5-DICHLOROPHENYL) CARBAMATE ON FIELD ISOLATES OF BOTRYTIS CINEREA.

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ABSTRACT

The [^{14}C]-carbendazim (MBC) binding activity of tubulin from field isolates of Botrytis cinerea has been determined in cell-free extracts by a modified charcoal assay, and traditional gel filtration. Benzimidazole sensitive (S) isolates showed high binding constants and maximum binding capacities, whereas binding was undetectable in highly resistant (R) and intermediate resistant (IR) isolates at a similar range of [^{14}C]-MBC concentrations. Differential affinity for MBC could be sufficient to account wholely for carbendazim sensitivity in B. cinerea. Negatively-correlated cross-resistance between MBC and ethyl N-(3,5-dichlorophenyl) carbamate (EPC) was observed in vitro for S and R, but not IR-isolates. However, [^{14}C]-EPC binding was not obtained in cell-free extracts from any strain. Although extreme lability or absence of a key binding factor cannot be discounted, this suggests that N-phenyl carbamate action on R-strains of B. cinerea is not analogous to the MBC-binding activity of S-isolates, but may involve a more subtle mechanism.

INTRODUCTION

Under suitable conditions, almost any temperate plant may be susceptible to infection by Botrytis cinerea. The disease is of particular economic importance on vines, ornamental crops and salad vegetables. The benzimidazole fungicide benomyl, together with its conversion compound carbendazim (methyl benzimidazol-2-yl carbamate; MBC; 'Bavistin'), initially showed effective antifungal activity against a wide range of pathogens, including B. cinerea. Resistance was soon reported, however, and has led repeatedly to breakdown of disease control since then.

The primary mode of action of carbendazim is generally considered to be binding to β-tubulin, leading to interference with microtubule assembly and hence cytoskeletal functioning. In the non-pathogenic filamentous fungus Aspergillus nidulans, binding studies with radiolabelled MBC have shown that sensitivity or resistance are correlated with a differential binding affinity of tubulin for the fungicide (Davidse, 1975; Davidse & Flach 1977). Resistance has been associated with a mutation in the β-tubulin gene in A. nidulans (Sheir-Neiss et al. 1978) and other benzimidazole sensitive organisms. Studies with plant pathogenic fungi have been rather limited, and the situation is very much less clearly defined. However, correlations between in vitro sensitivity and the affinity of tubulin for carbendazim

have been observed in Penicillium expansum (Davidse, 1982), Venturia nashicola (Ishii & Davidse 1986) and Fusarium oxysporum (Gasztonyi et al. 1987). In B. cinerea, tubulin-like protein has been identified through radiochemical-MBC binding assays of sensitive strains, and differential binding observed between field resistant and sensitive isolates (Gessler et al. 1980; Tripathi & Schlösser, 1982; Groves et al. 1988). In the work reported here, a modified charcoal assay procedure has been developed for B. cinerea extracts, and used to quantify the affinity of tubulin for MBC in various strains.

In B. cinerea and some other plant pathogenic fungi, negatively-correlated cross-resistance between carbendazim and N-phenyl carbamates has been reported for highly MBC sensitive and highly resistant isolates (Kato et al. 1984; Gasztonyi et al. 1986). Methyl N-(3,5-dichlorophenyl) carbamate (MDPC) induced morphological changes in germ tubes and arrested mitosis in benzimidazole resistant strains of B. cinerea in a manner similar to carbendazim on germ tubes of sensitive strains (Suzuki et al. 1984). Direct binding to resistant tubulin is a possible mode of action of N-phenyl carbamates. In the work reported here, cell-free extracts of B. cinerea have been tested for the ability to bind ethyl N-(3,5-dichlorophenyl) carbamate (EPC), which shows similar behaviour in vitro to the methyl ester.

MATERIALS AND METHODS

Isolates, culture conditions and preparation of mycelial extracts

Two carbendazim sensitive (S), three highly resistant (R) and two intermediate resistant (IR) field isolates of B. cinerea were kindly donated for this work (Table 1): strain nos. 1-4 by A.D.A.S., no. 5 from the Jealotts Hill collection, and nos 6-7 by the Food Research Institute, Norwich. Culture storage, conidial germling growth, mycelial extract preparation and soluble protein determination were as described (Groves et al. 1988). Inhibition of growth by 'Bavistin' and EPC was measured on plates of 2% dextrose, 0.4% yeast extract, 0.2% mycological peptone, 1.5% agar medium. Fungicides were added in ethanol to 0.1% (vol/vol). Concentrations effecting total inhibition of growth (MIC) and 50% reduction in radial growth (EC50) were estimated from plots of radial growth against logarithm of concentration.

Gel filtration assay

[^{14}C]-MBC (specific activity 30 mCi/mmol) or [^{14}C]-EPC (specific activity 26.7 mCi/mmol) in dimethyl sulphoxide (DMSO) was incubated with crude extract amended with 1 mM guanosine 5'-triphosphate (GTP) and 0.5% (vol/vol) solution PPL (40 mg/ml phenyl methyl sulphonyl fluoride, 0.6 mg/ml pepstatin A and 10 mg/ml leupeptin in DMSO) for 2 hours at 4°C; total DMSO in the incubation mixture was 1% (vol/vol). Binding was measured by gel filtration of 1.5 ml on a Sephacryl S-200 (Pharmacia) column (45 x 1.5 cm), as described by Groves et al. (1988). Protein elution was monitored continuously by u.v. absorption at 254 nm. Radioactivity in protein fractions, after correction for background, was considered to represent bound fungicide.

Charcoal assay

Binding of [^{14}C]-MBC was also measured by a charcoal adsoption assay. Crude extracts were incubated either with [^{14}C]-MBC or with excess (50 μM) unlabelled carbendazim (technical grade) for 2 hours at 4°C. The latter (control) samples were amended with [^{14}C]-MBC immediately prior to assay. Triplicate 250 μl aliquots of incubation mixtures were combined with equal volumes of activated charcoal suspension, at 6 mg/ml in elution buffer. After mixing (6 inversions/min) for exactly 10 mins at 4°C to permit adsorption of unbound MBC, suspensions were centrifuged at 11,600 x g for 5 min. The radioactivity in supernatants (200 μl) and incubation mixtures (50 μl) was assessed. The amounts of unadsorbed radioactivity in incubation mixtures containing bovine serum albumin (BSA) at 20 mg/ml and 0-10 μM [^{14}C]-MBC were systematically 30% less than those containing an additional 50 μM unlabelled MBC. Determinations of unadsorbed radioactivity in controls of B. cinerea extracts were therefore adjusted accordingly. The difference in amount of radioactivity between sample and corrected control was assumed to represent bound [^{14}C]-MBC. Free MBC was determined from the difference between bound and total MBC in the incubation mixture.

RESULTS AND DISCUSSION

Charcoal assay evaluation

The published charcoal assay (Davidse & Flach 1977) used [^{14}C]-MBC/bovine serum albumin incubation mixtures to determine the percentage of free MBC adsorbed. These controls were inappropriate for B. cinerea extracts, as conidial germlings produce variable (but generally large) quantities of extracellular polysaccharide which may reduce charcoal adsorption correspondingly. To overcome this, a novel 'internal control' has been developed: [^{14}C]-MBC binding is blocked with excess unlabelled MBC.

The MBC-binding activities of extracts from isolate 1 were determined in parallel gel filtration and charcoal assays. Charcoal assay estimates were typically about 10% higher at 5 μM [^{14}C]-MBC. Similar results were obtained in work with extracts from sensitive Aspergillus nidulans (Davidse & Flach 1977), and may be due to reduced dissociation of MBC from the binding-complex. The charcoal assay is less laborious, faster and more cost-effective than gel filtration, and is particularly suitable for parallel multiple determinations.

The presence of <1% DMSO in the buffers from extraction onward was essential for detection of binding activity. Substitution of DMSO with ethanol at extraction lead to 90% loss of activity; however, addition of ethanol to a DMSO-treated extract did not result in loss of activity. Hence, DMSO appears to stabilize the binding site. The stabilizing effect of DMSO on brain tubulin has been characterized (Algaier & Himes 1988).

Mode of action of carbendazim

The MBC-binding properties of extracts from seven strains of B. cinerea were investigated with the charcoal assay. Crude supernatants were incubated with [^{14}C]-MBC at 5 concentrations within the range 0.1 to 5 μM. At 5 μM, binding was detected only in extracts from the two highly MBC sensitive sensitive isolates (Table 1). Results from the sensitive isolates

TABLE 1

Comparison of fungicide growth inhibition and MBC binding:

Strain number	MIC (μM) MBC	MIC (μM) EPC	EC50 MBC	EC50 EPC	[^{14}C]-MBC bound (pmol/mg protein)	MBC class
1	0.5	>400	0.25	130	14.43 (1.30) *	S
2	>5000	8.0	>5000	0.65	0.36 (0.46)	R
3	0.5	>400	0.25	80	5.57 (3.33) *	S
4	>5000	4.0	>5000	0.65	0.42 (0.07)	R
5	>5000	4.0	>5000	0.65	0.19 (0.16)	R
6	>5000	>400	30	60	0.78 (0.11) *	IR
7	>5000	>400	80	80	1.01 (0.42) *	IR

Binding to crude extract preparations from seven strains of B. cinerea at 5 μM [^{14}C]-MBC. Mean (standard deviation) of bound MBC (charcoal assay) was determined in two or four* experiments.

TABLE 2

MBC-binding properties of Botrytis cinerea crude extracts, and comparison with those of three strains of Aspergillus nidulans:

Organism	Strain	EC50 (μM)	S_o (pmol/mg)	K (l/mol)	1/K (μM)
B. cinerea	1	0.25	16.0(1.5)	3.0(1.0)x10^6	0.3
B. cinerea	3	0.25	5.8(3.8)	3.0(1.4)x10^6	0.3
A. nidulans	186 +	1.5	26.4(1.1)	1.6(0.05)x10^6	0.6
A. nidulans	003 +	4.5	33.0(10.1)	4.5(1.1)x10^5	2.2
A. nidulans	R +	95	18.9(2.1)	3.7(0.5)x10^4	27
B. cinerea	2	>5000	-	-	-
B. cinerea	4	>5000	-	-	-

Mean (standard deviation) of K and S_o were determined in four experiments.

1/K = free MBC concentration at which 50% of binding sites are occupied.

\+ data from Davidse & Flach (1977)
\- data not calculable (see text)

displayed sigmoid form in plots of log [free MBC] vs. [bound MBC]. A plateau was observed in each case at 5 μM, due to the approach of the maximum binding capacity asymptote. It was therefore possible to determine the apparent binding constants (K) and maximum binding capacities (S_o) for these isolates. Results are summarized, and compared with those from three isolates of A. nidulans (Davidse & Flach 1977) in Table 2.

The apparent binding contant in strain 1 was even higher than that of the supersensitive mutant of A. nidulans. However, the maximum binding capacity was only slightly smaller than that of the three A. nidulans strains. In extracts of strain 3 values of K were as high as those of strain 1, but estimates of S_0 were lower and more variable. This could reflect variation in tubulin content between individual strain 3 extracts, or difficulty in stabilization of MBC binding - even in the presence of 1% (vol/vol) DMSO. Determination of S_0 should therefore be interpreted cautiously.

In B. cinerea strains 1 and 3, and the three A. nidulans strains, the concentrations of MBC effecting 50% reduction in radial growth were correlated with the concentrations of free MBC in extracts at which 50% of the binding sites were occupied (1/K). In contrast, no correlation was observed between EC50 and S_0. The affinity for MBC could reasonably be sufficient to account wholely for differences in sensitivity to MBC in B. cinerea.

As binding in extracts from strains 2, 4, 5, 6 and 7 was consistently low, and did not display a sigmoid form on plots of log [free MBC] vs. [bound MBC], it was not possible to seek values of K and S_0 from these isolates (Table 2). Lack of binding in the IR strains (6 and 7) suggests that intermediate resistance is probably associated with a decreased affinity for MBC. This refutes the possibilities of the co-expression of mutant and wild-type tubulins, or of an exclusively non-binding mechanism, such as differential permeability of hyphae to MBC, operating in these isolates. The mutations leading to MBC intermediate resistance and EPC sensitivity would appear to be biochemically distinct.

Mode of action of ethyl N-(3,5-dichlorophenyl) carbamate

The highly S and R-isolates of B. cinerea showed negatively-correlated cross-resistance with EPC (Table 1). The IR-isolates were as sensitive to EPC as the S-isolates, and formed a third distinct sensitivity class. In a number of [^{14}C]-EPC binding experiments, assessed by gel filtration, no carbendazim resistance-dependent EPC-binding activity was observed: extracts from both resistant and sensitive strains showed comparable elution profiles. Even with 50 µM [^{14}C]-EPC in the incubation mixture, no peak in radioactivity was detected in protein-containing fractions and 1% DMSO did not enhance binding. Although extreme lability or absence of a key binding factor cannot be discounted, this suggests that N-phenyl carbamate action on resistant strains of B. cinerea is not analogous to the MBC-binding activity of sensitive isolates, but may involve a more subtle mechanism.

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SELECTIVE EFFECTS OF PROPAMOCARB AND PROTHIOCARB ON THE FATTY ACID COMPOSITION OF SOME OOMYCETES

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ABSTRACT

The carbamate fungicides propamocarb and prothiocarb have been considered to act by affecting the biosynthesis of a membrane constituent in Pythiacious species. Fatty acids were analysed in mycelium of Pythium ultimum grown in the presence of propamocarb and prothiocarb, and in four Phytophthora species similarly treated with propamocarb. In each case an unusual fatty acid, was found to accumulate. This was identified as Δ^7-hexadecenoic acid which has not previously been reported in fungi. Its structure was confirmed by synthesis of the cis-isomer. It is not clear if production of Δ^7-hexadecenoic acid is causally related to the selective action of the two fungicides on oomycetes, but it was not formed in carbamate-treated ascomycete and basidiomycete fungi, nor in oomycetes treated with metalaxyl or cymoxanil. The acid had little effect on fungal growth when applied externally.

INTRODUCTION

Propamocarb (I) and prothiocarb (II) (Fig. 1) are carbamic acid esters developed by Schering AG to combat diseases caused by oomycetes, particularly Phytophthora and Pythium species (Sijpesteijn et al. 1974). They are especially effective when used as soil drenches or as seed/bulb treatments to control root and stem diseases. In practice, however, they have been rather overshadowed by the discovery of the phenylamides which are generally more active and have a broader spectrum of action against oomycetes (Schwinn & Urech 1986). Nonetheless, the development of resistance to the phenylamides, and the need to identify new target sites for effective fungicides, are powerful incentives for studies aimed at understanding how these carbamates exert their selective antifungal effects.

propamocarb prothiocarb

Figure 1. Structures of carbamate fungicides

Earlier studies have demonstrated that propamocarb or prothiocarb applied to Pythium species increased cell membrane permeability with a leakage of cell constituents (Papavizas et al. 1978). The carbamates were fungistatic rather than fungicidal, and mycelium treated with high dosages recommenced growth when transferred to new media. Because propamocarb caused leakage only when it was in contact with the fungus during growth, and not after application to the fully developed

mycelium, it has been inferred that it is likely to operate by affecting the biosynthesis of a membrane constituent rather than having a direct effect on the membrane itself (Langcake et al. 1984). Interestingly both growth inhibition and membrane leakage induced by propamocarb could be largely counteracted by addition of cholesterol to the medium (Papavizas et al. 1978).

In considering what biochemical process might be altered by these fungicides, we decided to take as a lead the mode of action of the thiocarbamate herbicides. Thiocarbamates are believed to inhibit long chain fatty acid biosynthesis in plants although the precise details are unclear (Harwood et al. 1987). Because these herbicides bear some structural similarity to the carbamate fungicides we decided to investigate possible effects of the fungicides on the fatty acid composition of susceptible fungi. Propamocarb was used in most of the experiments as mode of action studies on prothiocarb may be complicated by its known metabolism to ethyl mercaptan, which itself may be fungitoxic (Kerkenaar & Sijpesteijn 1977).

MATERIALS AND METHODS

Growth of fungi and treatment with fungicides

Unless otherwise stated the following procedure was adopted. Fungicides were incorporated into sterile glucose-yeast extract medium (20 ml) which was then inoculated with plugs of fungal mycelium grown on V-8 agar. The flasks were then incubated for 3 - 5 days at 25°C in darkness without agitation. After removal of the agar plugs, the mycelium was filtered off and freeze-dried.

Extraction and analysis of fatty acids

Freeze-dried fungal mycelium was extracted with chloroform/methanol (30 ml, 2 : 1 by vol) and the extracts filtered and evaporated. The residue was then hydrolysed with 5% ethanolic potassium hydroxide. After first washing this solution with hexane to remove neutral constituents, the total fatty acid fraction was obtained by acidifying the aqueous layer and then extracting with ethyl acetate. These extracts were dried over sodium sulphate, evaporated, and the fatty acids methylated with diazomethane in ether. Fatty acid methyl esters were analysed on a 25m x 0.25 mm RSL 500 BP column using helium carrier gas (0.75 kg cm^{-1}) with a programme of 170° to 200° at 2° min^{-1}. GC-mass spectra were obtained on a Kratos MS-80 coupled to this column.

The various lipid classes were isolated by preparative thin-layer chromatography of the chloroform/methanol extract on Merck silica gel plates after development with hexane/ether/acetic acid (80 : 30 : 1 by vol). Fatty acids were subsequently obtained from individual classes by hydrolysis as described above.

Identification of Δ^7-hexadecenoic acid

The methylated fatty acids (1 mg) from propamocarb treated Pythium ultimum were dissolved in pyridine/dioxane (1 : 8 by vol, 200 μl) and an osmium tetroxide/dioxane solution (2%, 40 μl) added. After work-up with sodium sulphite (Capella & Zorzut 1968), the diol thus formed was silylated with pyridine/N,0-bis(trimethylsily)acetamide (1 : 1 by vol, 60 μl) and analysed by GC-mass spectrometry.

Synthesis of the fatty acid was by the Wittig reaction. Nonyltriphenylphosphonium bromide was prepared by heating 1-bromononane and triphenylphosphine in benzene under reflux for 2 days. Treatment of this salt with butyl lithium in dry benzene under helium then generated the ylide (Fig. 2) which when reacted with methyl-7-oxoheptanoate afforded methyl cis- 7-hexadecenoate.

$$CH_3(CH_2)_7CH{=}PPh_3 + CHO(CH_2)_5CO_2CH_3 \longrightarrow CH_3(CH_2)_7CH{=}CH(CH_2)_5CO_2CH_3$$

Figure 2. Synthesis of methyl cis-7-hexadecenoate

RESULTS

Identification of Δ^7-hexadecenoic acid

An unknown fatty acid was induced in substantial amounts in P. ultimum following treatment with propamocarb (5 ppm; Table 1). The methyl ester had a retention time intermediate between those of palmitic (16 : 0) and palmitoleic (16 : 1 Δ^9) methyl esters. Treatment with osmium tetroxide followed by silylation produced a silylated diol (Capella & Zorzut 1968) which in the mass spectrometer produced fragment ions at m/z 215 and 231. This established the position of the double bond as Δ^7. Confirmation of structure was by synthesis using a Wittig reaction (Fig. 2). Synthetic methyl cis-7-hexadecenoate was identical to the methylated natural product in all respects.

TABLE 1

Total fatty acid composition of Pythium ultimum following treatment with four fungicides used to control oomycetes[a].

Treatment	Fatty acids (% of those identified)[b]								
	14:0	16:0	16:1(Δ^9)	16:1(Δ^7)	18:0	18:1	18:2	20:3	20:4
Untreated	20.5	29.7	9.9	trace	4.6	14.7	13.7	3.2	3.6
Propamocarb (5 ppm)	18.3	20.2	4.9	18.1	3.2	11.8	13.9	3.2	6.3
Prothiocarb (5 ppm)	15.2	23.2	5.6	18.2	3.9	16.2	9.8	3.7	4.2
Metalaxyl (0.005 ppm)	19.0	33.4	13.3	trace	2.4	10.1	13.1	3.5	5.0
Cymoxanil (25 ppm)	21.3	34.2	11.3	trace	2.5	11.9	10.8	2.3	5.6

[a] Data presented are mean of 3 experiments. Growth inhibition at the given dosages was in the range 30 - 70%.

[b] Only traces of other fatty acids were detected.

TABLE 2

Total fatty acid composition of Phytophthora species after treatment with propamocarb (100 ppm).

Treatment	Fatty Acids (% of those identified)[a]						
	14:0	16:0	16:1(Δ^9)	16:1(Δ^7)	18:0	18:1	18:2
P. megasperma	13.9	38.6	3.5	2.5	3.0	13.4	25.2
+ propamocarb (88)[b]	12.5	42.0	2.7	9.0	9.0	12.0	12.8
P. palmivora	39.1	31.5	8.7	1.5	1.5	7.2	10.5
+ propamocarb (50)	32.9	19.1	5.5	25.1	1.3	7.7	8.2
P. cinnamomi	18.6	46.6	3.6	–	2.1	15.7	13.3
+ propamocarb (29)	27.5	19.7	4.6	30.0	1.0	8.7	8.5
P. nicotianae	34.7	24.6	3.7	–	2.6	22.8	11.4
+ propamocarb (35)	33.2	23.9	3.1	7.1	3.4	21.5	7.7

[a] Small amounts of longer chain fatty chains were sometimes observed.

[b] Percentage growth inhibition.

TABLE 3

Effects of treatment period on the fatty acid composition of Pythium ultimum treated with propamocarb[a].

Time after treatment (h)	Fatty acids (% of those identified)								
	14:0	16:0	16:1(Δ^9)	16:1(Δ^7)	18:0	18:1	18:2	20:3	20:4
1 (0)[b]	17.5	38.1	4.3	trace	5.8	14.0	10.1	3.3	7.0
7 (38)	14.6	30.0	3.6	5.0	4.9	14.9	12.9	4.0	10.2
16 (0)	17.7	29.7	3.7	11.3	4.7	10.1	12.2	3.5	7.2
24 (29)	18.2	20.1	4.8	18.2	3.2	12.0	14.0	3.2	6.2

[a] Mycelium grown in static culture with 5 ppm propamocarb.

[b] Percentage growth inhibition.

Distribution of Δ^7-hexadecenoic acid among lipid classes

Δ^7-Hexadecenoic acid was found to be present at approximately the same relative amounts in the triacylglycerol, free fatty acid mono and/or diacylglycerol and phospholipid fractions of P. ultimum treated with propamocarb (5 ppm).

TABLE 4

Effect of fungicide dose on fatty acid composition of Pythium ultimum treated with propamocarb[a]

Dosage (ppm)	Fatty acids (% of those identified)								
	14:0	16:0	16:1(Δ^9)	16:1(Δ^7)	18:0	18:1	18:2	20:3	20:4
0 (0)[b]	22.3	28.1	6.2	trace	4.2	12.0	12.2	4.2	10.9
0.15 (32)	15.1	28.7	5.0	8.2	6.8	12.6	12.8	2.9	7.8
0.31 (10)	21.8	25.1	6.1	3.7	4.6	11.3	11.7	4.6	11.1
0.63 (58)	13.7	24.9	3.5	13.0	6.4	10.4	13.4	4.8	9.9

[a] Fungus grown in static culture for 5 days.

[b] Percentage growth inhibition.

Fatty acid composition of insensitive fungi treated with propamocarb

Growth of Ustilago maydis, Septoria tritici, Botrytis cinerea, Pyricularia oryzae and Pyrenophora teres was not inhibited by propamocarb (100 ppm) and Δ^7-hexadecenoic acid was not detected in either control or treated cells.

Fungitoxicity of Δ^7-hexadecenoic acid

When added as a 1% ethanolic solution to a glucose-yeast liquid medium synthetic Δ^7-hexadecenoic acid was inhibitory to P. ultimum at 100 ppm but not at lower concentrations. In this respect it was comparable to a number of other saturated and unsatured fatty acids.

DISCUSSION

The experiments reported here revealed that application of propamocarb and prothiocarb to P. ultimum produced changes in fatty acid composition at dosages which inhibited growth. No major changes in constitutive fatty acids were observed other than a decrease in the 16 : 0 (palmitic) acid (Table 1). However, the most marked change in the fatty acid profile was the appearance in large quantities of a new acid, found only in trace amounts in untreated mycelium. This was identified as Δ^7-hexadecenoic acid. We were unable to find previous reference to the occurrence of this acid in fungi, but trace amounts have been detected in animals and plants.

It is important to consider how this Δ^7 acid might be produced, and whether its presence or mechanism of formation is causally related to the mode of action of the carbamate fungicides. No increased production was found (Table 1) in P. ultimum mycelium treated with metalaxyl (mode of action - inhibition of RNA biosynthesis) or cymoxanil (mode of action - unknown), hence the acid does appear not to be simply a product of fungicide damaged mycelia.

Five species of higher fungi, insensitive to propamocarb, did not produce the Δ^7-hexadecenoic acid. By contrast four species of Phytophthora, a genus generally well controlled *in vivo* by the carbamate fungicides, all showed an increase (Table 2). However, in this and other experiments the correlation between Δ^7-hexadecenoic acid production and growth inhibition *in vitro* was poor (Tables 2, 3 and 4). A time course experiment (Table 3) demonstrated a gradual accumulation of Δ^7-hexadecenoic acid over a 24 h period while a dose-response study produced some evidence of a dose-dependency (Table 4).

Although fatty acids may have antimicrobial properties (Kerwin 1987) there appears no obvious reason why Δ^7-hexadecenoic acid should be especially fungitoxic. However, it is conceivable that its formation *in vivo* and incorporation into phospholipids may lead to the formation of cellular membranes with modified physicochemical properties which can, however, be stabilised by cholesterol thereby nullyfying the fungistatic effect (Papavizas *et al*. 1978).

One possible explanation for the production of Δ^7-hecadecenoic acid in oomycetes is the induction or activation of a specific Δ^7 desaturase enzyme acting on a C 16 : 0 substrate. An alternative hypothesis is that the Δ^7-hexadecenoic acid could be produced by incomplete β-oxidation of Δ^9-octadecenoic (oleic) acid. It remains to be established whether formation of the acid is causally related to the selective action of carbamate fungicides on oomycetes.

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ISOLATION, CHARACTERISATION AND GENETIC ANALYSIS OF MUTANTS OF *ASPERGILLUS NIDULANS* RESISTANT TO TOLCLOFOS-METHYL

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ABSTRACT

Spontaneous and U.V.-induced mutants of *Aspergillus nidulans* were recovered from both sectored colonies and monoconidial isolates growing on synthetic medium amended with 25-200 µg tolclofos-methyl/ml. They differed considerably in morphology, pigmentation, growth rate, conidiation and level of resistance to four fungicides. All mutants tested were cross-resistant to tolclofos-methyl, dichlobenil, quintozene and vinclozolin, they produced fewer conidia than their wild type parent, and were abnormally sensitive to medium supplemented with 0.4M NaCl or 0.7M glucose. Some mutants required the fungicides for maximum growth and conidiation. Mutants carried mutations in one of three fungicide resistance genes located on linkage groups III and V. Most mutant alleles were recessive but some were partially dominant to their wild type alleles.

INTRODUCTION

Tolclofos-methyl is used to control various soil-borne fungal diseases of vegetables and ornamentals, especially those caused by *Rhizoctonia solani*. Its mode of action is unknown, but is probably similar to that of the aromatic hydrocarbon and dicarboximide fungicides (Leroux & Fritz 1984). Fungi can develop resistance to these fungicides (Leroux & Fritz 1984), and a few of these resistant mutants have been explored genetically (Grindle 1987).

The aim of this investigation was to assemble a collection of stable, defined mutants of *A. nidulans* to probe the action of tolclofos-methyl and the genetics of resistance to this fungicide. The saprophytic fungus *A. nidulans* is of no importance in plant pathology, but nevertheless is an excellent "model" filamentous fungus for detailed genetic studies under laboratory conditions (Clutterbuck 1974).

MATERIALS AND METHODS

Isolates

The haploid laboratory strain ("wild type") from which all mutants were derived was a double auxotroph (*pro bi*) from the Glasgow collection. The haploid master strain E (MSE) with genetic markers on all eight linkage groups (McCully & Forbes 1965) was used to assign the fungicide resistance genes to particular linkage groups and to derive fungicide resistant haploid progeny with new combinations of marker genes for dominance and allelism tests.

Media and chemicals

All strains were grown at 37°C on a complete medium (CM) consisting of Czapek-Dox salts, 10 g/l glucose and 20 g/l agar, supplemented with hydrolysed casein, hydrolysed nucleic acids, yeast extract and vitamins. The sterilized medium was amended with three fungicides (added as suspensions in deionised water) and the chemically related herbicide dichlobenil (added as a solution in acetone) after the medium had cooled to 55°C. The formulations used were dichlobenil (technical grade), quintozene (Brassicol 20% a.i. WP), tolclofos-methyl (Rizolex 50% a.i. EC) and vinclozolin (Ronilan, 50% a.i. WP).

Isolation of mutants

Mutants were obtained by two methods. In method 1, mass conidial inocula were placed at the centre of Petri dishes of CM amended with 25 µg tolclofos-methyl (TM)/ml. After 3-7 days incubation, inocula were removed from sectors with abnormal morphology or improved growth and incubated on CM to obtain colonies for further analysis. In method 2, suspensions of conidia in dilute saline (1% w/v) were exposed to U.V. light until only 5% remained viable. Samples of control (not irradiated) and mutagenised (irradiated) conidia were added to bottles of cool (55°C) CM amended with 200 µg TM/ml, the medium was dispensed into Petri dishes, and the dishes incubated until the resistant conidia had formed colonies from which inocula could be removed for further analysis. Presumptive mutants obtained by methods 1 and 2 were tested rapidly on CM amended with TM to identify genuine mutants. These mutants were again tested rapidly on CM and CM amended with various concentrations of TM, quintozene and vinclozolin to record variations in growth rate, morphology, conidiation and levels of fungicide resistance. Selected mutants representing the diversity of phenotypes found by these rapid tests were analysed in more detail as described below.

Determinations of growth rate, conidiation, fungicide resistance and osmotic sensitivity

Monoconidial isolates obtained by incubating conidia on CM for 18h were transferred to the centres of dishes of CM and CM amended with 1, 2, 4, 8, 16, 32, 64 and 100 µg fungicide/ml, 0.4 M NaCl, and 0.7 M glucose. There were three replicates of each treatment.

Growth rates were determined from colony diameters after 48h and 96h incubation. These were used to calculate ED50 values (Effective Dose of fungicide, µg a.i./ml, that reduces growth by 50%) from the regression of growth rate on log fungicide concentration.

Conidial densities were determined from the numbers of conidia in discs (6 mm diam.) taken from 7-day-old colonies. Three discs from each colony were shaken vigorously in dilute Tween 80 detergent to obtain conidial suspensions for haemacytometer counts.

Genetic analyses

Methods for synthesising diploids, assigning genes to specific linkage groups by haploidisation of diploid cultures, and deriving

ascospores for meiotic analysis have been described (Clutterbuck 1974, Forbes 1959, Pontecorvo et al. 1953).

For dominance/recessivity tests, mutants carrying fungicide resistance genes (r^-) were combined with strains carrying wild-type alleles (r^+) to produce diploids ($r^-//r^+$). Haploids and diploids were grown for 72h on CM and CM amended with 1-100 µg TM/ml. The mutant allele r^- was considered to be recessive to r^+ if the $r^-//r^+$ diploid was phenotypically indistinguishable from the fungicide sensitive r^+ haploid. Otherwise, if the $r^-//r^+$ diploid was phenotypically indistinguishable from the fungicide resistant r^- haploid, the resistance gene was dominant.

For allelism tests, pairs of fungicide resistant mutants were intercrossed and ascospores incubated on CM and CM amended with TM to distinguish fungicide-resistant and -sensitive progeny. The mutants were considered to be allelic if less than 1% of the progeny were fungicide sensitive recombinants.

RESULTS AND DISCUSSION

Isolation of mutants

Colonies produced by mass-conidial inocula on CM amended with 25 µg TM/ml (method 1) were phenotypically unstable, producing 1-4 obvious sectors on each dish. Out of 96 such sectors taken from 50 colonies, 38 proved to be genuine TM-resistant mutants. Conidia embedded in CM amended with 200 µg TM/ml (method 2) produced 5 TM-resistant mutants from 5.6×10^6 control spores (mutation frequency = 0.9×10^{-6} viable conidia) and 53 mutants from 0.232×10^6 U.V.-irradiated spores (mutation frequency = 228×10^{-6} viable conidia).

Phenotypes of mutants

The resistant mutants varied considerably in morphology, pigmentation, growth rate, sporulation and levels of resistance to the fungicides. About 70% of the mutants showed high resistance to TM (ED50 > 100 µg/ml, compared to 13.4 µg/ml of the parent strain), high resistance to quintozene (ED50 > 100 µg/ml compared to 11.6 µg/ml) and low resistance to vinclozolin (ED50 > 16 < 32 µg/ml compared to 7.2 µg/ml). About 30% of the mutants were also very resistant to vinclozolin (ED50 > 100 µg/ml).

Representative mutants, designated USY-1 to -11, were analysed in more detail for differences in growth rate and sporulation on various media (Tables 1, 2). The main points to emerge from these data are as follows:

1. Most mutants (e.g. USY-3 & 4) are morphologically abnormal, grow as well as the "wild type" parent on CM, show high resistance to TM, dichlobenil and quintozene, high or low resistance to vinclozolin, and are abnormally sensitive to high concentrations of sodium chloride or glucose.
2. Mutants USY-6 & 9 are similar to those described above except that they grow very poorly (< 6 mm/24h) on CM and attain normal rates of growth (12-13 mm/24h) on CM amended with fungicide.

TABLE 1

Growth rate (increase in colony diam., mm/24h) and conidiation (conidia x $10^{-4}/mm^2$) of tolclofos methyl-resistant isolates of Aspergillus nidulans on basal medium (CM) and CM with various fungicides* and osmotica**

Isolate	Growth rate							Conidiation						
	CM	TM	Dic	Quin	Vin	NaCl	Gluc	CM	TM	Dic	Quin	Vin	NaCl	Gluc
USY-1	12.2	9.5	6.9	8.7	2.0	6.0	8.0	16.7	16.4	14.7	8.4	0.1	32.0	1.5
USY-2	11.6	10.5	4.5	9.2	1.5	5.4	3.8	3.0	11.3	9.9	-	-	4.0	0.2
USY-3	11.8	12.0	7.1	11.2	3.2	4.3	3.9	3.2	34.0	15.5	16.1	0.1	13.0	1.5
USY-4	13.1	12.5	11.9	11.7	13.6	4.8	6.4	3.0	12.5	1.6	11.7	16.6	13.0	0.8
USY-5	12.1	11.5	9.1	5.2	1.0	4.6	2.1	0.7	10.0	8.5	11.5	0.1	5.0	0.8
USY-6	5.8	12.0	8.9	12.8	4.0	4.4	2.9	0.1	26.6	44.0	23.9	0.1	12.6	0.2
USY-8	12.2	12.8	6.2	10.5	12.3	6.5	3.8	32.0	10.4	31.0	-	-	39.5	1.3
USY-9	2.5	11.0	2.6	13.2	3.3	7.4	9.0	0	0.2	0.5	-	-	13.6	0.3
USY-10	13.4	10.3	7.9	7.0	9.3	6.5	7.1	17.1	11.0	10.0	-	-	38.5	1.0
USY-11	12.6	11.7	7.8	12.4	2.0	4.9	3.8	7.4	42.5	24.0	21.1	0	9.5	6.6
wild type	13.0	3.5	0.8	1.8	0	8.5	15.5	96.0	0.6	0	0.1	0	77.0	26.8

The measurements are means of three replicate colonies.- not determined.

* 100 µg/ml tolclofos methyl (TM), 100 µg/ml dichlobenil (Dic), 100 µg/ml quintozene (Quin), 100 µg/ml vinclozolin (Vin) ** 0.4M sodium chloride (NaCl), 0.7M glucose (Gluc).

TABLE 2

Conidiation (conidia x $10^{-4}/mm^2$) of tolclofos-methyl-resistant mutants of Aspergillus nidulans on fungicide amended medium

Mutant	Fungicide	Concentration of fungicide (µg a.i./ml medium) 1	2	4	8	16	32	64
USY-1	tolclofos methyl	14.2	12.3	10.2	12.7	19.6	16.7	11.2
USY-4		15.9	20.4	12.0	15.7	17.0	15.7	15.0
USY-6		3.1	3.8	10.0	12.0	37.0	21.7	30.3
USY-9		10.1	15.0	5.4	3.4	3.0	0.5	0.5
USY-10		24.2	21.4	11.8	18.8	15.7	7.0	7.4
USY-1	vinclozolin	17.2	18.3	18.4	10.9	11.7	0.5	0.1
USY-4		8.4	13.7	10.0	10.3	13.6	18.4	16.4
USY-6		0.1	0.2	0.1	2.3	2.4	4.1	1.0
USY-9		0.4	0.2	0.3	4.8	4.5	3.3	0.7

3. All mutants produce fewer conidia than the wild type on CM and CM supplemented with sodium chloride or glucose, but more conidia than the wild type on CM amended with fungicide.
4. Mutants USY-6 and-9 are dependent on the fungicides for maximum conidiation.

Genotypes of mutants

Each of the mutant isolates carried a single mutant gene causing resistance to the fungicides. The mutant genes were completely recessive (USY-1, 4, 9, 10) or partially recessive (USY-2, 3, 8) or partially dominant (USY-5, 6, 11) to their wild type alleles. Three fungicide resistance genes have been located: mutants USY-1, 2 and 9 carry mutations in the same gene on linkage group III; mutant 5 carries a mutation in a different gene on LG III; mutant 10 carries a mutant gene on LG V.

Previous studies of A. nidulans mutants resistant to aromatic hydrocarbon fungicides identified a resistance gene on LG III (Van Tuyl 1977). We have not determined whether this gene is identical to either of the LG III genes detected in this investigation.

Studies of Neurospora crassa have shown that at least six different genes specify resistance to aromatic hydrocarbon and dicarboximide fungicides (Grindle 1984, 1987). Most N. crassa mutants resemble the A. nidulans mutants described here in that they are morphologically abnormal, produce few conidia, and are extremely sensitive to high concentrations of salts and sugars that raise the osmolarity of the growth media (Ellis et al. 1986, Grindle 1983). However, this study has revealed unique mutants that are dependent on low doses of fungicides in their growth medium for normal growth and conidiation. If phenotypically similar mutants of pathogenic fungi were to occur, this could pose significant problems in agriculture and horticulture.

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TUBULIN FROM BOTRYTIS CINEREA, AND THE POTENTIAL FOR DEVELOPMENT OF AN IMMUNODIAGNOSTIC FOR BENZIMIDAZOLE RESISTANCE.

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ABSTRACT

Fungal tubulin, the site of action of methyl benzimidazol-2-yl carbamate (carbendazim, MBC), has been partially characterized from field isolates of the plant pathogen Botrytis cinerea. The molecular weight of the tubulin dimer was determined by gel filtration of cell-free extracts after incubation with [^{14}C]-MBC: our estimate of 100-110,000 is comparable with published results. After partial purification based upon DEAE-Sephadex A-50, the α- and β-subunits from both highly MBC sensitive and tolerant isolates were identified by SDS-gel electrophoresis and Western immunoblotting: molecular weights were approximately 54,000 and 52,000 respectively, slightly smaller than the analogous bovine brain tubulin subunits. The potential use of tubulin enriched fractions from B. cinerea for induction and screening of monoclonal antibodies useful in MBC resistance diagnosis is discussed.

INTRODUCTION

An economically effective strategy to deal with fungicide resistant pathogens depends upon their recognition before they become established and widespread. The differentiation of fungicide resistant and sensitive strains of a pathogen relies currently on in vitro assays using fungicide amended agar plates, in vivo assays involving fungicide-treated leaves, or slide germination tests of spores in the presence of fungicide. The first two methods both require isolation of pure test cultures and are slow to set up and carry out. The latter is far too insensitive to detect low levels of resistant biotypes, and is still slow. It is often impossible to forecast with accuracy the extent or rapidity of spread of resistant isolates on a regional or international basis, or to apply effective alternative control before further substantial crop losses have been suffered. The introduction of a cheap and rapid diagnostic test for low proportions of resistant biotypes - ideally germinating airborne spores -is therefore a pressing need in field pathology.

Botrytis cinerea was selected for initial development of a field diagnostic test for benzimidazole resistance, being an economically important pathogen, growing readily in vitro. It has a substantial history of MBC resistance worldwide, and resistant spore populations have been shown to persist well after cessation of fungicide application.

The biochemical basis of benomyl resistance is relatively well understood. The binding affinity of the microtubule protein tubulin for [^{14}C]-MBC has been shown to correlate closely with the sensitivity of the strain for the fungicide (Davidse & Flach, 1977; Gessler et al. 1980; Groves et al. 1988). The development of MBC resistance in strains of normally sensitive species, such as B. cinerea, is ascribed to a subtle alteration in the structure of tubulin. This results in decreased affinity for carbendazim, but without adverse effect on microtubule function. It is this structural difference that may offer a basis for immunochemical differentiation of MBC sensitive and resistant biotypes.

This paper reports initial work toward development of a serological method for rapid detection of low proportions of MBC resistant conidia in B. cinerea populations. Tubulin from B. cinerea has been partially characterized through an immunochemical identification of the α- and β-subunits in a partially purified fraction, combined with a radiochemical detection of dimeric tubulin, as achieved by Gessler et al. (1980). The possible usefulness of the tubulin enriched preparation in monoclonal antibody production and screening is assessed, and alternative future approaches to development of an immunodiagnostic are proposed.

MATERIALS AND METHODS

Isolates and culture conditions

Both B. cinerea strain 1 (EC50 = 0.25 μM MBC) and strain 2 (EC50 >5000 μM MBC) were isolated from infected Rhododendron spp. in a Surrey glasshouse, and kindly supplied by A.D.A.S.. The original cultures were preserved on silica gel at -22°C, and revived as required on a medium of 2% dextrose, 0.4% yeast extract, 0.2% mycological peptone (DYP), 1.5% agar. For spore preparation, mycelial discs were transferred to 3% malt extract, 0.5% mycological peptone agar, and sporulation encouraged with 12 hours near u.v. light irradiation per day from days 4-7. All plates were grown at 20°C. When matured, spores were washed free, strained and stored at -22°C.

Preparation of mycelial extracts and protein determination

Conidial germlings were generated for 20-24 hours at 25°C in litre baffled Erlenmeyer shake flasks containing 250 ml DYP and 10^6 spores/ml. Rinsed germlings were placed in a previously cooled 'X-Press' cell disintegrator (LKB) with 0.4 ml homogenization buffer per gram wet weight mycelium. Three passages through the apparatus gave total breakage. Homogenization buffers are detailed below. Homogenates were centrifuged at 32,500 x g for 10 mins at 4°C, and the supernatant further centrifuged for 30 min. Protein was determined by absorption at 595 nm according to Bearden (1978), with bovine serum albumin as standard.

Molecular weight determination of the tubulin dimer

Homogenization buffer consisted of 100 mM piperazine-N,N'-bis [2-ethanesulphonic acid]) (PIPES-Na), pH 6.9, containing 200 mM NaCl, 5 mM $MgCl_2$, and 2 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA), amended with 1 mM guanosine 5'-triphosphate (GTP) and 0.5% (vol/vol) solution PPL (40 mg/ml phenyl methyl sulphonyl fluoride, 0.6 mg/ml pepstatin A and 10 mg/ml leupeptin) in dimethyl sulphoxide (DMSO).

[^{14}C]-MBC (specific activity 30 mCi/mmol) was dissolved in DMSO and incubated for 2 hours at 4°C with crude extract, containing a further 1 mM GTP and 0.5% PPL; total DMSO in the incubation mixture was 1% (vol/vol).

The molecular weight of the MBC-tubulin complex was determined by gel filtration on a Sephacryl S-200 (Pharmacia) column (45 x 1.5 cm), equilibrated with elution buffer of 50 mM PIPES-Na, pH 6.9, 100 mM NaCl, 2.5 mM $MgCl_2$, 1mM EGTA, 0.005% solution PPL in DMSO and 0.045% solution PP (no leupeptin) in DMSO. Protein elution was monitored continuously by u.v. absorption at 254nm. Fractions (1ml) were collected, and radioactivity assessed by liquid scintillation spectrometry. The mean disintegrations per minute were calculated, after correction for background. Radioactivity in protein fractions was considered to represent bound fungicide. Marker proteins were: cytochrome c (horse heart), MW 12,400; carbonic anhydrase (bovine erythrocyte), MW 29,000; albumin (bovine serum), MW 66,000; alcohol dehydrogenase (yeast), MW 150,000; and β-amylase (sweet potato), MW 200,000.

Tubulin enrichment and immunochemical detection

Homogenization buffer was 100 mM PIPES-Na, pH 6.9, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.2% Nonidet P-40, 1 mM GTP and 0.5% (vol/vol) PPL in ethanol. The crude extract was applied to DEAE-Sephadex A-50 (Pharmacia), equilibrated with 0.2M NaCl elution buffer, and washed with 0.2 M then 0.4 M NaCl buffer. Elution buffer was 50 mM PIPES-Na, pH 6.9, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.05 mM ethylene diamine tetra-acetic acid, di-sodium salt (EDTA), 1 mM DTT, 0.1 mM GTP and 0.05% PPL in ethanol, with NaCl as appropriate. The 0.4-0.9 M NaCl fraction was collected, and concentrated by addition of ammonium sulphate (enzyme grade) at 0.423 g/ml. After 30 min stirring on ice, the preparation was centrifuged at 32,500 x g for 10 min at 4°C.

The pellet was redissolved for SDS-electrophoresis (Laemmli, 1970), which was performed at 30mA/gel, with acrylamide / bis-acrylamide concentrations (wt/vol) of 8% / 0.214% (resolving gel) and 4% / 0.107% (stacking gel). Proteins were transferred to BA85 nitrocellulose sheets (Towbin et al. 1979), and treated with tris buffered saline plus 0.1% (vol/vol) Tween 20 (TBST) for 30 mins. Strips were cut and probed with one of several monoclonal or polyclonal antisera, then with an appropriate affinity purified peroxidase conjugated second antibody (Sigma). All antisera were prepared in TBST containing 1% (wt/vol) casein. Between each step, strips were washed six times, the fourth TBST wash containing 1M NaCl and 0.5% Tween 20 to reduce background. Colour was developed with a freshly prepared nickel enhanced diaminobenzidine reagent. Molecular weight standards (Fig. 2) were detected with Aurodye Forte (Janssen).

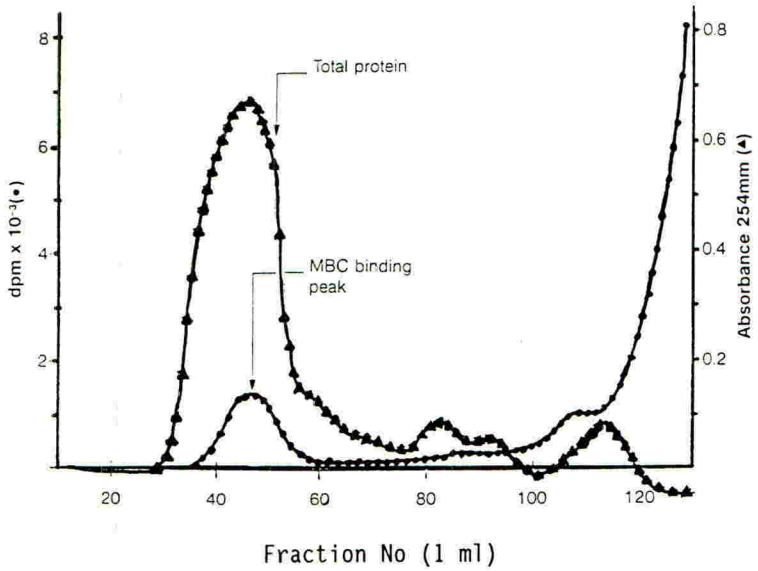
RESULTS AND DISCUSSION

Tubulin dimers: radiochemical detection

Gel filtration of crude supernatant from strain 1 with [^{14}C]-MBC showed radioactivity eluted as a single symmetric peak in protein-containing fractions (Fig. 1). In three independent gel filtration assays, different incubation mixtures gave peak protein-bound radioactivities at elution volumes corresponding to molecular weights of c. 102,000, 105,000 and 109,000. These are similar to earlier published estimates for fungal

FIG. 1

Radiochemical detection of B. cinerea tubulin dimers:



Gel filtration of crude extract (strain 1), after incubation with [^{14}C]-MBC: absorbance due to protein (▲); radioactivity (•)

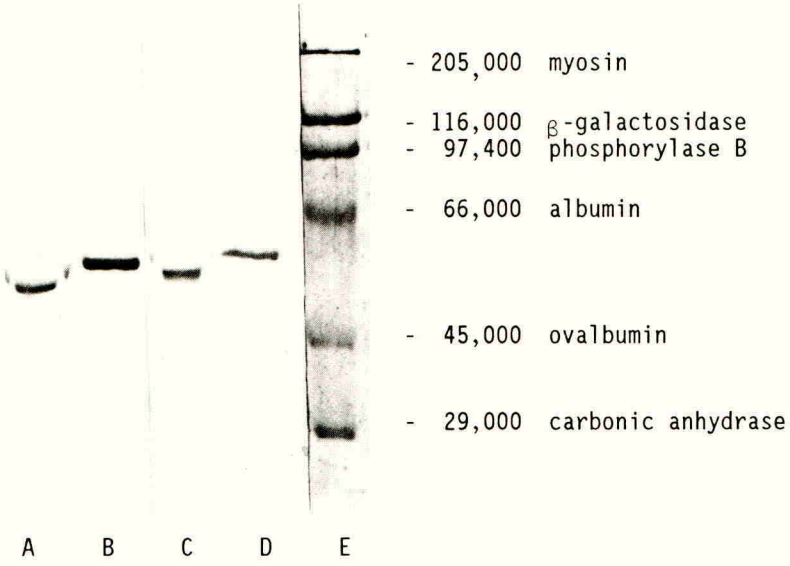
tubulins (Davidse & Flach 1977; Gessler et al. 1980). The corresponding fractions with strain 2 crude extracts contained negligible radioactivity. Assuming 1 mole of fungal tubulin binds 1 mole of MBC, and the molecular weight of the B. cinerea tubulin dimer is 105,000, then tubulin is c. 0.17% (wt/wt) of soluble protein in a typical crude extract. This compares favourably with published estimates of 0.1% and 0.2 - 0.4% for B. cinerea (Gessler et al. 1980) and Aspergillus nidulans (Davidse & Flach 1977) respectively, and is equivalent to about 400-500 µg tubulin per 25 g (wet wt) of disrupted conidial germlings.

Tubulin subunits: immunochemical detection

After partial purification, the α- and β-tubulin subunits from both B. cinerea strains were detected with two well characterised monoclonal antisera (Fig. 2): YOL1/34, anti-yeast α-tubulin (Kilmartin et al. 1982) and KMX-1, anti-Physarum β-tubulin (Birkett et al. 1985). Identification of both subunits was confirmed with a polyclonal anti-yeast tubulin IgG fraction. Their molecular weights were c. 54,000 and 52,000 respectively. These values were slightly smaller than our estimates of the corresponding bovine brain tubulins: c. 57,000 and 55,000. An additional B. cinerea α-tubulin band was sometimes detected at c. 57,000. No reaction was observed on Western blots of crude extracts due to their low tubulin content, but tubulins were identified in 0.2-0.4 M NaCl DEAE-Sephadex eluates. Recent experiments with extracts of strain 1 have shown that peak elution of MBC-binding activity from DEAE-Sephadex occurred at c. 0.3 M NaCl, and c. 80% of the activity had

FIG. 2

Immunochemical detection of B. cinerea tubulin subunits.



Lanes: A,C - B. cinerea tubulin
B,D - Bovine brain tubulin
E - MW standards

Stain: A,B - KMX-1 anti β-tubulin
C,D - YOL1/34 anti α-tubulin
E - 'Aurodye Forte'

eluted by 0.4M NaCl. This peak is lower than was found with A. nidulans (Davidse & Flach 1977). Fungal tubulins are clearly difficult to purify effectively.

Potential for immunodiagnostic development

Our consistent and reliable immunochemical detection of B. cinerea tubulin on Western blots is an essential foundation toward the use of tubulin as an antigen. However, three aspects will require further attention before a reliable immunoassay can be developed:

(1) A one-step purification of B. cinerea tubulin was not possible. An effective second stage purification is required to follow DEAE-Sephadex.
(2) Partially purified B. cinerea tubulin has not yet been detected in an enzyme-linked immunosorbent assay (ELISA) screen, though a highly purifed brain tubulin preparation was detected quantitatively (Groves, unpublished results).
(3) The antigenicity of B. cinerea tubulin, and the epitopic availability of the MBC-binding site are uncertain.

Alternative approaches to immunodiagnostic development should be considered. Highly purified tubulins have been reported from yeast and Physarum through in vitro assembly of microtubules; however, neither of these MBC-sensitive organisms are typical 'fungi', and may not contain a similar MBC-binding site to plant pathogens. In recent years, fungal tubulin sequence data have been published, and the amino acid substitution for resistance identified in a filamentous fungus, Neurospora crassa (Orbach et al. 1986). Such data could be utilized to generate appropriate synthetic peptide antigens.

Biotype-specific anti-tubulin antibodies would be a very valuable research tool: it would be possible to investigate the number of resistant epitopes within and between isolates, to compare the epitopes of MBC resistant mutants with those of the naturally resistant genera, and hence to develop a field diagnostic test for low levels of MBC resistant spores.

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COMPARATIVE STUDY OF THE BIOLOGICAL PROPERTIES OF PHYTOPHTHORA INFESTANS FIELD ISOLATES DIFFERING IN SENSITIVITY TO METALAXYL

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ABSTRACT

Phytophthora infestans isolates, obtained from potato fields in different regions of the USSR, were assayed for metalaxyl sensitivity using a leaf disc test. Isolates differed considerably in metalaxyl sensitivity. Sporulation of metalaxyl sensitive isolates was completely inhibited at concentrations < 1ppm metalaxyl; isolates with intermediate resistance sporulated at 100 ppm, but were inhibited at 1000 ppm metalaxyl; and isolates with high level resistance were able to grow and sporulate at 1000-2000 ppm. These sensitivity differences did not change after 12 - 15 passages on fungicide free medium (about 1.5 years). Passage through leaf discs on high metalaxyl concentrations did not increase the resistance of intermediate isolates. The fitness of resistant isolates was equal to that of the sensitive isolates.

INTRODUCTION

In recent years, intensive application of metalaxyl and related phenylamide fungicides to control Oomycetes has led to significant changes in sensitivity of pathogen populations in many countries (Davidse *et al*. 1981, Dowley & O'Sullivan 1981, Cooke & Logan 1983). These changes resulted frequently in considerable crop damage because of the failure of these fungicides to control potato late blight (*Phytophthora infestans*). Phenylamide fungicides have been used for several years against potato late blight in the Soviet Union, and development of resistant pathogen populations can be expected in the country. The results of metalaxyl sensitivity assays for 25 *P. infestans* field isolates, and a comparative study of their fitness is described in this paper. The infection ability, sporulation intensity, period of incubation and some other biological properties were used as an index of each isolates' fitness (Dyakov 1983).

METHODS

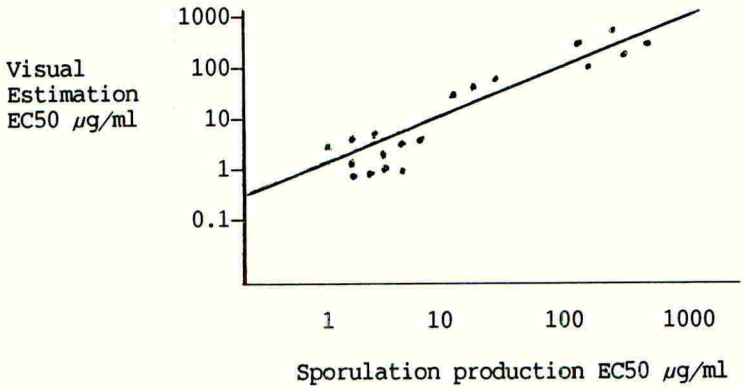
Isolates were collected from potato fields in various regions of the USSR between 1983 - 1985. Some of these isolates (R1-R8) were obtained from plants treated with metalaxyl (Ridomil 25 WP, 0.250 kg/ha a.i.), or from infected tubers after harvesting. All isolates were maintained on oat agar medium and were periodically passaged through leaves and tuber slices. The sensitivity of the isolates to metalaxyl was determined using a leaf disc test (Carter & Brent 1981, Schwinn & Sozzi 1982). Discs (15 mm diam.) were cut from potato leaves (cv. Lorch) and placed upside down on fungicide solutions (15 ml) in 9 cm petri dishes (five discs per dish). Metalaxyl concentrations ranged

from 0.01 ppm - 2000 ppm. Discs in control dishes floated on distilled water. Leaf discs were inoculated with drops of spore suspension (10 μl) containing 500 - 800 sporangia. After 7 - 8 days, at room temperature, the results were recorded using scale: 0 - no sporulation; 1; 2; 3; 4 = 1 - 25, 25 - 50, 50 - 75, 75 - 100% respectively of the disc surface covered with sporulation. In addition to a visual estimation, sporangial quantity per cm of disc was counted in a Goryaev camera, and this helped to confirm the visual method.

On the basis of results obtained by the two methods EC50 values were calculated (Gar 1963). Comparison of data shows high correlation level (Fig. 1) and confirms significance of visual test.

FIGURE 1

EC50 values for P. infestans isolates, differing in metalaxyl sensitivity.



RESULTS AND DISCUSSION

All isolates tested could be classified in one of three groups depending on sensitivity. The first group (EC50 = 0.01 - 0.1 ppm) included the majority of isolates tested; the second group, included isolates - R5, R6, R7 (EC50 = 13.9 - 50.6 ppm) and the third one - R1-R4, R8 isolates (EC50 > 300 ppm).

Metalaxyl totally suppressed the sporulation of the first group at 0.01 - 1 ppm. The second group of isolates failed to sporulate at 100 ppm metalaxyl. The third group of isolates sporulated at 1000 ppm. Nevertheless, there was some variation in metalaxyl sensitivity within each group.

TABLE 1

Metalaxyl sensitivity of P. infestans isolates.

Isolates	Region	Year of collection	EC50 ppm	95% Confidence intervals
220	Moscow	1983	0.02	0.019–0.03
290	–"–	1983	0.02	0.01–0.03
123	–"–	1983	0.04	0.03–0.05
D–85	–"–	1985	0.02	0.01–0.04
L–85	–"–	1985	0.05	0.03–0.08
182	Zakarpatie	1983	0.07	0.05–0.09
264	Byelorussia	1983	0.03	0.02–0.06
214	Estonia	1983	0.02	0.01–0.07
238	Latvia	1983	0.02	0.01–0.05
348	–"–	1983	0.02	0.01–0.03
324	Lithuania	1983	0.08	0.05–0.1
328	–"–	1983	0.1	0.07–0.2
303	Lvov	1983	0.001	0.0002–0.04
304	–"–	1983	0.03	0.02–0.05
N–85	Novosibirsk	1985	0.02	0.01–0.03
R1	Moscow	1985	322.0	231.8–441.1
R2	–"–	1985	1394.7	725.2–2649.9
R3	–"–	1985	1967.6	1337.9–2754.6
R4	–"–	1985	1379.3	786.2–1848.2
R5	–"–	1985	13.9	8.3–21.5
R6	–"–	1985	50.6	37.9–75.4
R7	–"–	1985	28.5	15.5–52.4
R8	–"–	1985	2092.2	1339.0–3054.6

Comment: For 236 (Latvia, 1985) and K–R (Primorsky region, 1979) isolates EC50 values were < 0.001 ppm.

The occurence of different metalaxyl resistant groups of isolates is of some interest, as a similar pattern of variation has been found in groups of such pathogens as Septoria nodorum (MBC, Horsten 1979) and Plasmopara viticola (metalaxyl, Herzog & Schuepp 1985). In our experiments sensitivity of isolates remained unchanged after 12 - 15 passages on metalaxyl-free oat medium (1.5 years). Attempts to increase the resistance of intermediate isolates by passages through leaf discs treated with higher metalaxyl concentrations failed, indicating the stability of the sensitivity groupings.

The role of intermediate isolates in the development of metalaxyl resistant populations is not clear since some of these isolates can not overcome the field dose of the fungicide applied as a spray. Such isolates are only able to develop on treated plants after some decline in metalaxyl levels as a result of metabolism within plants.

No significant differences between metalaxyl sensitive and resistant isolates in infection ability, latent period, sporulation and other fitness parameters were observed when spore suspensions of several isolates were inoculated onto leaves (cv. Lorch).

Some metalaxyl resistant isolates caused more disease, but statistical analysis failed to confirm any link between EC50 values and infection ability.

Metalaxyl resistance was not connected to any decrease in fitness. These results explain the rapid increase of metalaxyl resistance in P. infestans, and it can be expected that resistant forms will remain in the population after withdrawal of the fungicide.

TABLE 2

Biological properties of P. infestans isolates differing in sensitivity to metalaxyl.

Isolates	Latent period hours	Necrosis number per leaf	Necrosis area cm^2	Sporulation productivity sporangia x $10^3/cm^2$	Colony diameter cm	Sporangial viability %
Sensitive isolates						
L-85	64	14	0.8	45.9	6.5	83
D-85	64	11	0.3	15.6	8.0	73
N-85	69	8.3	0.1	32.5	7.0	86
K-R	88	24.0	0.02	27.5	5.9	75
238	69	29.0	0.2	42.5	5.7	63
303	69	23.3	0.3	20.6	3.6	82
214	69	11.0	0.5	48.7	5.3	73
182	88	13.0	0.05	78.1	4.9	87
Average	72	16.7	0.3	38.9	5.9	77.8
Intermediate isolates						
R5	64	19.7	0.6	30.1	6.6	82
R6	64	90.7	0.6	33.1	4.7	83
R7	64	61.3	0.8	26.8	5.2	73
Average	64	57.0	0.7	30.0	5.5	79.3
Resistant isolates						
R1	69	25.3	0.3	33.4	8.5	81
R2	64	60.7	0.3	41.5	8.3	87
R3	64	18.0	0.7	42.9	7.4	88
R4	64	15.0	0.4	42.1	5.4	79
R8	64	57.0	0.3	27.5	4.2	81
Average	65	35.2	0.2	37.5	6.8	83.2
LSD 5%		30.6	0.2	16.6	0.8	16.2

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