Neth. J. Pl. Path. 87 (1981) 11-24

Resistance to acylalanine fungicides in Phytophthora megasperma f.sp. medicaginis

L. C. DAVIDSE

Laboratory of Phytopathology, Agricultural University, Wageningen, the Netherlands

Accepted 24 March 1980

Abstract

Isolates of *Phytophthora megasperma* f. sp. *medicaginis* resistant to the systemic fungicide metalaxyl were obtained by mycelial adaptation and by mass selection from zoospores either untreated or treated with UV irradiation or with the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. In an assay with one-week-old lucerne seedlings all of 19 spontaneously obtained isolates showed a relatively low degree of resistance and were less virulent than the original isolate. Only one of these isolates showed resistance in vivo in this test. From 176 highly resistant isolates obtained after mutagenic treatment, 81 were as virulent as the orginal isolate and 33 of the latter displayed a considerable degree of resistance in vivo.

Metalaxyl at a concentration of a.i. in soil of 20 $mg.l^{-1}$ failed to control root rot of 7-8 week old lucerne plants inoculated with mycelial fragments of virulent resistant isolates. Under similar conditions root rot caused by the original isolate was completely prevented even at a concentration of a.i. of 2.5 $mg.l^{-1}$.

The resistance to metalaxyl appeared to be highly stable since virulent and resistant strains did not loose their resistance after 12 infection cycles in a seedling assay in the absence of the fungicide, neither did mixed populations of a resistant and a sensitive isolate under similar conditions.

The likelihood of development of resistance to acylalanine fungicides under practical conditions is discussed.

Additional key words: Medicago sativa, resistance to systemic fungicides, Ridomil, metalaxyl, furalaxyl.

Introduction

Acylalanines have recently been introduced as systemic fungicides with highly selective activity against fungi of the order Peronosporales (Schwinn et al., 1977a, b; Urech et al., 1977; Staub et al., 1978). Their efficacy in the control of numerous diseases caused by these fungi has repeatedly been shown, and in some cases their use might lead to a major breakthrough in chemical control methods.

Although the mechanism of action of the acylalanine fungicides has not yet been elucidated, it might be highly specific in view of the selectivity and systemic behavious of these compounds. Therefore, as has been encountered with other specifically-acting fungicides, development of resistance might be a potential threat for a long-lasting useful life of the acylalanines.

In this study the potential of *Phytophthora megasperma* f. sp. *medicaginis*, the

causal organism of lucerne root rot, to develop resistance to metalaxyl was evaluated. Several methods are described to obtain resistant strains of this fungus and to test their fitness and resistance in pathogenicity experiments.

Materials and methods

Fungus

P. megasperma f. sp. *medicaginis* isolate 7x, kindly supplied by D. P. Maxwell, University of Wisconsin, Madison, WI, USA and originally isolated from soil of the *Phytophthora* root rot nurseries in St. Paul, MN, USA, was used. The organism was maintained on V8-agar (Ribeiro, 1978) or in soybean broth (2 beans/50 ml distilled water) at either room temperature or 24°C. Zoospores were obtained by growing the organism on V8-agar in 9-cm-diameter Petri-dishes at 24°C for 4-5 days followed by 2-3 days at 30°C. Agar was removed from around the edge of each colony and the plates were flooded with 15-20 ml of sterilized distilled water and incubated at 15°C for 15-18 h. To obtain mycelial inoculum for pathogenicity experiments, the fungus was grown as still cultures in 100 ml of clarified V8-broth (Ribeiro, 1978) in 300 ml Erlenmeyer flasks at 25°C for 10-15 days.

The mycelial mats were harvested by filtering through four layers of cheese cloth mounted over a Büchner funnel, washed three times with tap water and finally pressed between several layers of paper towels to about 70-75% moisture (dry weight determinations were made on a subsample afterwards). The inoculum was prepared by fragmenting 5 grams of mycelium in a Waring Blendor for 10 sec in 100 ml tap water. The inoculum concentration was then adjusted to the desired value by dilution of the suspension in tap water.

Selection of resistant isolates

Resistant isolates of *P. megasperma* f. sp. *medicaginis* were obtained in two ways: a) by growing isolate 7x on V8-agar containing metalaxyl (0.5-2 $\mu g.ml^{-1}$) for 2-4 weeks and subculturing mycelium, which showed reduced sensitivity, on media with metalaxyl at higher concentrations and b) by mass selection on V8-agar amended with 2-20 μg metalaxyl per ml from encysted zoospores either untreated or treated with UV-radiation or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Zoospores were induced to encyst by adding 1 ml of undiluted, sterilized and clarified V8-broth to 8 ml of a zoospore suspension in a plastic Petri-dish (9 cm diameter). While encysting the zoospores attached to the bottom of the dish and started to germinate within 1 h. The cysts were exposed to the mutagenic treatments within 30 min after encystment. UV-irradiation was carried out with a Hanovia portable UV-lamp at 254 nm at a distance of 2 cm for 2-20 min. MNNG was added tot the dishes as an aqueous solution at a final concentration of 30 $\mu g.ml^{-1}$. After 5-20 min the liquid was removed and the cysts were washed with 5 ml clarified V8-broth for 10-15 min. After washing the cysts were overlayered with *c.* 10 ml molten and cooled V8-agar containing metalaxyl (2-20 $\mu g.ml^{-1}$). After the medium had solidified, another 10 ml of the same medium was added. The plates were incubated at 24°C and scored for the presence of colonies for a period of up to 4 weeks. Transfers were made first on V8-agar

amended with 2 μg metalaxyl per ml and thereafter on fungicide-free media.

Growth inhibition experiments

The degree of resistance of the isolates was determined by measuring the radial growth of mycelium on clarified V8-agar plates containing acylalanines at various concentrations.

Metalaxyl or furalaxyl was added to the molten agar as a 100- or 1000-fold concentrated solution of the technically pure products in ethanol. The experiments were carried out at least in triplicate.

Chemicals

Metalaxyl and furalaxyl were kindly provided by Ciba-Geigy Ltd., Basel, Switzerland and Ligtermoet Chemie B.V., Rotterdam, the Netherlands as technically pure products and commercial formulations (Ridomil 25 WP and Fongarid 50 WP, respectively). MNNG was purchased from Fluka AG, Buchs, Switzerland.

Pathogenicity experiments

The virulence of the resistant mutants was determined in two assays. For the first one, which is a modification of the seedling assay of Irwin et al. (1979), 0.2 g lucerne seeds (cv. Vernal, Old Seeds Co., Madison, WI, USA or cv. Orca, Cebeco-Handelsraad, Rotterdam, the Netherlands) were sown on 450 g of a sand-perlite (1 : 1, v/v) mix with a moisture content of 20-25% in 1-l wax-treated paper cups and covered with 150 g of the same mix. The cups were covered with a plastic Petri-dish and placed in a growth room with a 16-h photoperiod (12000 Lux) at 22.5°C. Seven days after sowing, the mix was saturated with water and pieces of a 9- or 10-day-old Petri-dish culture of the isolate to be tested were placed between the seedlings. Usually one culture was used per cup. In some experiments, inoculation was carried out by adding a mycelial suspension to the mix before planting and saturation of the mix after seven days. Zoospore suspensions and infected seedlings were also used as inoculum. The percentage of seedlings killed was estimated daily. For determining in vivo resistance of the isolates a similar procedure was followed except that two days before inoculation the seedlings were drench-treated with 150 ml of a Ridomil 25 WP suspension (100 μg a.i.ml^{-1}).

In the second assay for virulence 6-8 weeks old plants growing in peat or in a peat-sand (1 : 1, v/v) mix in 1-l clay pots (3-5 plants per pot), placed in saucers were used. The plants were placed in a growth room at 20°C or 22.5°C under the same light conditions as described above and were fertilized two times with 0.3 g of a soluble fertilizer (NPK 16 + 21 + 27) per pot. Inoculation was done by pouring 50 ml of a mycelial suspension containing 0.625 g wet weight of mycelium on the suface of the soil of each pot. The inoculum was mixed with the upper 1-2 cm of the soil and washed in by watering from the top until the saucers were half-filled with water. The soil was kept saturated for three days and then the pots were placed on 5-cm pieces of a PVC-tube (5 cm diameter) and allowed to drain for four days with watering from the top only when the surface appeared dry. After this period the pots were

placed back in the saucers and flooded again. This cycle of three days of flooding and four days of normal watering was maintained for at least four weeks at a temperature of 22.5°C after which the plants were uprooted and individually rated for disease severity of the root system using a scale of 1 to 5, according to Frosheiser and Barnes (1973): 1 = no symptoms, 2 = no obvious root lesions but most fine roots destroyed leaving small black spots at point of attachment, 3 = distinct localized lesions on tap root or one or two secondary roots with tip rot or both, 4 = part of the upper third part of the tap root rotted or nearly all of the lower two-third part rotted and 5 = nearly all of the tap root rotted. For determining in vivo resistance in this assay the plants received a soil drench (200 ml per pot) of Ridomil 25 WP two days before inoculation giving a final concentration of a.i. in soil of *c.* 20 mg.l^{-1}. There were at least three replicates per treatment with 3-5 plants per pot. Individual disease severity indexes (DSI) were statistically processed with a Kruskal-Wallis test and multiple comparisons were made between mean ranks of treatments.

Results

Sensitivity of Phytophthora megasperma f.sp. medicaginis to metalaxyl

The wild type sensitivity of *P. megasperma* f. sp. *medicaginis* was assessed with isolate 7x by measuring radial growth of mycelium on clarified V8-agar and in a spore germination assay. The results are given in Table 1.

Metalaxyl did not inhibit spore germination but was highly inhibitory towards mycelial growth. At 0.05 μg.ml^{-1}, the lowest concentration tested, germ tube elongation was initially unaffected, but, after a certain length was attained, growth stopped almost completely. On the basis of these results, a concentration of at least 2 μg metalaxyl.ml^{-1} was used for selection of resistant mutants.

Table 1. Effect of metalaxyl on growth of *P. megasperma* f. sp. *medicaginis,* isolate 7x.

Metalaxyl concentration (μg/ml^{-1})	% inhibition	
	germ tube growth[1]	mycelial growth[2]
0	0	0
0.05	11	38
0.2	22	67
0.5	29	90
2.0	28	100
5.0	36	100

[1] 6 h incubation in soybean broth.
[2] 8 days incubation on clarified V8-agar.

Tabel 1. Effect van metalaxyl op de groei van P. megasperma f. sp. medicaginis, isolaat 7x.

Table 2. Selection and resistance of metalaxyl-adapted isolates of *P. megasperma* f. sp. *medicaginis*.

Isolate	Selection procedures				Average colony diameter (mm) at various metalaxyl concentrations ($\mu g.ml^{-1}$) after 11 and 19 days of incubation				
	conc.[1]	days[2]	conc.[1]	days[2]	11 days			19 days[3]	
					0	5	50	5	50
MET-139	0.5	18	0.5	36	39	5[4]	5	5	5
MET-140	0.5	18	0.5	36	32	14	5	35	27
MET-141	0.5	18	0.5	36	43	17	14	55	48
MET-142	0	18	0.5	36	45	5	5	25	5
MET-143	0	18	0.5	36	62	5	5	5	5
MET-144	2	33	2	21	31	10	5	49	20
MET-145	2	33	2	21	39	17	10	68	53
MET-146	2	33	5	21	25	11	9	45	30
MET-147	2	33	5	21	33	15	9	75	70
7x	0	10	0	10	57	5	5	5	5
7x	0	10	0	10	58	5	5	5	5

[1] Concentration ($\mu g.\ ml^{-1}$) of metalaxyl in selection medium.
[2] Length of incubation period before next transfer was made.
[3] After 19 days the plates (9 cm diam) without metalaxyl were completely overgrown.
[4] Diameter of agar plug with inoculum.

Tabel 2. Selectie en resistentie van aan metalaxyl-geadapteerde isolaten van P. megasperma f. sp. medicaginis.

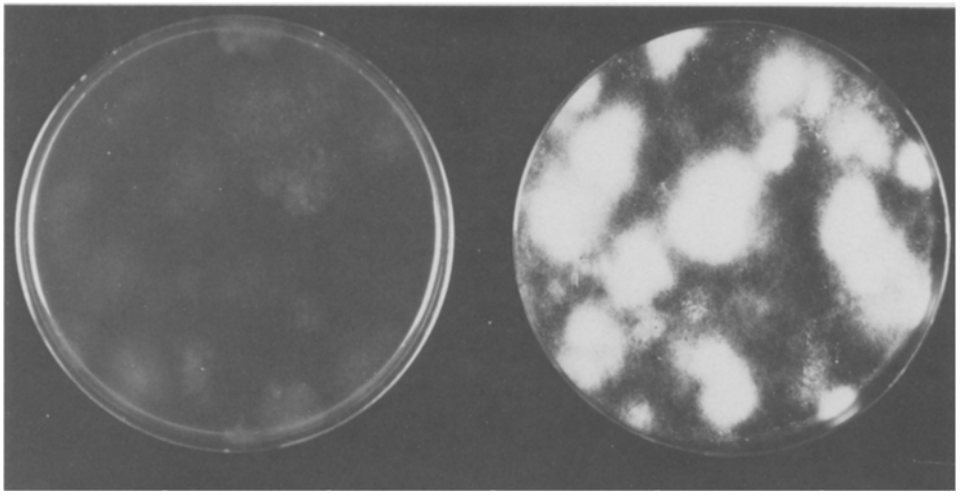
Selection of resistant isolates

A. Adaptation. Mycelium readily adapted to metalaxyl when growing at sublethal concentrations. Sectoring of faster growing mycelium frequently occurred and when this mycelium was transferred to media with metalaxyl at higher concentrations, it showed a reduced sensitivity. Even at an initially completely inhibitory concentration of 2 $\mu g.\ ml^{-1}$ this phenomenon was observed after five weeks. In this way a number of strains was selected. The reduced sensitivity to metalaxyl of some of them is shown in Table 2, together with their selection procedures.

As is evident from Table 2, a certain degree of resistance was attained with seven isolates. All these seven isolates showed a reduced growth rate on medium without metalaxyl and several of them had much less aerial mycelium than isolate 7x.

B. Mass selection. Mass selection from encysted zoospores proved to be an easy method to obtain large numbers of metalaxyl resistant isolates. When cysts not being exposed to any mutagenic treatment were overlayered with metalaxyl-amended agar, several colonies appeared on the surface of the agar after 11-26 days

Fig. 1. Development of metalaxyl-resistant colonies of *P. megasperma* f. sp. *medicaginis* after mass selection from c. 10^6 encysted zoospores on V8-agar containing 2 μg metalaxyl. ml^{-1}. Left, untreated zoospores; right, zoospores treated with 30 μg MNNG.ml^{-1} for 10 min.



Figuur 1. Ontwikkeling van kolonies van P. megasperma f. sp. medicaginis met resistentie tegen metalaxyl na massaselectie uit ca. 10^6 ingekapselde zoösporen op V8-agar met 2 μg metalaxyl. ml^{-1}. Links, onbehandelde zoösporen; rechts, zoösporen behandeld met 30 μg MNNG.ml^{-1} *gedurende 10 min.*

of incubation (Fig. 1). Colonies even developed on media containing 20 μg metalaxyl.ml^{-1}. In Table 3 the results are given from three experiments in which resistant-strains were obtained.

The number of isolations that could be made was highly dependent on the duration of the incubation period. Similar to the isolates obtained by adaptation these

Table 3. Isolation of metalaxyl-resistant isolates of *P. megasperma* f.sp. *medicaginis* by mass selection from untreated encysted zoospores.

Exp.	Metalaxyl[1] (μg.ml^{-1})	Total number of zoospores	Incubation period (days)	Number of isolates[2]	Mutants per 10^7 zoospores
1	2	29.6×10^6	11	1	0.3
2	2	10.8×10^6	14	5	5
3	20	2.3×10^6	26	12	52

[1] Concentration in selection medium.
[2] Isolates which were able to grow after isolation on V8-agar at 2 μg metalaxyl.ml^{-1}.

Tabel 3. Isolatie van tegen metalaxyl resistente isolaten van P. megasperma f. sp. medicaginis met behulp van massaselectie uit onbehandelde ingekapselde zoösporen.

Table 4. Isolation metalaxyl-resistant isolates of *P. megasperma* f.sp. *medicaginis* by mass selection from MNNG-treated encysted zoospores.

Exp.	Metalaxyl[1] ($\mu g.ml^{-1}$)	Total number of zoospores	Incubation period (days)	Number of isolates[2]	Mutants per 10^7 zoospores
1	2	15.4×10^6	14	69	45
2	2	38.9×10^6	14	52	13
3	2	59.2×10^6	11	127	21

[1,2] See Table 3.

Tabel 4. Isolatie van tegen metalaxyl resistente isolaten van P. megasperma f. sp. medicaginis met behulp van massaselectie uit met MNNG behandelde ingekapselde zoösporen.

isolates also showed a reduced growth rate and/or had less aerial mycelium than isolate 7x which suggests that in both cases the resistance is of a similar type.

Exposure of encysted zoospores to UV-irradiation for 2-3 min did not significantly increase the yield of resistant isolates. Out of 22×10^6 treated cysts overlayered with V8-agar, amended with 2 μg metalaxyl.ml^{-1}, seven colonies developed after 22 days. Upon transfer to a similar medium they continued growth but, like the isolates obtained from non-irradiated cysts their growth rate on fungicide-free media was reduced.

Treatment of encysted zoospores with MNNG resulted in the rapid development of many resistant colonies on V8-agar containing 2 μg metalaxyl.ml^{-1} (Fig 1, Table 4). Since so many colonies developed no attempt was made to isolate them all. After passage on V8-agar amended with 2 μg metalaxyl.ml^{-1} and transfer to fungicide-free media about half of the isolates showed a growth rate and colony morphology similar to that of the original isolate. The growth rate of the other isolates was lower.

Degree of metalaxyl resistance

Although all isolates obtained were able to grow on clarified V8-agar amended with 2 μg metalaxyl.ml^{-1} and thus appeared to be less sensitive than the original isolate, there was a considerable difference in degree of resistance of the isolates obtained by adaptation and mass selection from untreated cysts on one hand and isolates obtained by mass selection from MNNG-treated cysts on the other hand (Table 5).

ED_{50}-values for inhibition of mycelial growth on V8-agar of metalaxyl-resistant isolates obtained by adaptation (n = 9) and mass selection from untreated cysts (n = 13) were alle below 5 μg metalaxyl. ml^{-1}, whereas those of randomly chosen isolates obtained by mass selection from MNNG-treated cysts (n = 6) were all well above 100 $\mu g.ml^{-1}$. It is evident from this behaviour that resistant isolates of *P. megasperma* f. sp. *medicaginis* belong to two categories. Isolates of the first one are characterized by a relatively low level of resistance and have a reduced mycelial growth rate, whereas those of the second category attain a much higher level of resistance, which is accompanied in half of them with a normal growth rate and colony morphology. The isolates of this category can easily be obtained by mass selec-

Table 5. Degree of resistance to metalaxyl of the least sensitive isolates obtained by different selection procedures.

Isolate	Number of isolates tested	Origin[1]	Incubation period (days)	% inhibition of mycelial growth at different metalaxyl concentrations ($\mu g.ml^{-1}$)				
				2	5	10	50	100
7x	–	–	8	100	nd[2]	nd	nd	nd
MET-141	9	adaptation	11	74	68	74	76	nd
MET-161	13	m.s., –	5	nd	68	nd	nd	nd
MET-5	6	m.s., MNNG	8	7	3	0	−4	4

[1] m.s., – = mass selection from untreated cysts.
m.s., MNNG = mass selection from MNNG-treated cysts.
[2] nd = not determined.

Tabel 5. Graad van metalaxyl-resistentie van de minste gevoelige isolaten verkregen met behulp van verschillende selectieprocedures.

tion from MNNG-treated cysts. Eight metalaxyl-resistant isolates of the second category were also examined on resistance to furalaxyl. The ED_{50}-values for inhibition of mycelial growth on V8-agar of all strains were above 100 μg furalaxyl.ml^{-1} whereas mycelial growth of the original isolate 7x was already completely inhibited at a concentration of 5 μg furalaxyl.ml^{-1}.

Virulence and resistance in vivo of metalaxyl-resistant isolates

Although the potential of *P. megasperma* f. sp. *medicaginis* to develop resistance to acylalanine fungicides has unequivocally been demonstrated in the experiments described above, virulence, fitness and resistance in vivo of the resistant isolates will ultimately determine whether such isolates have a change to survive in natural populations. These properties, therefore, were examined and compared with those of the original isolate 7x in a number of experiments.

The seedling assay proved to be a valuable method to assess the virulence of the resistant isolates. By using agar cultures as inoculum, their ability to sporulate is indirectly examined, since zoospores are the infective propagules in this assay. Resistance in vivo in this test can easily be assessed by incorporating the fungicide into the mix.

Data about virulence and resistance in vivo of a large number of resistant isolates are summarized in Table 6. All six isolates obtained by adaptation and all thirteen isolates obtained by mass selection from untreated cysts showed a reduced virulence as compared with that of isolate 7x or were avirulent. The percentage of diseased seedlings increased with time and after six days two isolates obtained by adaptation and three obtained by mass selection were able to cause a complete damping-off of untreated plants. Only one of these isolates displayed resistance in vivo causing a 90-100% damping-off of the metalaxyl-treated plants six days after inoculation.

Table 6. Virulence and resistance in vivo of resistant isolates in the seedling assay.

Origin[1]	Number of isolates tested	Days after inoculation	% of isolates classified into three classes[2] according to their virulence on control and metalaxyl-treated plants					
			control plants			treated plants[3]		
			1	2	3	1	2	3
adaptation	6	3	0	33	67	0	0	100
		6	33	33	33	17	0	83
m.s., –	13	3	0	38	62	0	0	100
		6	23	46	31	0	0	100
m.s., MNNG	176	3	46	35	19	19	23	58
		6	67	25	8	39	17	44

[1] See Table 5.
[2] Classification according to percentages of plants killed (P). Class 1, 90 <P <100; class 2, 0 <P <90; class 3, P = 0. Isolate 7x caused a 90-100% kill three days after inoculation.
[3] Metalaxyl was applied as a soil drench of Ridomil 25 WP at a final concentration of 20 mg per kg mix, two days befor inoculation. This treatment gave complete control of damping-off caused by isolate 7x

Tabel 6. Virulentie en resistentie in vivo van resistente isolaten in de zaailingentoets.

Half of the MNNG-induced mutants were as virulent as isolate 7x and 19% of them caused a complete damping-off of metalaxyl treated plants within three days.

These data again indicate that a difference exists between isolates obtained by adaptation and mass selection from untreated cysts and those obtained after mutation induced with MNNG.

The virulence of seven isolates showing resistance in the seedling assay has also been compared with that of isolate 7x in the mature-plant assay. Table 7 gives the results of two experiments carried out under slightly different conditions.

Only MET-184 in both experiments and MET-271 in experiment 2 were significantly less virulent on untreated plants than isolate 7x. Metalaxyl completely failed to control root rot caused by all seven isolates, whereas disease caused by isolate 7x could be completely prevented by fungicide concentrations of a.i. in soil as low as 2.5 $mg.l^{-1}$ (data not shown). So the resistance of the isolates obtained with MNNG was also fully expressed in the mature-plant assay and the majority of the isolates tested was as virulent as the original isolate 7x.

Since the inoculum levels in both the seedling assay and the mature-plant assay might have been too high to detect small differences in virulence between the isolates, the effect of different inoculum levels was studied in the seedling assay. To this end, mycelial suspensions of different concentrations were mixed with the sand-perlite mix before sowing and after six days the mix was saturated with water. Under these conditions there was no difference between the emergence of the seedlings in infested and non-infested mix. The number of plants killed was determined daily and Table 8 gives the results of two experiments seven days after saturation. Al-

Tabel 7. Root disease severity index of mature control and metalaxyl-treated 'Vernal' plants inoculated with metalaxyl-resistant isolates and isolate 7x.

Isolate[1]	Average disease severity index[2]			
	Experiment 1[3]		Experiment 2[3]	
	control plants	treated plants[4]	control plants	treated plants[4]
MET-52	4.9 a[5]	4.0 a	4.2 a b c d	4.2 a b
MET-184	2.7 b	3.2 a b	3.3 d	3.3 b c
MET-191	4.2 a b	5.0 a	4.1 b c d	4.3 a b
MET-210	4.6 a	4.7 a	4.9 a b c	4.8 a
MET-234	4.1 a b	4.6 a	4.3 a b c d	4.2 a b
MET-271	4.9 a	3.9 a	4.0 c d	3.9 a b
MET-277	4.7 a	4.8 a	5.0 a	4.7 a b
7x	5.0 a	1.0 b	4.9 a b	1.0 c

[1] Isolates were obtained by MNNG treatment. The inoculum quantity varied between 159-232 (exp. 1) and 158-173 (Exp. 2) mg dry weight mycelium per pot.
[2] Scored 1-5. 1 = no disease symptoms. 5 = severely diseased. See Materials and Methods.
[3] In exp. 1 8-week-old plants grown up at 22.5 °C in peat-sand (1:1, v/v) (3 plants/pot, 3 pots /treatment) and in Exp. 2 7-week old plants grown up at 20 °C in peat (5 plants/pot, 3 pots treatment) were used. After inoculation the pots were placed at 22.5°C.
[4] Metalaxyl was applied as a soil drench of Ridomil 25 WP at a final concentration of *c.* 20 mg per 1 soil two days before inoculation.
[5] Values within a column followed by the same letter are not significantly different at P = 0.05 (Kruskal-Wallis test with multiple comparison of treatments). Non-inoculated plants had an average disease severity index of 1.0.

Tabel 7. Aantastingsgraad van volwassen, al of niet met metalaxyl behandelde 'Vernal' planten welke waren geïnoculeerd met tegen metalaxyl resistente isolaten en isolaat 7x.

though a considerable variation exists between replicates, it seems justified to say that in both experiments at least one resistant isolate showed a virulence comparable with that of 7x. MET-184 was less virulent in both experiments which corresponds to its lower virulence in the mature root assay.

Stability of resistance and competitive ability of metalaxyl-resistant isolates

The seedling assay was also used to determine the competitive ability of metalaxyl-resistant isolates (MET-52, MET-184, MET-191 and MET-210) in mixed populations with isolate 7x in the absence of the fungicide. To this end 6-day-old seedlings (cv. Orca) growing together in one cup were inoculated with 50 000 zoospores of one of the resistant isolates as well as with 50 000 zoospores of isolate 7x. In control experiments seedlings were inoculated with 100 000 zoospores of the various isolates separately. Before inoculation, the mix was saturated and kept saturated during the experiment. Three days after inoculation seedlings were used as inoculum for a second set of plants. This procedure was repeated ten more times with alternating incubation periods of three and four days. After each cycle the presence of the

Table 8. Damping-off of lucerne seedlings caused by metalaxyl-resistant isolates and isolates 7x at different inoculum levels.

Experiment	Isolate[1]	Highest inoculum level (mg dry wt per cup)	Percentage of plants killed at various inoculum levels[2]					
			1/1	1/4	1/16	1/64	1/256	1/1 + metalaxyl[3]
1	MET-52	87	100	95 ± 5[4]	4 ± 2	4 ± 5	0	100
	MET-184	83	100	16 ± 7	0	2 ± 2	0	97 ± 2
	MET-191	93	100	93 ± 5	36 ± 39	0	0	100
	MET-210	95	100	92 ± 8	15 ± 6	1 ± 1	0	100
	MET-271	103	100	78 ± 4	15 ± 12	0	0	99 ± 1
	7x	97	100	100	69 ± 16	1 ± 1	3 ± 2	0
2	MET-52	108	100	16 ± 2	22 ± 22	0	0	100
	MET-184	100	91 ± 6	0	0	0	0	98 ± 1
	MET-191	112	100	1 ± 1	0	0	0	100
	MET-210	108	100	99 ± 1	40 ± 19	63 ± 38	0	100
	MET-271	115	100	85 ± 1	5 ± 6	0	0	100
	7x	114	100	99 ± 1	26 ± 16	8 ± 6	10 ± 2	0

[1] Isolates were obtained after MNNG treatment.

[2] Twenty ml of mycelial suspensions containing varying amounts of mycelium (1/1 = 0.3 g wet weight per 20 ml) were mixed with 0.6 kg of the sand-perlite mix before sowing. After six days the mix was saturated with water and seven days later the healthy and diseased seedlings were counted.

[3] Metalaxyl was applied as a soil drench of Ridomil 25 WP at a final concentration of 20 mg per kg mix at the time of sowing.

[4] Standard deviation of 3 replicates.

Tabel 8. Aantasting van luzerne zaailingen door metalaxylresistente isolaten en isolaat 7x bij verschillende inoculumdichtheden.

resistant isolate in the population was determined by transferring seedlings to a test set of seedlings which, one day before inoculation, had been metalaxyl-treated by applying a soil drench to a final concentration of 20 mg per kg mix. All treatments were carried out in duplicate.

During the course of the experiment no apparent reduction in rate of damping-off of test seedlings occurred with the isolates MET-52, MET-191 and MET-210, either mixed with 7x or separately. Usually a complete damping-off was observed seven days after inoculation. The ability of the mixture of MET-184 and 7x to cause damping-off of the test seedlings started to decline in the 4th cycle but increased again after the 6th cycle and after the 9the cycle again a complete damping-off occurred. MET-184 alone did not show any decrease of virulence towards metalaxyl-treated seedlings although the rate at which damping-off of test seedlings as well as of untreated seedlings occurred was lower than that with the other isolates. Isolate 7x was never able to cause disease of the metalaxyl-treated test seedlings. Since in

this experiment a complete infection cycle is completed every 3-4 days the presence of the resistant mutants in the mixed populations after 12 infection cycles indicates that the fitness of the resistant isolates is not significantly reduced. The fluctuating behaviour of the mixture of MET-184 and 7x cannot be explained.

Discussion

Development of resistance in *P. megasperma* f. sp. *medicaginis* to the acylalanine fungicide metalaxyl could easily be demonstrated in laboratory experiments. Two different types of resistance were found. The first type is characterized by a relatively low degree of resistance and isolates of this type could be obtained by adaptation of mycelium or mass selection from zoospores. They are less virulent than the original isolate, show a reduced mycelial growth rate, and hardly display resistance in vivo. In this respect isolates of this type have similar characteristics as mutants of other fungi which are resistant to inhibitors of stereol biosynthesis (Fuchs et al., 1977; De Waard and Gieskes, 1977; Brown and Hall, 1979), pimaricin (Dekker and Gielink, 1979a) or pyrazophos (Dekker and Gielink, 1979b). The reduced fitness and virulence which accompany this type of resistance might suggest a decreased membrane permeability being responsible for metalaxyl resistance in these isolates of *P. megasperma* f. sp. *medicaginis* since permeability changes often disturb other processes as well

The second type of resistance found is characterized by a high degree of resistance. A considerable number of isolates of this group are as virulent as the original isolate and display a high degree of resistance in vivo. A change at the target site of metalaxyl caused by mutation might be responsible for this type of resistance. Since these mutants were only obtained by mutagenic treatment of encysted zoospores with MNNG one might argue whether this type of resistance will also arise spontaneously. However, chemical mutagens affect mutation rates by increasing the frequencies of DNA replication errors and misrepair of genetic damage, processes which are also the basis of spontaneous mutations, so it is unlikely, that MNNG induces a specific type of mutation, which will not occur spontaneously. The spontaneous mutation rate, however, is obviously much lower than that after mutagenic treatment. The numbers of zoospores used in the mass selection procedure without artificial induction of mutations were apparently too low for obtaining isolates with the second type of resistance. The high yield of these mutants after MNNG treatment is probably due to the fact that this replication fork specific mutagen (Drake and Baltz, 1976) acted during a period of active DNA synthesis in the early phases of germination of the zoospores.

Resistance to acylanine fungicides has not yet been found under practical conditions. Staub et al (1979) described isolates of *Phytophthora infestans* with decreased sensitivity to acylalanine fungicides in vitro, but these isolates were usually less virulent and/or fully sensitive to fungicide treatment on potato and tomato plants. These isolates, however, were obtained by adaptation and mass selection without mutagenic treatment and are likely to be of the same type as those of *P. megasperma* f. sp. *medicaginis* obtained by similar methods. Whether resistant isolates of *P. infestans* obtained by artificial induction of mutations behave differently remains to be investigated.

In general, our results indicate that, in order to make a reliable statement about the likelihood of resistance development to new fungicides several methods of induction of mutations should be used. When a mutagenic treatment is applied, it should be as mild as possible to reduce the number of additional mutations and large numbers of mutants should be tested for virulence and resistance in vivo.

In view of our results development of resistance to acylalanine fungicides may occur in practice. Therefore, measures should be taken to keep the selection pressure as low as possible, especially when acylalanine fungicedes are used to control pathogens with a high reproduction rate, a short infection cycle and aerial dispersal. Time will tell whether these precautions will be successful.

Note added in proof

In recent papers (Malathrakis, N.E., 1980. Proc. 5th Congr. Mediterr. Phytopath. Union, Patras, Sept. 21-27: 145-146; Pappas, A.C., 1980. Proc. 5th Congr. Mediterr. Phytopath. Union, Patras, Sept. 21-27: 146-148; Reuveni, M., Eyal, H. & Cohen, Y., 1980. Plant Dis. 64: 1108-1109 and Davidse, L.C., 1980. Gewasbescherming 11: 205-207) the development of resistance to metalaxyl under field conditions in *Pseudoperonospora cubensis* and *Phytophthora infestans* have been described. It indicates that model systems as the one presented here can be of great value in estimating the risk of development of resistance to fungicides before they are widely used in practice.

Acknowledgements

The author expresses appreciation to Oda Gerritsma for able technical assistance; to Heleen Krüse for carrying out the experiment on stability of the resistance and to Prof. Dr J. Dekker, Dr A. Fuchs and Dr M. A. de Waard for critical reviews of the manuscript.

Samenvatting

Resistentie tegen acylalanine fungiciden in Phytophthora megasperma f.sp. medicaginis

Van *Phytophthora megasperma* f. sp. *medicaginis,* een wortelpathogeen van luzerne, konden tegen metalaxyl resistente isolaten worden verkregen na adaptatie van mycelium en selectie uit zoösporen die al dan niet behandeld waren met het chemisch mutagens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Alle 19 spontaan verkregen isolaten vertoonden een lage graad van resistentie en hun virulentie in een zaailingentest was lager dan van het oorspronkelijke isolaat. Slechts één van deze isolaten vertoonde resistentie in vivo. Bij toetsing van 176 isolaten met een hoge graad van resistentie die na een mutagene behandeling waren verkregen bleek dat 81 isolaten even virulent waren als het oorspronkelijke isolaat en dat er 33 een aanzienlijke graad van resistentie in vivo te zien gaven.

Metalaxyl in een a.i. concentratie van 20 $mg.l^{-1}$ grond kon het optreden van wortelrot bij 7-8 weken oude luzerne planten niet verhinderen, wanneer deze werden geinoculeerd met gefragmenteerd mycelium van resistente isolaten. Onder dezelfde

omstandigheden kon het oorspronkelijke isolaat reeds volledig worden bestreden bij een a.i.-dosering van 2.5 $mg.l^{-1}$.

Resistentie tegen metalaxyl bleek een zeer stabiele eigenschap te zijn omdat bij een viertal getoetste stammen de resistentie niet verdween na 12 infectiecycli in een zaalingentest bij afwezigheid van het fungicide.

Onder deze omstandigheden bleek ook dat de resistente stammen niet verdwenen uit mengpopulaties van een gevoelige en een resistente stam hetgeen erop wijst dat de virulentie van de resistente isolaten van hetzelfde niveau is als dat van het oorspronkelijke isolaat.

Op grond van de gevonden resultaten mag de mogelijkheid tot resistentieontwikkeling tegen acylalanine fungiciden in de praktijk aanwezig worden geacht.

References

Brown, I. F. & Hall, H. R., 1979. Induced and natural tolerance to fenarimol (EL-222) in *Cladosporium cucumerinum* and *Venturia inaequalis*. Phytopathology 69: 914. (Abs.).

Dekker, J. & Gielink, A. J., 1979a. Acquired resistance to pimaricin in *Cladosporium cucumerinum* and *Fusarium oxysporum* f. sp. *narcissi* associated with decreased virulence. Neth. J. Pl. Path. 85: 67-73.

Dekker, J. & Gielink, A. J., 1979b. Decreased sensitivity to pyrazophos of cucumber and gherkin powdery mildew. Neth. J. Pl. Path. 85: 137-142.

Drake, J. W. & Baltz, R. H., 1976. The biochemistry of mutagenesis. A. Rev. Biochem. 45: 11-37.

Frosheiser, F. I. & Barnes, D.K., 1973. Field and greenhouse selection for *Phytophthora* root rot resistance in alfalfa. Crop Sci. 13: 735-738.

Fuchs, A., Ruig, S. P. de, Tuyl, J. M. van & Vries, F. W. de, 1977. Resistance to triforine: a non-existent problem? Neth. J. Pl. Path. 83 (Suppl. 1): 189-205.

Irwin, J. A. G., Miller, S. A. & Maxwell, D. P., 1979. Alfalfa seedling resistance to *Phytophthora megasperma*. Phytopathology 69: 1051-1055.

Ribeiro, O. K., 1978. A source book of the genus *Phytophthora*. Gantner Verlag, Germany.

Schwinn, F. J., Staub, T. & Urech, P. A., 1977a. A new type of fungicide against diseases caused by Oomycetes. Meded. Fac. Landbouwwet. Rijksuniv. Gent 42: 1181-1188.

Schwinn, F. J., Staub, T. & Urech, P. A., 1977b. Die Bekämpfung falscher Mehltau-Krankheiten met einem neuen Wirkstoff aus der Gruppe der Acylalanine. Mitt. biol. Bundanst. Ld-u. Forstw. Berlin-Dahlem 178: 145-146.

Staub, T., Dahmen, H. & Schwinn, F. J., 1978. Biological characterization of uptake and translocation of fungicidal acylalanines in grape and tomato plants. Z. PflKr. PflSchutz 85: 162-168.

Staub, T., Dahmen, H., Urech, P. & Schwinn, F. J., 1979. Failure to select for in vivo resistance in *Phytophthora infestans* to acylalanine fungicides. Plant Dis. Reptr 63: 385-389.

Urech, P. A., Schwinn, F. J. & Staub, T., 1977. CGA 48988, a novel fungicide for the control of late blight, downy mildews and related soil-borne diseases. Proc. Br. Crop Prot. Conf. 1977: 623-631.

Waard, M. A. de & Gieskes, S. A., 1977. Chracterization of fenarimol-resistant mutants of *Aspergillus nidulans*. Neth. J. Pl. Path. 83 (Suppl. 1): 174-188.

Address

Laboratory of Phytopathology, Agricultural University, Binnenhaven 9, 6709PD Wageningen, The Netherlands.