Resistance of *Botrytis cinerea* to Dichlofluanid in Greenhouse Vegetables

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ABSTRACT

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During a survey in Crete in 1986, samples of various vegetables infected by *Botrytis cinerea* were taken from 76 greenhouses, and the sensitivity of the fungus to dichlofluanid was tested by a spore germination test. Also, 52 single-spore isolates obtained from 10 greenhouses were tested for their sensitivity to dichlofluanid with respect to mycelial growth, spore germination, and disease control by foliar sprays. Thirteen single-spore isolates of *B. cinerea*, obtained from vineyards in which no control against gray mold had ever been applied, were compared with the vegetable isolates. On the basis of the inhibitory concentration (IC) of dichlofluanid for mycelial growth, the greenhouse single-spore isolates were divided into four classes with IC values of 3, 9, 27, and ≥ 81 μg/ml respectively. The mean ED_{50} values of dichlofluanid for the inhibition of spore germination for the isolates of each class and the group of vineyard isolates were: class 1, 0.063 ± 0.016; class 2, 0.058 ± 0.016; class 3, 0.043 ± 0.016; class 4, 0.267 ± 0.147 and vineyard isolates, 0.056 ± 0.025 μg/ml. The mean ED_{50} values for the protection of young bean plants (*Vicia faba* L.) by foliar sprays for the same isolates were: class 1, 65.0 ± 10.4; class 2, 7.7 ± 39.4; class 3, 110.3 ± 75.0; class 4, 369.5 ± 177.8 and vineyard isolates, 65 ± 22.1 μg/ml. The isolates of classes 1, 2, and 3 were as sensitive to dichlofluanid as the vineyard isolates, while those of class 4 were significantly less sensitive. In a greenhouse experiment, dichlofluanid, captan, and chlorothalonil applied at label rates were not effective against gray mold in cucumber caused by an isolate of class 4 with a dichlofluanid ED_{50} value of 0.150 μg/ml for spore germination. Fifty-five of the 76 samples obtained during the survey were classified in the least sensitive class 4. This accounts for the reduced effectiveness of this fungicide against gray mold that had been noticed by the growers.

Gray mold, caused by *Botrytis cinerea* Pers. ex Fr. is the most severe disease of greenhouse crops in Crete during the winter. Because the control of environmental conditions in most plastic greenhouses is not possible and cultivars of the greenhouse vegetables tomato, cucumber, pepper, melon, and eggplant resistant to gray mold are not available, control is based on the frequent application of chemicals. As a result, strains resistant to benzimidazoles and dicarboximides have been selected (8,10) and dichlofluanid and the similar-acting phthalimides or chlorothalonil (7) have been used as alternatives. These have been considered low-risk fungicides from the resistance viewpoint (7), but growers report that they are ineffective against gray mold. Because dichlofluanid has been used in greenhouses in Crete for 20 years, we considered that strains of *B. cinerea* resistant to this fungicide might have been selected as well. Work on resistance to this fungicide is presented here.

MATERIALS AND METHODS

In a survey carried out during the spring of 1986 in the main vegetable-growing areas of Crete, five gray mold infected plant parts were randomly collected from each of 76 greenhouses with tomatoes (*Lycopersicon esculentum* Mill.), cucumbers (*Cucumis sativus* L.), melons (*Cucumis melo* L.), aubergines (*Solanum melongena* L.), and peppers (*Capsicum annuum* L.) within an area of 1,000 m^2 in each greenhouse. The infected material was left for 1 day in a room with natural light to promote sporulation. Spores were collected and their sensitivity to dichlofluanid was tested with the spore germination test described in this paper.

Based on that test, 26 single-spore isolates were selected that had a low sensitivity to dichlofluanid and 26 with a high sensitivity. These 52 isolates were further tested by mycelial growth, spore germination, and disease control by foliar spray tests, and were compared with 13 single-spore isolates obtained from four samples taken from four vineyards, in which no control against gray mold had ever been applied.

Mycelial growth tests. Petri dishes containing 20 ml of potato-dextrose agar (PDA) amended with 0, 0.3, 1, 3, 9, 27, and 81 μg/ml of dichlofluanid (Euparen 50WP) were centrally inoculated with 5-mm plugs from the periphery of 3-day-old cultures and incubated at 22 C. The radial growth of the colony was measured in two orthogonal directions 48 and 72 hr later, and the lowest concentration that inhibited mycelial growth from the disks was recorded for each isolate.

Spore germination tests. Petri dishes each containing 12 ml of dextrose agar amended with 0.0, 0.015, 0.031, 0.062, 0.125, 0.250, 0.500, and 1.0 μg/ml of dichlofluanid were used. In each of two petri dishes with each concentration of dichlofluanid, 1 ml of spore suspension containing approximately 2.10^5 spores/ml was evenly spread and incubated at 22 C. The spore suspensions were obtained directly from the infected plant parts, in the case of the greenhouse survey, or from 8-day-old cultures on PDA at 22 C, in the case of the single-spore isolates. To promote sporulation, the cultures were continuously irradiated with a pair of Philips type BLB fluorescent 20W lamps that were 20 cm above them. When germ tubes in the control unamended agar were about 30–40 μ long (about 5 hr incubation), a few drops of formalin were added to each petri dish to stop germination. Germinated spores out of 100 on each plate were counted, and the regression of percent inhibition, corrected by Abbott's formula (2), on dichlofluanid concentration was calculated for each isolate.

Disease control in inoculated broad beans. Pot-grown broad bean (*Vicia faba* L.) plants with two pair of fully developed leaves were punctured with a hot needle at 10 points evenly distributed on the total leaf area. Ten of these plants for each isolate to be tested were sprayed to runoff with 0.0, 12.5, 25.0, 50.0, 100.0, 200.0, 400.0, or 800.0 μg/ml of dichlofluanid using a hand sprayer. As soon as they were dry, they were sprayed to runoff with a spore suspension, produced as previously described and containing 1.10^6 spores/ml of each isolate, and put in a moist chamber at 20–22 C. Three to four days after incubation, when the control leaves had more than 70% of the puncture points infected, the severity of the infection at each puncture was rated on a 0–5 visual scale (0 = no infection and 5 = diameter of the infected tissue, about 1 cm). The regression of percent protection, corrected by Abbott's formula (2), on dichlofluanid concentration was calculated for each isolate.

Disease control in an inoculated greenhouse chamber crop. The effectiveness of dichlofluanid (Euparen 50WP), captan (Captan 50WP), chlorothalonil (Daconil 75WP), guazatine (Befran 25AS), and vinclozolin (Ronilan 50WP) against an isolate of *B. cinerea* resistant to benzimidazoles, dicarboximides, and dichlofluanid was tested in a greenhouse experiment. Treatments were arranged in a randomized block design with four replicates. Experimental plots were 4.5 ×

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1.8 m. Cucumber (*Cucumis sativus* L.) were planted in two rows of seven plants each. Fungicides were applied to runoff with a knapsack sprayer, at 7- or 15-day intervals, beginning 15 March. Label rates were used as follows: dichlofluanid, 1.20 g/L; captan, 1.70 g/L; chlorothalonil, 1.50 g/L; vinclozolin, 0.50 g/L; and guazatine, 0.25 g/L. When plants were fully developed, about 10 L of fungicide suspension was used for the four plots of each treatment, equivalent to a rate of 2,000 L/ha. A spore suspension of a greenhouse isolate resistant to benzimidazoles, dicarboximides, and dichlofluanid containing about 2.10^4 spores/ml, from an 8-day-old culture, was applied with a mist sprayer over the treated plots at the time the first flowers opened. To facilitate infection, plants were misted with water twice daily. Infection was rated three times during the growing season by counting the leaf nodes with infected flowers and the total leaf nodes of each plant. The percent leaf nodes with infected flowers was then calculated for each plot. For the statistical analysis the percent infected leaf nodes (N) was transformed into angle $\Phi = \sin^{-1}N^{1/2}$, and the means were compared using Duncan's multiple range test ($P = 0.05$).

Persistence of resistance in culture. Six greenhouse isolates with reduced sensitivity to dichlofluanid and four single-spore isolates, also with reduced sensitivity, were subcultured every 3 days on PDA at 22 C, seven and 12 times, respectively, and their sensitivity to dichlofluanid was tested by spore germination test at every third subculturing.

RESULTS

Greenhouse samples. Of 76 samples infected with *B. cinerea* from 76 greenhouses, 21 yielded conidia that gave about 20% germination at 0.125 μg/ml of dichlofluanid and 0% germination at 0.250 μg/ml. A few conidia from all the remaining 55 samples germinated at 0.250 μg/ml of dichlofluanid.

Single spore isolates. *Mycelial growth tests.* Mycelial inhibition began at 0.3 μg/ml of dichlofluanid for 31 of the single-spore isolates and at 1.0 μg/ml for the remaining 21. Colonies grew regularly at lower concentrations, but at higher concentrations there was either no growth at all, or, more frequently, sectors appeared the second day of incubation that overgrew slower-growing mycelium. For this reason, ED_{50} values were not easy to calculate and the inhibitory concentration (IC) that inhibited mycelial growth of the original colony was selected to characterize the sensitivity of each isolate. The 52 single-spore isolates were divided into four sensitivity classes with IC = 3, 9, 27, and ≥81 μg/ml of dichlofluanid. The number of the isolates in each class was 7, 13, 7, and 25, respectively. The IC value for mycelial growth for the 13 vineyard isolates varied from 9 to 27 μg/ml of dichlofluanid. Radial growth of the mycelium of one representative isolate from each sensitivity class and of a representative vineyard isolate are shown in Table 1. The four groups of isolates from greenhouse vegetables were treated as distinct classes in the following reports.

Spore germination tests. Spore germination began after 3 hr incubation and about 90% of the spores had developed germ tubes 30–40 μ long after 5 hr incubation. The highest dichlofluanid concentration at which spores of 26 of the 52 single-spore isolates from greenhouses, as well as all the vineyard isolates, germinated was 0.125 μg/ml. At 0.015–0.031 μg/ml, germination was equal to the control. No germination occurred at 0.250 μg/ml. In contrast, some spores of the remaining 26 single-spore isolates germinated at 1.0 μg/ml of dichlofluanid, and at 0.062–0.125 μg/ml germination was equal to the control. The respective regression equations of percent inhibition on dichlofluanid concentration fitted the data well with correlation coefficients (r = 0.85 to 0.99, n = 2) significant at $P = 0.05$. The mean ED_{50} and ED_{95} values derived from the regression equations are presented in Table 2. These spore-germination values for the greenhouse single-spore isolates with IC for mycelial growth 3, 9, and 27 μg/ml do not differ significantly among themselves, but they are significantly lower than the corresponding values for the isolates with IC ≥ 81 μg/ml. Similarly, ED_{50} and ED_{95} values for the inhibition of spore germination for the 13 vineyard single-spore isolates do not differ significantly from those of the isolates with IC for mycelial growth 3, 9, and 27 μg/ml, but they are significantly lower than those for the isolates with IC ≥ 81 μg/ml, $P = 0.05$. Dosage-response lines of one representative isolate from each class and vineyard isolates with observed data are shown in Figure 1. All the remaining isolates of the same class behaved in a similar way.

Disease control in broad beans. Infection of young broad bean plants by *B. cinerea* became visible the second day of inoculation and by the third day more than 70% of puncture sites had lesions. For the isolates with high ED_{50} values for the inhibition of spore germination, the least infection occurred at 800 μg/ml of dichlofluanid, and at 50 μg/ml it was nearly equal to the control. For the isolates with low ED_{50} values, the least infection occurred at 200 μg/ml of dichlofluanid, and at 12–25 μg/ml it was

Table 1. Radial growth (mm) of mycelium of four greenhouse isolates of *Botrytis cinerea*, representative of four different sensitivity classes, and one vineyard isolate at various concentrations of dichlofluanid

	Dichlofluanid concentration (μg/ml)						
Isolates	**0.00**	**0.33**	**1**	**3**	**9**	**27**	**81**
Greenhouse isolates							
Class 1 (IC = 3)[y]	50	37	23	5*[z]	5*	...	...
Class 2 (IC = 9)	80	73	55	42	5*	5*	5*
Class 3 (IC = 27)	71	68	63	45	31	5*	5*
Class 4 (IC = 81)	64	62	59	51	42	42	5*
Vineyard isolate	75	65	45	32	5*	5*	...

[y] IC = dichlofluanid concentration (μg/ml) that inhibits mycelial growth from agar disks, but not from sectors.
[z] * = Diameter of the original inoculum.

Table 2. Mean ED_{50} and ED_{95} values (μg/ml) derived from regression equations of several concentrations of dichlofluanid for inhibition of spore germination and protection of *Vicia faba* by foliar sprays for several single-spore isolates of *Botrytis cinerea* having different inhibitory concentrations (IC)[y] of dichlofluanid against mycelial growth

Isolates	**Concentration**	**Inhibition of spore germination**	**Protection by foliar sprays**
Greenhouse isolates			
IC = 3	ED_{50}	0.063 ± 0.016[z] (n = 7)	65.0 ± 10.4 (n = 5)
	ED_{95}	0.214 ± 0.092 (n = 7)	303.0 ± 168.9 (n = 5)
IC = 9	ED_{50}	0.058 ± 0.016 (n = 13)	77.7 ± 39.4 (n = 6)
	ED_{95}	0.198 ± 0.045 (n = 13)	290.3 ± 195.1 (n = 6)
IC = 27	ED_{50}	0.043 ± 0.016 (n = 7)	110.3 ± 75.0 (n = 3)
	ED_{95}	0.184 ± 0.034 (n = 7)	320.3 ± 123.1 (n = 3)
IC ≥ 81	ED_{50}	0.267 ± 0.147 (n = 25)	369.5 ± 177.8 (n = 14)
	ED_{95}	0.852 ± 0.359 (n = 25)	1,653.4 ± 944.5 (n = 14)
Vineyard isolates			
IC = 3–27	ED_{50}	0.056 ± 0.025 (n = 13)	65.0 ± 22.1 (n = 9)
	ED_{95}	0.170 ± 0.055 (n = 13)	221.8 ± 85.8 (n = 9)

[y] Dichlofluanid concentration (μg/ml) that inhibits mycelial growth from agar disks, but not from sectors.
[z] Mean ± standard deviation.

equal to the control. The regression equations of dichlofluanid concentrations on percent disease control by foliar sprays for single-spore isolates fitted the data well with correlation coefficients (r = 0.86 to 0.98, n = 10) significant at P = 0.05. The mean ED_{50} and ED_{95} values derived from the regression equations are presented in Table 2. The dosage-protection values for the isolates with IC = 3, 9, and 27 μg/ml are not significantly different among themselves, but they are significantly lower than the corresponding values for the isolates with IC ≥81 μg/ml. Similarly, the mean ED_{50} and ED_{95} values for 13 vineyard isolates do not differ significantly from the corresponding values of the isolates with IC = 3, 9, and 27 μg/ml, but are significantly lower than those for the isolates with IC ≥81 μg/ml. Dosage-protection lines of one representative isolate from each class and from the vineyard isolates with observed data are shown in Figure 2. All the remaining isolates of the same class behaved in a similar way.

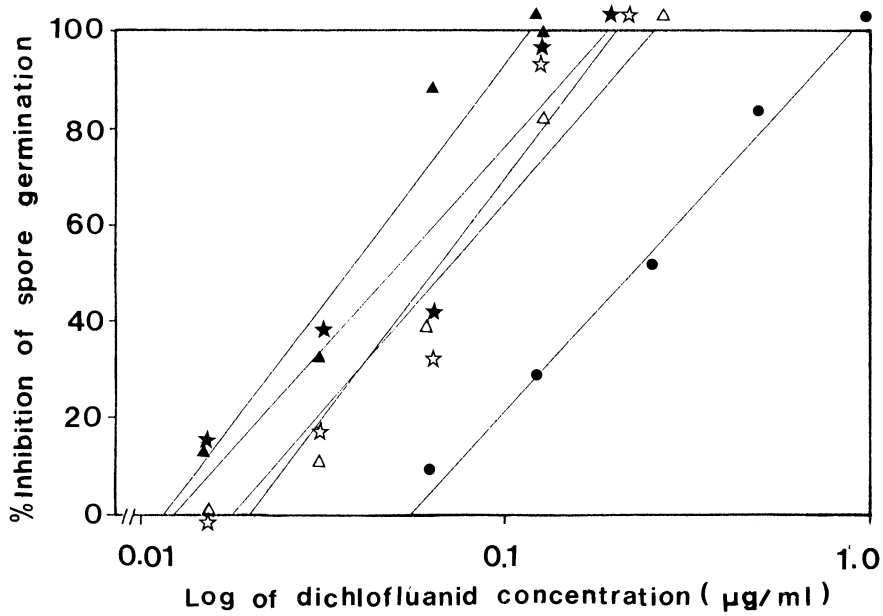

Fig. 1. Representative dichlofluanid dosage-response curves for spore germination of four classes of greenhouse isolates of *Botrytis cinerea*, with inhibitory concentration for mycelial growth 3 (open triangle), 9 (open star), 27 (closed star), and ≥81 μg/ml (closed circle), respectively, and one vineyard isolate (closed triangle). The linear regression equations are: IC = 3, $Y = 151.8 + 87.3 \log X$; IC = 9, $Y = 166.4 + 95.8 \log X$; IC = 27, $Y = 159.8 + 82.8 \log X$; IC ≥81, $Y = 105.3 + 82.1 \log X$; and vineyard isolates, $Y = 197.0 + 101.7 \log X$ (Y = percent inhibition of spore germination and X = dichlofluanid concentration).

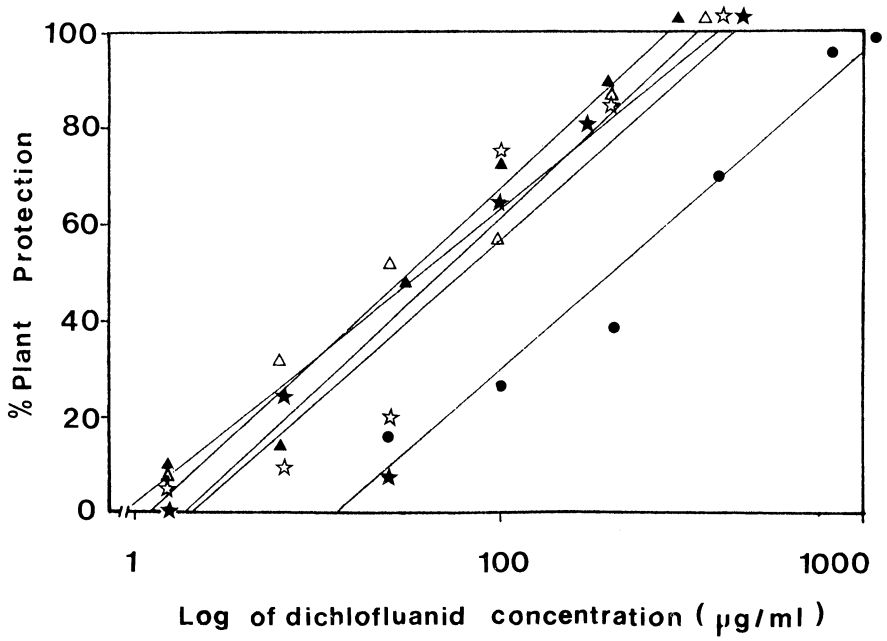

Fig. 2. Representative dichlofluanid dosage-response curves for plant protection by foliar sprays of four classes of greenhouse isolates, with inhibitory concentration for mycelial growth 3 (open triangle), 9 (open star), 27 (closed star), and ≥81 μg/ml (closed circle), respectively, and one vineyard isolate (closed triangle) of *Botrytis cinerea* to dichlofluanid. The linear regression equations are: IC = 3, $Y = 84.5 + 72.9 \log X$; IC = 9, $Y = 56.0 + 59.9 \log X$; IC = 27, $Y = -104.9 + 80.4 \log X$; IC ≥81, $Y = -106.6 + 67.9 \log X$; and vineyard isolates, $Y = 72.8 + 70.4 \log X$ (Y = percent disease protection and X = dichlofluanid concentration).

Disease control in an inoculated greenhouse cucumber crop. Gray mold appeared in the cucumber plants 10 days after artificial inoculation in all treatments, mostly on the first senescent flowers. Except for guazatine, there were no consistent significant differences between fungicides and the control throughout the growing season (Table 3).

Persistence of resistance. Four of the six mass greenhouse isolates and three of the four single-spore isolates that were subcultured retained the same level of sensitivity up to the seventh and 12th subculture, respectively. The other two greenhouse isolates and one single-spore isolate started to respond with a higher sensitivity from the third subculture. At the sixth subculture, their sensitivity was equal to the reference sensitive isolate (ED_{50} = 0.056 μg/ml).

DISCUSSION

The inhibitory concentration of dichlofluanid for mycelial growth, its ED_{50} and ED_{95} values for inhibition of spore germination, and disease control by foliar sprays for the greenhouse single-spore isolates tested indicate that these isolates can be divided into two categories: 1) those with IC for mycelial growth ≤27 μg/ml that had ED_{50} and ED_{95} values for the inhibition of spore germination and disease control by foliar sprays equal to the vineyard wild isolates, and 2) those isolates with IC for mycelial growth ≥81 μg/ml that had ED_{50} and ED_{95} values for the inhibition of spore germination and disease control by foliar sprays significantly higher than those of the vineyard isolates. For practical purposes, isolates of the second category are resistant to dichlofluanid. Preliminary tests and the results of the greenhouse experiment (Table 3) indicate that the same strains are also resistant to captan, which has a mode of action similar to dichlofluanid (6,7). My data also indicate that resistance is stable in culture. The mean ED_{50} values of the resistant isolates for the inhibition of spore germination is about 4.5 times higher than the ED_{50} values for the sensitive isolates. Because dichlofluanid affects mainly spore germination, it was doubtful that such a low level of resistance could substantially alter the effectiveness of dichlofluanid in practice, but the results of the greenhouse experiment indicate that dichlofluanid, captan, and chlorothalonil had no efficacy against gray mold, even at the

beginning of the experiment when the inoculum was very low. Considering the 20 years of history of efficacy of dichlofluanid against *B. cinerea* (9), I conclude that its failure in this experiment was because the isolate used to initiate infection had now become resistant.

The data provide convincing evidence that in 55 greenhouses out of 76 surveyed, strains of *B. cinerea* resistant to dichlofluanid predominated, which explains the growers' failures to control gray mold with dichlofluanid. Such extensive field resistance to dichlofluanid in greenhouses in Crete combined with the resistance to benzimidazoles that appeared in early 1970 (8) and with the resistance to dicarboximides that appeared in 1981 (10) makes gray mold control very difficult.

As far as we know, this is the first report of field resistance to dichlofluanid. Pepin and MacPherson (11) reported strains of *B. cinerea* resistant to captan, and Barak and Edgington (1) found they were also cross-resistant to the other phthalimides, folpet and captafol, as well as to the similar-acting fungicides thiram, chlorothalonil, and ethylene thiuram monosulfide. Dichlofluanid also has the same mode of action as these fungicides.

For many years it has been considered that the probability was very low for fungi to mutate at the many sites affected by dichlofluanid and similar-acting fungicides. Thus, no resistance to these fungicides was expected in the field (3,6). Nevertheless, the reported resistance of *Pyrenophora avenae* Ito & Kuribay. to organomercurials (5), *Venturia inaequalis* (Cooke) Wint. to dodine (4,12,13), and now *B. cinerea* to dichlofluanid presents increasing evidence that under certain conditions (e.g., prolonged application of the same fungicide, applications in greenhouses), fungi with many cycles during the growing season may develop resistance even to multisite fungicides. According to Georgopoulos (3), that might happen if sites differed in sensitivity. In this case, mutational alteration to the most sensitive one might give an increment of resistance. The low level of resistance observed in these cases (13) supports the above hypothesis.

Table 3. Effectiveness of different fungicides against an isolate of *Botrytis cinerea* resistant to benzimidazoles, dicarboximides, and dichlofluanid in the control of gray mold in greenhouse cucumber

Treatment[y]	Rate (g/L a.i.)	Infected leaf nodes		
		21 April	30 April	12 May
Guazatine	0.25	1.8 b[z]	2.9 c	7.3 c
Captan	1.70	5.2 ab	10.1 ab	20.0 ab
Chlorothalonil	1.50	6.6 a	14.6 ab	22.7 a
Dichlofluanid	1.20	4.9 ab	8.1 abc	18.1 ab
Vinclozolin	0.50	2.9 ab	5.2 bc	12.2 bc
Control		4.8 ab	12.2 a	19.0 ab

[y]Guazatine, captan, chlorothalonil, and dichlofluanid were applied at 7-day intervals and vinclozolin at 15-day intervals.

[z]Numbers followed by the same letter within columns are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

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