

REACTION OF FIELD POPULATIONS OF SUNFLOWER DOWNY MILDEW (*Plasmopara halstedii*) TO METALAXYL AND MEFENOXAM

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Received: August 22, 2004

Accepted: May 22, 2005

SUMMARY

Seed treatment with phenylamide fungicides (metalaxyl and mefenoxam) provides an effective chemical control of sunflower downy mildew caused by the oomycete *Plasmopara halstedii*. Resistance of the fungus to metalaxyl has been reported in Spain and differences of disease incidence (DI) caused by field populations (FPs) have been observed. The sensitivity of several FPs to seed treatment with metalaxyl was compared. Different groups of FPs were discriminated on the basis of DI caused on sunflower plants. None of the FPs was completely controlled by the fungicide at the dose used. In addition, the efficacy of metalaxyl and mefenoxam against FPs resistant to metalaxyl were evaluated. Three genotypes of sunflower were treated with the fungicides at two different doses. Neither metalaxyl or mefenoxam nor doses had a significant effect on the DI caused by the FPs, suggesting the absence of effect of the double quantity of active enantiomer provided by mefenoxam as compared with metalaxyl. Nevertheless, significant differences on DI were observed among genotypes. The different DIs could be related to: a) different germination rates and, consequently, size of the radicle in the moment of inoculation, b) higher amount of fungicide provided to the confectionery genotype, which has a larger size of seed.

Key words: fungicide resistance, *Helianthus annuus* L., mefenoxam, metalaxyl, phenylamide fungicides, sunflower downy mildew

INTRODUCTION

Sunflower downy mildew is caused by the fungus *Plasmopara halstedii* Farl. Berl. & de Toni, an obligate parasite which infects seedlings during the early stages of development, usually from sowing to two leaves. Systemic infection is characterized by symptoms of chlorotic mosaic bordering the veins of the leaves as well as

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stunting of the plants. Optimum conditions for the disease, such as high inoculum concentrations, abundant rain and temperatures of 15-18°C can cause preemergence or postemergence damping off of the young plants.

The incorporation of genes of resistance to *P. halstedii* is a common way to control the disease. These genes are effective against one or more races of the fungus, but they are overcome by more virulent races of the fungus after continuous crop of resistant hybrids. Besides genetic strategy the disease can also be chemically controlled through metalaxyl and mefenoxam. Metalaxyl is a phenylamide fungicide which provides systemic protection against oomycetes. In sunflower, and due to the early infection of the host, metalaxyl has been used as seed dressing for the control of downy mildew since 1980 (Iliescu, 1980; Melero-Vara *et al.*, 1982). Mefenoxam was marketed ten years later and it contains the biologically active enantiomer of the racemic fungicide metalaxyl (Shetty, 1998). Both fungicides are used worldwide against sunflower downy mildew, although metalaxyl has recently been prohibited in Spain.

In different Peronosporales, insensitivity to metalaxyl appearing after continuous foliar applications of the fungicide has been widely reported in several crops (Cohen and Reuveni, 1983; Crute, 1987; 1992; Katan and Basi, 1981; Klein, 1994; Lamour and Hausbeck, 2000; Parra and Ristaino, 1998; Wicks *et al.*, 1994; Wiglesworth *et al.*, 1988). In sunflower, since it only comes in contact with *P. halstedii* once every season, its single application was thought to reduce the probability of building up resistance in the fungus. However, the occurrence of resistant isolates of the pathogen has been reported under conditions of greenhouse and field (Oros and Virányi, 1984; Molinero-Ruiz *et al.*, 2003). Besides, the resistance of the fungus has been found at doses of metalaxyl lower than 2 g a. i./kg seed in Hungary, the USA and Turkey (Gulya *et al.*, 1997; Gulya *et al.*, 1999; Virányi *et al.*, 1992) and at commercial doses of 2 g a.i./kg seed in France and Spain (Albourie *et al.*, 1998; Molinero-Ruiz *et al.*, 2003). Some authors have reported the rapidity with which resistance to phenylamide fungicides has arisen in natural populations of oomycetes (Parra and Ristaino, 1998).

Nowadays the treatment of commercial sunflower hybrids with mefenoxam is a requirement for all the hybrids grown in Spain. However, resistance to mefenoxam has been already reported in natural populations of the oomycete *Phytophthora capsici* (Parra and Ristaino, 1998; Lamour and Hausbeck, 2000).

The objectives of this work were to compare the pathogenicity of different field populations (FPs) of *P. halstedii* on metalaxyl treated seed, and to assess if differences in protection conferred by metalaxyl and mefenoxam occur when resistance to metalaxyl is observed in the pathogen.

MATERIALS AND METHODS

Pathogenicity of field populations of *P. halstedii* on metalaxyl treated seed

In a preliminar test, disease incidences (DI) between 18 and 100% (data not shown) were recorded on sunflower plants from seed treated with metalaxyl and inoculated with different FPs of *P. halstedii*. Thus, experiment 1 was established to

compare the reaction to metalaxyl of nine FPs collected in different locations and with different virulences (Molinero-Ruiz *et al.*, 2002).

Seed of the susceptible sunflower oilseed hybrid cultivar PRO9103 were dressed with Allegiance 28% LS (Gustafson Inc., McKinney, 75070 TX, USA) at 0.3 g a.i./kg seed. The experimental unit consisted of one tray (25 × 25 × 6 cm) filled with sand:perlite (2:3 v), in which 40 seeds were sown. Three replications were arranged in a complete randomized design, being the FP of *P. halstedii* the factor to be analyzed (Table 1). Each FP was inoculated to three trays of metalaxyl-treated seed and three trays of nontreated seed (control). For the control, sunflower seed were dressed with the same volume of water than the volume of fungicide used for the metalaxyl treated seed.

Table 1: Field populations (FP) of *Plasmopara halstedii* used in Experiment 1

Reference of FP	Location*	Virulence
15	Sevilla, S Spain	100
16	Guadalajara, C Spain	100
25	Gerona, NE Spain	703
27	Cádiz, S Spain	100
66	Córdoba, S Spain	100
76	Córdoba, S Spain	100
86	Cádiz, S Spain	100
96	Cuenca, C Spain	100
106	Sevilla, S Spain	100

* NE=Northeast, S=South, C=Center

Three days after sowing, when seeds had germinated but had not emerged yet, soil drench inoculation was performed in the corresponding trays by irrigation with 100 ml of a suspension of sporangia in distilled water (20×10^3 per ml). Afterwards, the trays were kept in a greenhouse at 24-31°C with 16 h photoperiod of 350 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$. Watering was done daily for 10-12 additional days until the first pair of leaves were 1-2 cm long. Trays were then transferred to a dark room at 16°C and 100% RH for 14-16 h to ensure pathogen sporulation. DI was recorded as percentage of plants showing sporulation of the fungus on the cotyledons and/or true leaves. DI in seedlings from treated seed inoculated with the different FPs tested was corrected (CDI) according to the values obtained for nontreated controls ($\text{CDI} = (\text{DI treated} \times 100) / \text{DI nontreated}$). Analysis of variance was performed with transformed values $[(\% \text{ CDI} + 0.5)^{0.5}]$ (Steel and Torrie, 1988) and means were compared by a Fisher's protected LSD test ($P=0.05$).

Assessment of the efficacy of mefenoxam against field populations of *P. halstedii* with resistance to metalaxyl

The protection of metalaxyl and mefenoxam was assessed using two FPs: one of them resistant to metalaxyl (FP 48, collected in Fargo ND) and the other intermediately sensitive (FP 56, from Cordoba, Spain) in experiments 2 and 3, respectively. In both experiments oil hybrids PRO9103 and CAR270 and the confectionery hybrid RH3701, all genetically susceptible to the disease, were used. Artificial inoculation of the host, growth conditions during and after inoculation and disease eval-

uation procedures were similar to those described for experiment 1. Seed was treated using metalaxyl as Allegiance 28% LS or mefenoxam as Apron-XL 32% LS (Novartis Corporation, Greensboro, 27409 NC, USA). The doses of 2.0 g a.i./kg seed and 0.3 g a.i./kg seed, used in Europe and in the USA and Canada, respectively, were compared for each hybrid-formulation combination. A complete randomized factorial design was used, with hybrid, fungicide formulation and dose of application as the main factors. The experimental unit consisted of one tray (40 seeds), and four replications for each combination of treatments were established. Angular transformation was applied to values of DI in each tray before analysis of variance was performed (Steel and Torrie, 1988). When F test was significant ($P < 0.05$), mean values were compared according to Fisher's protected LSD test ($P = 0.05$) and orthogonal contrasts were made to analyze the interaction between factors.

RESULTS

Pathogenicity of field populations of *P. halstedii* on metalaxyl treated seed

Three groups of FPs of *P. halstedii* were discriminated by the analysis, since significant differences were shown ($P < 0.0001$). Two FPs caused 90 and 95% CDI and four caused CDI from 50 to 60%. The three remaining FPs, including the only FP of race 703 (Table 1), caused low CDI (4-8%) (Figure 1). None of the FP tested was completely controlled by metalaxyl at the dose of the treatment.

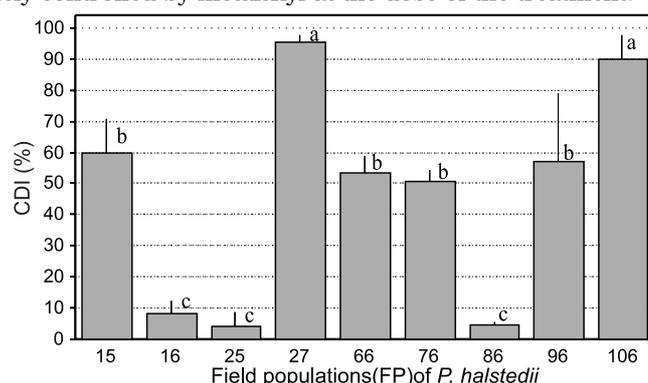


Figure 1: Incidence of disease in sunflower hybrid CAR270 caused by *Plasmopara halstedii*. Seed was treated with 0.3 g a.i./kg seed of metalaxyl and inoculated with different field populations (FP) using the soil drench method (Experiment 1). Different letters show significantly ($P < 0.05$) different values according to Fisher's protected contrast of Least Significant Differences. Vertical bars represent standard errors.

Assessment of the efficacy of mefenoxam against field populations of *P. halstedii* with resistance to metalaxyl

Neither treatment with metalaxyl or mefenoxam nor doses had a significant effect on the DI caused by FP 48 and FP 56. However, the highest sensitivity of FP

56 to metalaxyl compared with that of FP 48 was confirmed, since mean DI were 91 and 95% (experiment 2) and 54 and 56% (experiment 3) for the doses of 2.0 and 0.3 g a.i./kg seed, respectively (Tables 2 and 3). FP 56 also showed to be more sensitive to mefenoxam than FP 48: mean values of DI 94 and 95% in experiment 2 and 50 and 58% in experiment 3 were recorded at the doses of 2.0 and 0.3 g a.i./kg seed, respectively (Tables 2 and 3).

Table 2: Disease incidence (DI) on sunflower hybrids PRO9103, RH3701 and CAR270 treated with metalaxyl and mefenoxam at two different doses and inoculated with FP 48 of *Plasmopara halstedii*. Experiment 2

Hybrid ^x	Fungicide ^y	Dose (g a.i./kg seed) ^z	Disease incidence (%)
PRO9103 A	Metalaxyl	0.3	98
		2.0	95
	Mefenoxam	0.3	96
		2.0	96
RH3701 AB	Metalaxyl	0.3 a	93
		2.0 b	86
	Mefenoxam	0.3 a	96
		2.0 b	87
CAR270 B	Metalaxyl	0.3	94
		2.0	92
	Mefenoxam	0.3	92
		2.0	99

^{x, z} Different letters show significant ($P < 0.05$) differences

^y Seeds were treated with Allegiance 28% LS (metalaxyl) or Apron-XL 32% LS (mefenoxam)

Table 3: Disease incidence (DI) on sunflower hybrids PRO9103, RH3701 and CAR270 treated with metalaxyl and mefenoxam at two different doses and inoculated with FP 56 of *Plasmopara halstedii*. Experiment 3

Hybrid ^x	Fungicide	Dose (g a.i./kg seed) ^y	Disease incidence (%)
PRO9103 A	Metalaxyl	0.3	73
		2.0	81
	Mefenoxam	0.3	78
		2.0	70
RH3701 B	Metalaxyl	0.3	47
		2.0	54
	Mefenoxam	0.3	46
		2.0	56
CAR270 C	Metalaxyl	0.3	42
		2.0	35
	Mefenoxam	0.3	50
		2.0	22

^x Different letters show significant ($P < 0.05$) differences

^y Seeds were treated with Allegiance 28% LS (metalaxyl) or Apron-XL 32% LS (mefenoxam).

In experiment 2 hybrid RH3701 showed significantly ($P < 0.0045$) lower DI when 2.0 g a.i./kg seed doses had been applied (86 and 87% in treatments with met-

alaxy and mefenoxam, respectively) (Table 2). When FP 56 was inoculated, the hybrid PRO9103 showed a significantly ($P < 0.0001$) high incidence of disease independently upon the fungicide or the dose of the treatment. The mean DI in PRO9103 was 75%, while hybrid CAR270 showed the lowest incidence of downy mildew: mean 37% (Table 3).

DISCUSSION

Despite the previous identification of only one isolate of *P. halstedii* resistant to the commercial dose of metalaxyl out of 52 tested (Molinero-Ruiz *et al.*, 2003), the results of this paper show the frequent occurrence of resistance to the fungicide in FPs when low doses are used. This seems to indicate that resistant populations of the fungus occur naturally in the soil. Resistance to phenylamide fungicides prior to their commercial use has been detected in *Pseudoperonospora cubensis*, *Phytophthora infestans* and *Plasmopara viticola* fungicides (Gisi *et al.*, 2000). Thus, resistant genotypes might evolve by naturally occurring random mutations in the absence of metalaxyl, selection by fungicide treatment increasing their frequency in the FP (i.e. fungal genotypes in the field). Besides, phenylamide fungicides provided good control of *P. infestans* in spite of the presence of resistant sub-populations of the pathogen (Gisi *et al.*, 2000; Gisi and Cohen, 1996).

The inocula used in the present work were obtained as a mixture of sporangia on diseased plants. Single-sporangium isolates of each FP, obtained from the infection of individual susceptible plants with a single sporangium, could help to ascertain the homogeneous or heterogeneous reaction of different components of the same FP to phenylamide fungicides. Role of oospores in sexual reproduction is the first step in the study of the nature of fungus resistance to phenylamide fungicides (Spring and Zipper, 2000). The resistance to metalaxyl has been reported to be incompletely dominant in other oomycetes: *P. infestans*, *P. capsici*, *P. sojae*, and *Bremia lactucae* (Gisi *et al.*, 2000; Lamour and Hausbeck, 2000).

Different frequencies of resistant populations of *P. halstedii* found in the present work compared with that of Molinero-Ruiz *et al.* (2003) could be due to: 1) dose of metalaxyl applied or 2) inoculation method used (whole seedling inoculation and soil drench). However, our experience shows that no difference is found on the efficacy between both methods of inoculation of the fungus in order to induce disease on sunflower.

Apparently, there is no relation between the virulence of FPs and their reaction to mefenoxam, although the set of FPs tested were not representative of the range of virulences identified in Spain (Molinero-Ruiz *et al.*, 2002).

The protection level achieved by the seed treatment of the three hybrids used in experiments 2 and 3 was not affected by either the fungicide or the dose used, suggesting the absence of effect of the "double quantity" of active enantiomer caused by mefenoxam when it is applied at the same dose as metalaxyl. The results also reveal

the existence of resistance to mefenoxam in *P. halstedii* from Spain as well as from the USA. Isolates of *Peronospora parasitica* and *P. capsici* showing resistance to mefenoxam have also been reported (Parra and Ristaino, 1998; 2001; Visunavat *et al.*, 1998). The lower DI on hybrid RH3701 treated with 2.0 g a.i./kg seed of metalaxyl and inoculated with FP 48 (Table 2) could be due to a higher amount of fungicide provided to each individual seed, since oilseed hybrids have smaller seed than confectionery hybrids. Also, differences of DI in the hybrids inoculated with FP 56 (Table 3) could be related to their different germination rates, which may result in lower infection when the soil drench inoculation method is used.

The management of sunflower downy mildew rely on the use of hybrids with genetic resistance as well as on the treatment with mefenoxam. The monitoring of the distribution and frequency of FP of *P. halstedii* resistant to phenylamides must be continuous, and fungicides with different modes of action are needed in fields where resistance is observed.

CONCLUSIONS

In the spring of 2003 the comercialization of sunflower seed treated with metalaxyl has become obligatory for the seed companies. In a previous work only one of 52 isolates of *P. halstedii* from Spain was resistant to metalaxyl at the labeled dose (Molinero-Ruiz *et al.*, 2003). This isolate was collected in a sunflower field in which sunflower is being treated with metalaxyl for the last twenty years, implying a strong pressure on the fungus.

Our results reveal that, although metalaxyl keeps being effective if it is used at the labeled dose, resistance to the fungicide is observed in FPs if doses as low as 0.3 g a.i./kg seed are used. Besides, mefenoxam has been proved to have the same efficacy as metalaxyl for the control of the disease when both fungicides are used at the same dose. However, it does not provide control of FPs of the fungus with resistance to metalaxyl. Thus, resistance to mefenoxam also occurs in FPs which are resistant to metalaxyl.

A continuous monitoring of the sensitivity of *P. halstedii* to mefenoxam is needed, since treated sunflower seed will be used in Spain every growing season and selection by fungicide treatment might occur in FPs of the fungus.

ACKNOWLEDGMENTS

We acknowledge the support of the Spanish Education and Science Office (M.E.C.) and the National Institute for Agricultural Research (I.N.I.A.). We thank Gustafson Inc. and Novartis Corporation for supplying the fungicides used, and we gratefully acknowledge Teresa Olmo and Robert A. MacArthur for technical support.

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REACCIÓN DE LAS POBLACIONES CAMPESTRES DE TIZÓN (*Plasmopara halstedii*) EN GIRASOL A METALAXIL Y MEFENOXAM

RESUMEN

El tratamiento de la semilla con fungicidas de fenilamido (metalaxil y mefenoxam) garantiza una protección química eficaz contra el tizón de girasol, causado por la oomycete *Plasmopara halstedii*. La resistencia del hongo a metalaxil, fue observada en España, tanto como la presentación diferencial de la enfermedad debido a la existencia de las poblaciones campestres del hongo. Comparamos la sensibilidad de varias poblaciones del hongo campestres al tratamiento de la semilla con metalaxil. Diferentes grupos de las poblaciones campestres eran separados sobre la base de la presentación de la enfermedad en las plantas de girasol. Ninguna población campestre había sido totalmente controlada por las dosis de fungicida utilizadas. Aparte de ello, evaluamos también la eficacia de metalaxil y mefenoxam, en relación con las poblaciones campestres, que anteriormente habían demostrado resistencia a metalaxil. Tres grupos de genotips fueron tratados con fungicidas en dos dosis. Ni las dosis de metalaxil ni de mefenoxam, tenían significativa influencia en la aparición de la enfermedad causada por parte de las poblaciones campestres, lo que indica que no había influencia de la doble dosis de enantiomer activo del mefenoxam en relación con metalaxil. No obstante, fueron observadas significativas diferencias en la aparición de la enfermedad entre genotipos. Las diferencias en la presentación de la enfermedad podían explicarse: a) por los diferentes niveles de germinación, en consecuencia, por el tamaño del raíz en el momento de infección, b) por mayor cantidad de fungicida con el cual fue tratado el genotipo de consumo, que tenía semilla de mayor tamaño.

RÉACTIONS DES POPULATIONS CHAMPÊTRES DE ROUILLE (*Plasmopara halstedii*) DU TOURNESOL AU METALAXYL ET AU MEFENOXAM

RÉSUMÉ

Le traitement des graines au moyen des fongicides de phénylamide (metalaxyl et mefenoxam) assure un contrôle chimique efficace de la rouille causée par l'oomycète *Plasmopara halstedii*. Une résistance du champignon au metalaxyl est apparue en Espagne et des différences d'incidence de la maladie ont été observées dans les populations champêtres. Nous avons comparé la sensibilité de plusieurs populations de champignons champêtres au traitement des graines au metalaxyl. Différents groupes de populations champêtres ont été mis à part selon le critère d'apparition de la maladie sur les plantes de tournesol. Aucune population champêtre n'a été complètement contrôlée par le fongicide. De plus, nous avons évalué l'efficacité du metalaxyl et du mefenoxam par rapport aux populations champêtres qui avaient montré antérieurement une résistance au metalaxyl. Trois groupes de génotypes ont été traités au

moyen des fongicides en deux doses. Ni le metalaxyl ni le mefenoxam ni les doses n'ont eu un effet significatif sur l'apparition de la maladie provoquée par les populations champêtres, ce qui montre que la double dose d'énantiomère actif du mefenoxam par rapport au metalaxyl n'a pas eu d'effet. Cependant des différences significatives dans l'apparition de la maladie parmi les géotypes ont été observées. On a pu expliquer des différences dans l'apparition de la maladie : a) par des niveaux différents de germination et conséquemment par la dimension du radicule au moment de l'inoculation, b) la plus grande quantité de fongicide administrée au géotype de confection dont la graine était de plus grande dimension.