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Resistance to metalaxyl in isolates of the sunflower pathogen *Plasmopara halstedii*

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Abstract

Plasmopara halstedii isolates showing an atypical reaction to metalaxyl were collected in France, in 1995 and 1996, and tested in the laboratory for their level of sensitivity to this fungicide. Primary and secondary infections caused by one of these isolates were not controlled by the metalaxyl concentration registered for seed treatment. The EC_{50} of this isolate was 12 800 mg a.i. kg^{-1} compared with 22 mg a.i. kg^{-1} for sensitive isolate, indicating a 582-fold decrease in sensitivity to the compound. There was no reduction in the agressiveness of the resistant isolate. Using other anti-oomycete fungicides, it appeared that propamocarb, contact fungicides (fluazinam, folpet, mancozeb) and the mixed formulations dimethomorph + mancozeb, cymoxanil + mancozeb and ofurace + folpet were effective against primary infections made with metalaxyl resistant and sensitive isolates, but not against secondary infections. Metalaxyl mixed with fluazinam, folpet or mancozeb was more effective against primary infections with the resistant isolate than metalaxyl alone. The EC_{50} of five other isolates ranged from 5 800 to 32 900 mg a.i. kg^{-1}, indicating a variability in metalaxyl sensitivity of resistant sunflower downy mildew isolates. This is the first report of physiological resistance to metalaxyl in *Plasmopara halstedii*.

Introduction

Downy mildew, *Plasmopara halstedii* (Farl.) Berl. et de Toni, of sunflower, *Helianthus annuus* L., is a major disease of this crop. Most important yield losses are due to primary infections through the roots (Allard, 1979) and early secondary infections (aerial) which result in systemic infection of plants by the fungus (Regnault and Tourvieille, 1991).

This obligate parasite is specific to sunflower. It shows a number of physiological races which can be distinguished by their differential virulence on sunflower genotypes (Gulya et al., 1991). Race A is the most widespread of the five races known in France (Roeckel-Drevet et al., 1997). Use of resistant cultivars is the most effective method to control this pathogen (Vear, 1978) but as genetic resistance of sunflower may be overcome by new physiological races (Carson, 1981), chemical control by seed treatment with metalaxyl is widely used. Metalaxyl is a systemic phenylamide fungicide which is particularly effective against host-dependant growing mycelium of *P. halstedii* (Mouzeyar et al., 1995). Its common use in France since 1990 has meant that losses caused by this disease have been insignificant despite the spread of new races showing virulence on most cultivars (Penaud, 1994). However, in 1995 and 1996 (Penaud et al., 1997), prospecting in France showed downy mildew isolates which exhibited, in the laboratory, atypical responses to metalaxyl.

This led us to study the metalaxyl sensitivity level of these isolates by a method based on that of Melero-Vara et al. (1982). The metalaxyl sensitivity level and sporulation capacity of an atypical isolate collected in 1995 (ApR2) were compared with those of a sensitive isolate (A). In addition, the existence of several members of Peronosporales showing cross-resistance between metalaxyl and related fungicides (Cohen and

Reuveni, 1983, Diriwächter et al., 1987, Samoucha and Cohen, 1984) led us to test the sensitivity of ApR2 to some other anti-oomycete fungicides.

Materials and methods

Host-parasite system

Sunflower genotypes used were a population cultivar Peredovik susceptible to all known French downy mildew races, two commercial hybrids, Fantasol and Albena, and an experimental hybrid EL64 , the last three susceptible to race A.

Plasmopara halstedii isolates of race A used as inoculum were the isolate A, sensitive to metalaxyl, collected in 1988 (Tourvieille et al., 1988) and maintained on EL64 seedlings, and six isolates resistant to metalaxyl, ApR2 and ApR4, ApR5, ApR6, ApR7, ApR8 collected in many areas of France respectively in 1995 and 1996 using the protocol described by Penaud et al. (1997). These isolates were maintained on Peredovik seedlings treated with the registered rate of metalaxyl, 2 100 mg active ingredient (a.i.) kg^{-1}. The maintenance cycle for both sensitive and resistant isolates consisted every two weeks, of using the spores produced on seedlings from the previous infection cycle to infect a new series of germinated seeds.

Evaluation of metalaxyl sensitivity of P. halstedii *isolates*

Fungicide-free seeds (100 g) were treated by shaking them for 1 min in glass jar of water (30 ml) containing the appropriate weight of metalaxyl (as Apron 35) to give the following rates 0, 0.21, 2.1, 21, 210, 2 100 and 21 000 mg a.i. kg^{-1} seed and dried at room temperature (Melero-Vara et al., 1982).

Artificial infections through roots were prepared as follows: seeds were allowed to germinate in sterile moist filter paper for 48 h. They were then inoculated with *P. halstedii* using the method described by Cohen and Sackston (1973). For each metalaxyl concentration, batches of 20 germinated seeds were placed in pill boxes and just covered with a suspension of 10^5 zoosporangia ml^{-1} of distilled water for 5 h at 18 $\pm$ 1°C. Each batch was planted in 1 litre pots filled with soilless compost (16 seedlings per pot with undamaged root initials). The seedlings were then placed in a growth chamber at 18 $\pm$ 1°C, with a relative humidity of 70%, a photoperiod of 16 h and a light intensity of about 200 μE $m^{-2}s^{-1}$.

Eleven days after infection, after an abundant watering, the seedlings were covered with transparent polythene bags to provide a saturated atmosphere. Three days later, the percentage of seedlings bearing zoosporangia on their cotyledons and/or young leaves was noted. This percentage was calculated, for each metalaxyl concentration and each downy mildew isolate, for 20 replicates of 16 seedlings. This large number of replications made it necessary to carry them out over 10 maintenance cycles, with 2 replicates per cycle.

Artificial aerial contaminations were prepared as follows: Peredovik seeds were metalaxyl-treated and allowed to germinate as previously. For each metalaxyl concentration, 8 seedlings were planted in 1 litre pots filled with soilless compost. After the first pair of leaves had expanded, a suspension of 5×10^4 zoosporangia ml^{-1} of distilled water was applied on leaves with a very small spray so as to distribute inoculum homogeneously. The pots were then covered with transparent polythene bags to maintain a saturated atmosphere for 48 h. Thirteen days after infection, after 24 h in a saturated atmosphere, the percentage of seedlings bearing chlorotic spots and zoosporangia on the leaves was noted. This percentage was calculated, for each downy mildew isolate and each metalaxyl concentration, for 7 replicates of 8 seedlings.

Metalaxyl efficacy against primary and secondary infections of the downy mildew isolates was characterised by metalaxyl effective concentrations giving 50% (EC_{50}) and 95% (EC_{95}) inhibition of fungal development. The resistance factor was the ratio between the EC_{50} of the isolates tested and the EC_{50} of the reference isolate (A) which was metalaxyl sensitive. As data were proportions of plants showing sporulation, they were analysed by a generalized linear model (glm), using the canonical links and variance functions for binomial distribution. This method is also called the log-logistic regression, since the metalaxyl concentrations are transformed using a log-scale. A dose-response model was fitted to observed data and its significance tested through the analysis of deviance, which measures the likelihood of the model (Collett, 1991). A Chi-square test was performed to assess whether the deviance value showed significant departure from the null hypothesis, which was rejected when $P(\chi^2) < 0.05$ as useral. Approximate confidence intervals of EC_{50} and EC_{95} were computed according to Collett (1991).

Table 1. List of fungicide formulations used in experiments with primary and secondary *Plasmopara halstedii* infections

Proprietary name	Active ingredient(s)	Application rate g a.i. kg^{-1} seed	Formulation	Manufacturer
Apron 35	Metalaxyl 35%	2.1	WS	Ciba-Geigy
Apron 35 + Sagiterre	Metalaxyl 35% + Fluazinam 500 g l^{-1}	2.1 + 2.5	WS + SC	Ciba-Geigy
Sagiterre	Fluazinam 500 g l^{-1}	2.5	SC	Ciba-Geigy
Apron 35 + Dithane M45	Metalaxyl 35% + Mancozeb 80%	2.1 + 10.5	WS + WP	Ciba-Geigy
Dithane M45	Mancozeb 80%	10.5	WP	Ciba-Geigy
Apron 35 + Foltane	Metalaxyl 35% + Folpet 50%	2.1 + 9	WS + WP	Ciba-Geigy + Sipcam
Foltane	Folpet 50%	15.0	WP	Sipcam
Milfuram	Ofurace	1.2	WP	AgrEvo
Vamin TS	Ofurace 80 g l^{-1} + Folpet 600 g l^{-1}	1.2 + 9.0	FS	AgrEvo
Fulvax	Cymoxanil 6% + Mancozeb 70%	0.9 + 10.5	WP	Ciba-Geigy
Forum PM	Dimethomorph 50%	4.5	WP	Cyanamid Agro
Acrobat M	Dimethomorph 9% + Mancozeb 60%	2.25 + 15.0	WP	Cyanamid Agro
Aliette	Fosetyl-Al	4.0	WP	Rhône-Poulenc Agro
Previcur N	Propamocarb HCl 722 g l^{-1}	7.22	SL	AgrEvo

Quantification of sporulation

For each metalaxyl concentration and each downy mildew isolate (A and ApR2), cotyledons bearing sporulation were cut, placed in a jar with 10–60 ml of distilled water, and shaken thoroughly. The jar was then immersed in ultrasonic-cell for about 10 s to obtain a zoosporangia suspension. Zoosporangia were counted using a aemocytometer. Mean zoosporangia numbers per cotyledon were calculated for 10 replicates. Results were expressed as zoosporangia number per cotyledon. Comparisons of means were made using the Newman-Keuls test at P = 0.05. As variances were heterogeneous, these data were first converted with a square root function.

Sensitivity of A and ApR2 isolates to different anti-oomycete fungicides

Fourteen commercial anti-oomycete fungicide formulations were used (Table 1). Applications were made to sunflower seeds of the hybrid Fantasol by Cargill France and of the hybrid Albena by SPV (Service de la Protection des Végétaux). The use of different hybrids in this study was not significant because a study on commercially metalaxyl treated seeds of 42 different sunflower hybrid varieties not containing any race A resistance genes, indicated that there were no interaction between host genotype and the isolate ApR2 (data not shown). The efficacy of these products was tested on primary and secondary infections caused by isolates A and ApR2 according to the method described above. For each fungicide and downy mildew isolate, there were 4 replicates. Comparisons of means were made using the Newman-Keuls test at P = 0.05, after data convertion with an arcsine square root function.

Results

Metalaxyl sensitivity level of resistant isolate (A) compared with that of sensitive isolate (ApR2)

The different maintenance cycles had no significant effect on the proportion of seedlings showing sporulation after primary infections with isolates A ($P(\chi^2)$ = 0.64) and ApR2 ($P(\chi^2)$ = 0.59). Thus there was no modification in action of metalaxyl on these isolates during infection cycles. Replications were therefore considered independently of maintenance cycle.

For all metalaxyl rates, the percentage of seedlings showing sporulation was greater with the isolate ApR2 than with isolate A (Figure 1). Only the resistant isolate induced sporulation on seedlings treated with 2 100 and 21 000 mg metalaxyl kg^{-1} seed. The latter concentration induced phytotoxicity on cotyledons in some cases but did not modify the normal growth of the seedlings. Adjusted values of EC_{50} and EC_{95} of the sensitive isolate were respectively 22 (confidence interval: $19 < 22 < 27$) and 510 ($360 < 510 < 710$) mg a.i. kg^{-1}. This isolate was thus controlled effectively by the registered rate of metalaxyl (2 100 mg a.i. kg^{-1}). For the isolate

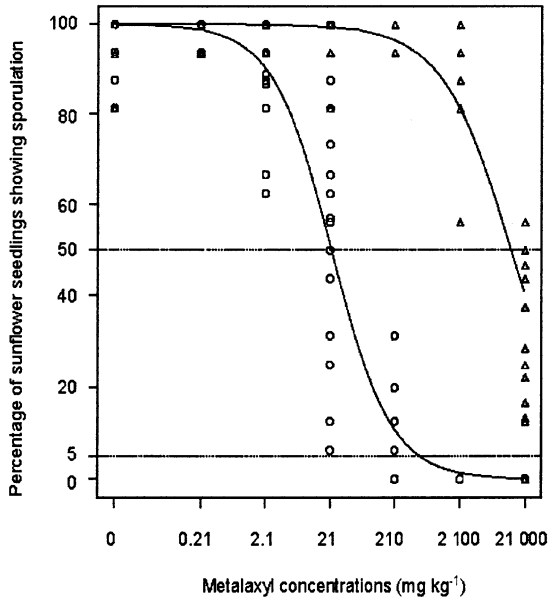

Figure 1. Comparison of the percentage of sunflower seedlings (cv. Peredovik) treated with seven metalaxyl concentrations, showing sporulation after primary infections with *Plasmopara halstedii* A and ApR2. ○ and △ indicate values for replicates infected with isolates A and ApR2 respectively. In some cases, several replicates have the same value.

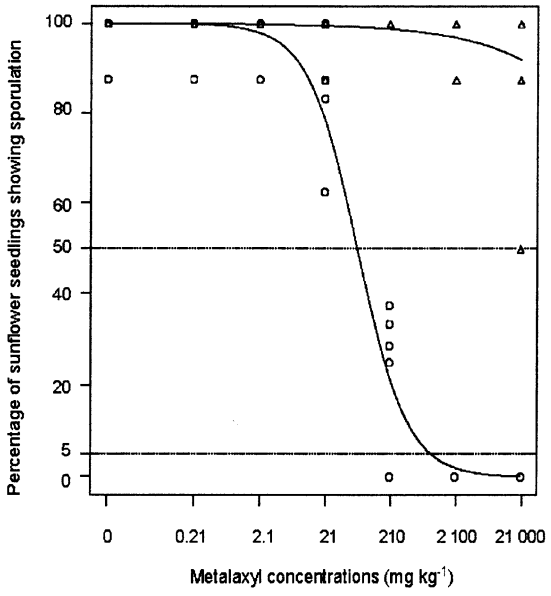

Figure 2. Comparison of the percentage of sunflower seedlings (cv. Peredovik) treated with seven metalaxyl concentrations, showing sporulation after secondary infections with *Plasmopara halstedii* A and ApR2. ○ and △ indicate values for replicates infected with isolates A and ApR2 respectively. In some cases, several replicates have the same value.

ApR2, the EC_{50} was 12 800 (10 000 < 12 800 < 16 400) mg a.i. kg^{-1} but the EC_{95} could not be calculated with reliability because there were 25% of sporulating seedlings with 21 000 mg a.i. kg^{-1}. The resistance factor was thus 582 (526 < 582 < 607), indicating that the ApR2 isolate was 582 times more resistant to metalaxyl than the isolate A (Figure 1).

After secondary infections caused by the isolate A, no symptom was noted for 2 100 and 21 000 mg a.i. metalaxyl kg^{-1} seed but the percentage sporulation was higher than after primary infections for the other metalaxyl rates (Figure 2). Adjusted values of EC_{50} and EC_{95} of the sensitive isolate were respectively 67 (45 < 67 < 99) and 920 (440 < 920 < 1 900) mg a.i. kg^{-1}. Thus, this isolate was effectively controlled by the 2 100 mg kg^{-1} metalaxyl concentration. These concentrations could not be calculated with reliability for the isolate ApR2 because 91% of seedlings showed disease symptoms with the 21 000 mg a.i. kg^{-1} indicating that the EC_{50} and EC_{95} were higher than 21 000 mg a.i. kg^{-1} (Figure 2).

Quantification of sporulation

A Newman-Keuls test indicated that mean zoosporangia numbers were not statistically different at P=0.05 between A and ApR2 isolates for metalaxyl concentrations lower than 2.1 mg a.i. kg^{-1} (Figure 3). In contrast, a significant difference was noted for higher or equivalent concentrations and a decrease of zoosporangia number when fungicide concentration was increased. Without metalaxyl, zoosporangia numbers produced by A and ApR2 were not statistically different. Thus, while the isolates produced different amounts of sporulation in the presence of fungicide, they were similar without fungicide.

Sensitivity of A and ApR2 isolates to different anti-oomycete fungicides

Results are presented in Table 2. Among fourteen formulations tested in primary infections, only dimethomorph and fosetyl-al were not entirely effective against the sensitive isolate. With isolate ApR2, only propamocarb and use of contact fungicide (fluazinam, folpet, mancozeb), alone or in mixture with other fungicide, prevent sporulation after primary infection. Resistance

Table 2. Mean percentages (± standard deviation) of sunflower seedlings (cv. Albena and Fantasol) treated with different fungicide formulations (Table 1), showing sporulation after primary and secondary infections of A and ApR2 *Plasmopara halstedii* isolates. Values labelled by the same letter within any columns are not significantly different at P = 0.05

Treatments	Primary infections				Secondary infections			
	A		ApR2		A		ApR2	
Untreated control	93.8 ± 5.1	a	93.8 ± 8.8	a	96.9 ± 6.3	a	100.0	a
Metalaxyl	0.0	e	93.8 ± 5.1	a	0.0	e	100.0	a
Metalaxyl + Fluazinam	0.0	e	6.3 ± 8.8	e	0.0	e	100.0	a
Fluazinam	9.4 ± 3.6	de	9.4 ± 3.6	de	100.0	a	100.0	a
Metalaxyl + Mancozeb	0.0	e	7.8 ± 11.8	e	0.0	e	100.0	a
Mancozeb	3.1 ± 6.3	e	6.3 ± 8.8	e	100.0	a	100.0	a
Metalaxyl + Folpet	0.0	e	0.0	e	0.0	e	100.0	a
Folpet	4.7 ± 6.0	e	3.1 ± 3.6	e	100.0	a	100.0	a
Ofurace	7.8 ± 3.1	de	87.5 ± 14.4	a	68.5 ± 17.7	b	100.0	a
Ofurace + Folpet	0.0	e	3.1 ± 6.3	e	65.6 ± 27.7	b	96.9 ± 6.3	a
Cymoxanil + Mancozeb	0.0	e	0.0	e	100.0	a	100.0	a
Dimethomorph	21.9 ± 23.1	cd	28.1 ± 14.9	cd	100.0	a	100.0	a
Dimethomorph + Mancozeb	0.0	e	0.0	e	100.0	a	100.0	a
Fosetyl-Al	20.3 ± 3.1	cd	34.4 ± 16.5	c	90.6 ± 18.8	a	100.0	a
Propamocarb HCl	0.0	e	0.0	e	75.0 ± 10.2	b	100.0	a

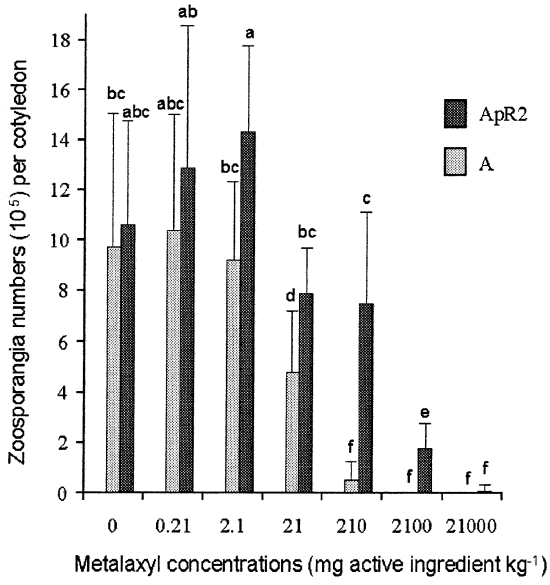

Figure 3. Effect of metalaxyl on the number of zoosporangia (10^5) produced by a metalaxyl sensitive (A) and a metalaxyl resistant (ApR2) isolates of *Plasmopara halstedii*. The vertical lines represents the standard deviation of the means of 10 experiments per isolate. Values labelled by the same letter are not significantly different at P=0.05.

to metalaxyl of ApR2 was coupled with resistance to ofurace. Secondary infections with ApR2 were not controlled by any of the fungicides and only metalaxyl alone or in mixture controlled aerial infection by isolate A. The results obtained with the registered rate of metalaxyl confirmed that this fungicide was totally effective in controlling primary and secondary infections of the sensitive isolate but not of isolate ApR2.

Metalaxyl sensitivity level of resistant isolates collected in 1996

The five isolates were inhibited only by high rates of metalaxyl and none was controlled by the registered rate (Table 3). Only the EC_{50} of ApR5 was statistically different from that of the other isolates. It was the least metalaxyl sensitive, with a resistance level 2.6 times greater than that of the ApR2 isolate. The EC_{50} of other isolates did not differ statistically.

Discussion

This is the first report of *P. halstedii* isolates showing such a high level of metalaxyl resistance in the laboratory. Viranyi et al. (1992) previously observed differences in tolerance to metalaxyl seed treatment between downy mildew isolates, but the metalaxyl concentrations used were 1 or 2 mg kg^{-1}seed whereas the registered rate is 2 100 mg kg^{-1}.

Table 3. Metalaxyl concentrations inhibiting of 50% (EC_{50}) growth of ApR2, ApR4, ApR5, ApR6, ApR7 and ApR8 *Plasmopara halstedii* isolates (95% confidence interval)

Isolates	EC_{50} (mg active ingredient kg^{-1})		
ApR2	10 000 <	12 800	< 16 400
ApR4	5 000 <	7 100	< 10 100
ApR5	16 900 <	32 900	< 64 300
ApR6	6 300 <	9 700	< 14 900
ApR7	3 900 <	5 800	< 8 700
ApR8	5 800 <	9 300	< 15 200

The ApR2 resistance level is similar to that of other metalaxyl resistant oomycete plant pathogens. For example, Klein (1994) reported *Pseudoperonospora humuli* strains showing a resistance factor close to 500. This high level of metalaxyl resistance in ApR2 excluded any possibility of controlling it by increasing the rate of metalaxyl.

Since there was no increase in the resistance factor after ten ApR2 maintenance cycles on seeds treated with metalaxyl at the registered rate and that some isolates collected in 1996 had a similar level of resistance, it cannot be considered that laboratory multiplication cycles caused any selection for resistance.

The metalaxyl resistant isolates used in this study showed a variability which has also been noted in some other oomycetes. Staub and Sozzi (1981) reported *Plasmopara viticola* strains showing resistance factors ranging between 100 and 1 000. In *P. humuli* strains, the resistance factors ranged between 60 and more than 600 (Klein, 1994).

This *P. halstedii* metalaxyl resistance appeared in France 6 years after generalized use of the fungicide. In other oomycetes, such resistance appeared more rapidly after the first use of this fungicide. For example, metalaxyl resistant strains of *P. viticola* appeared in France only two years after first fungicide use (Staub and Sozzi, 1981). This slow appearance of resistance in *P. halstedii* may be because metalaxyl is exclusively used as a seed treatment and is therefore only applied once in each sunflower cultural cycle.

Although the sporulation capacity of sensitive and resistant isolates was affected by metalaxyl concentrations higher than 2.1 mg a.i. kg^{-1}, an increase of metalaxyl concentration reduced the number of spores produced but did not completely block sporulation of resistant isolates.

For *P. halstedii*, metalaxyl resistance could affect both systemic invasion by mycelium and zoosporangia production. This phenomenon is also found in *Phytophthora parasitica* (Ferrin and Wadsworth, 1992). However, Coffey and Young (1984) showed that sporangia production in *Phytophthora infestans* was inhibited in both sensitive and resistant isolates. In the absence of metalaxyl, ApR2 sporulated as well as the sensitive isolate, thus the acquisition of resistance did not disturb the sporulation capacity of ApR2. As the sporulation capacity of an isolate is an indication of its competitiveness (Ferrin and Wadsworth, 1992), ApR2 appears likely to remain in *P. halstedii* populations containing metalaxyl sensitive isolates. Studies on the stability of resistance are in progress to determine the probability of survival of resistant isolates in the absence of metalaxyl.

Results obtained with several other anti-oomycete fungicides against A and ApR2 isolates showed that use of propamocarb and most mixtures with contact fungicides (folpet, mancozeb, fluazinam) controlled ApR2 primary infections efficiently. Several studies have shown the efficacy of mixtures of metalaxyl with contact fungicides against metalaxyl resistant isolates of other oomycetes. Thus, Moss (1987) showed that a mixture of metalaxyl and mancozeb gave very good control of a metalaxyl resistant isolate of *Pseudoperonospora cubensis*. Pappas (1985) observed the same phenomenon with metalaxyl resistant isolates of *P. infestans*. However, Clerjeau (1994) showed that a mixture of metalaxyl and a contact fungicide gave poor control of metalaxyl resistant strains of *P. viticola*. Here, metalaxyl + mancozeb in mixture did not totally control ApR2 infections but was more effective than metalaxyl alone.

In this study, only mixtures containing metalaxyl were effective against secondary infections, but these were ineffective when the infection was caused by a metalaxyl resistant isolate. The other mixtures were totally ineffective. Secondary infections can be controlled by seed fungicide treatment only if the fungicide is systemic. Thus, addition of a contact fungicide, although controlling metalaxyl resistant primary infections, is of no value against metalaxyl resistant secondary infections since it is not translocated to the aerial plant parts. Other systemic fungicides tested (ofurace, dimethomorph, propamocarb) were ineffective against secondary infections. Ofurace did not control a metalaxyl resistant infection as there is cross-resistance between these two products, a phenomenon described in most of oomycetes resistant to

phenylamides (Gisi and Cohen, 1996). Dimethomorph and propamocarb are not related to metalaxyl, so there was no cross-resistance between these products and metalaxyl because they were ineffective agaisnt secondary infection whatever the downy mildew isolate. In order to explain this phenomenon, we can suppose that either these fungicides (and ofurace in sensitive situation) were less effective on *P. halstedii* host-dependant stages than metalaxyl, this hypothesis was confirmed for ofurace and propamocarb by Viranyi and Oros (1991), and/or these products did not move into the sunflower aerial parts in sufficient concentration to confer protection, this phenomenon was described in sunflower for an other systemic fungicide, carbendazim, in the chemical control of *Sclerotinia sclerotiorum* (Lamarque et al., 1985).

The appearance of *P. halstedii* isolates showing a high level of metalaxyl resistance in laboratory indicates that this pathogen, already known to show changes in virulence, allowing it to overcome host-plant resistance, has the capacity to overcome the most effective of currently available fungicides, metalaxyl. Use of seed treatment with both metalaxyl and a contact fungicide would make it possible (i) to allow much slower evolution of resistant fungus population (Staub and Sozzi in Gisi and Cohen, 1996), (ii) to avoid scattering of resistant isolates by infected seeds in uninfected countries, (iii) to limit primary attacks whatever the composition of pathogen population and so limit sources of inoculum for aerial contamination. Nevertheless this would not protect against aerial contamination by spores from infected volunteer plants. It is likely that contact fungicides used alone in foliar sprays would make it possible to limit metalaxyl resistant aerial infections. However, this practice would be costly for farmers and at the present time, no fungicide has been authorized as a foliar spray against *P. halstedii* in France. Loss of efficiency in the field, has not yet been observed, but the risk exists and it is essential to continue monitoring for the occurrence and selection of resistant isolates.

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