Metalaxyl Resistance in French Isolates of Downy Mildew

Resistance to Metalaxyl Seed Treatment in *Plasmopora halstedii*

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ABSTRACT

Six isolates of *Plasmopara halstedii* (sunflower downy mildew) showing 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. The EC50 of these isolates ranged from 5800 to 32900 mg a.i. metalaxyl kg^{-1}. For one of these isolates, ApR2, studies showed that acquisition of resistance was linked with neither reduced competitiveness nor reduction in agressivity. However, this resistance was not stable without metalaxyl. Studies on commercially metalaxyl treated seeds of 42 sunflower hybrids susceptible to race A indicated that there were no interaction between host genotype and the ApR2 isolate.

INTRODUCTION

Downy mildew, *Plasmopara halstedii* (Farl.) Berl . et de Toni, of sunflower, *Helianthus annuus L.*, is a disease present in France since 1966 (Louvet and Kermoal, 1966). Two methods exist to control this parasite : the use of genetically resistant cultivars and seed treatment with metalaxyl, [N - (2, 6 - dimethyl - phenyl) - N - (2' - methoxyacetyl) - alanine methyl ester]. Since the common use of this fungicide in 1990, this disease have caused no losses despite the spread of new races showing virulence on most cultivars (Penaud, 1994). However, in 1995 and 1996, prospection in France showed downy mildew isolates which exhibited atypical responses to metalaxyl, in the laboratory (Penaud *et al.*, 1997). This led us to study the metalaxyl sensitivity level of these isolates, in particular one of them denoted ApR2. Previously, Viranyi and Oros (1991) and Mouzeyar *et al.* (1995) showed that host dependant stages of cycle life of *P. halstedii* were more sensitive to

metalaxyl than host independent stages. Thus, it was possible that sunflower genotype could influence the metalaxyl sensitivity level of this atypical isolate. This hypothesis was tested. Lastly, it was interesting to test stability of metalaxyl resistance of this isolate without fungicide, its sporulation capacity (compared with those of a metalaxyl sensitive isolate) and its capacity to survive in mixture with a metalaxyl sensitive isolate.

MATERIALS AND METHODS

Host-parasite system

The sunflower genotype used was the population variety Peredovik susceptible to all known downy mildew races.

P. halstedii isolates of race A used as inoculum were the isolate A, sensitive to metalaxyl, collected in France in 1988 (Tourvieille *et al.*, 1988) and maintained on EL64 (experimental INRA hybrid) seedlings, six isolates insensitive to metalaxyl, ApR2 collected in France in 1995 and ApR4, ApR5, ApR6, ApR7, ApR8 collected in France in 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}.

Metalaxyl sensitivity of the six atypical *P. halstedii* isolates

The metalaxyl sensitivity level of these isolates was evaluated according to the method described in previous paper (Albourie *et al.*, 1998). The insensitivity factor was the ratio between the EC50 of the isolates tested and the EC50 of the reference isolate (A) which was metalaxyl sensitive.

Relationship between ApR2 isolate and sunflower genotypes

Plant breeders in French seed companies supplied us with 42 different commercial sunflower hybrids. All were known to be susceptible to race A. For each genotype, untreated seedlings and seedlings treated with the registered rate of metalaxyl (treatment realised by plant breeders) were infected through roots like previously. The mean rate of seedlings showing sporulation on their cotyledons and / or their leaves was noted for each genotype.

Stability of ApR2 metalaxyl resistance

To follow the stability in time of ApR2 metalaxyl resistance, untreated Peredovik seedlings were infected by this isolate following the method described previously (Albourie *et al.*, 1998). After 15 days, a suspension of 10^5 zoosporangia ml^{-1} produced from sporulated cotyledons of untreated seedlings, was used to infect other seedlings grown from seeds either

untreated or treated with the registered rate of metalaxyl. This maintenance cycle was repeated 11 times.
During each cycle, the mean rate of seedlings treated with 2 100 mg a.i. kg^{-1}, showing sporulation on cotyledons and / or their leaves was calculated on 10 replicates of 12 seedlings.
At the same time, we followed, after each maintenance cycle on Peredovik seedlings treated with the registered rate of metalaxyl, the mean rate of seedlings showing sporulation on their cotyledons and / or their leaves.

Quantification of sporulation
For each metalaxyl concentration and each downy mildew isolate (A and ApR2), cotyledons bearing sporulations were cut, placed in a jar with 10-60 ml of distilled water, and shaken thoroughly. The jar was then immersed in ultrasonic-cell during about 10 s to obtain a zoosporangia suspension. Zoosporangia were counted using a hemocytometer. For each replicate of each treatment, two counts were carried out but only the average was used. Mean zoosporangia numbers per cotyledon were calculated on 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 converted with a square root function.

Competitiveness of ApR2 isolate
Untreated Peredovik seedlings were infected through roots by a 10^5 zoosporangia ml^{-1} suspension which contained a mixture of A and ApR2 isolates in proportion to 1:1, 2:1, 3:1, 4:1, 10:1, 20:1 and 30:1 respectively.
As previously, 15 days after infection, zoosporangia were collected in order to produce a suspension of 10^5 zoosporangia ml^{-1} used to infect other untreated and metalaxyl treated seedlings. For each cycle, the mean rate of seedlings treated with 2 100 mg a.i. metalaxyl kg^{-1} and showing sporulation on their cotyledons and / or their leaves was calculated on 2 replicates of 12 seedlings.

RESULTS

Metalaxyl sensitivity of atypical *P. halstedii* isolates (Table 1)
The six isolates studied showed weak metalaxyl sensitivity levels. None was controlled by the registered rate. Only the EC50 of ApR5 was statistically different from that of the other isolates. It was the least metalaxyl sensitive with a resistance factor equal to 1500 (confidence interval : 890 < 1 500 < 2 400). The EC50 of other isolates did not differ statistically.

Table 1. 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})
A	19< 22 < 27
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

Table 2. Behaviour after primary *Plasmopara halstedii* infections of 42 sunflower hybrids with or without seed treatment at the registered rate of metalaxyl.

Hybrids	Without seed treatment	Metalaxyl seed treatment
Resistant to A and ApR2	0	0
Resistant to A and susceptible to ApR2	0	37
Susceptible to A and ApR2	42	5
Total	42	42

Quantification of sporulation (Figure 1)

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}. 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 if there was no fungicide.

Relationship between ApR2 isolate and sunflower genotypes (Table 2)

All genotypes used were susceptible to A isolate of *P. halstedii*. On the whole, the seed treatments made by plant-breeders were correct : 6 of them showed 100 % of efficiency in their seed treatment against the isolate A.

We note rates of sporulated seedlings ranging between 36 and 100 % with the ApR2 isolate. Thus there was no significant interaction between the metalaxyl resistant isolate and sunflower hybrids used in this study.

Stability of ApR2 metalaxyl resistance (Figure 2)

When the ApR2 isolate was maintained without fungicide, the rate of sporulated treated seedlings decreased after the sixth maintenance cycle, to 44 % during the twelfth cycle.

In contrast, when this isolate was maintained on seeds treated with the registered rate of metalaxyl, the proportion of sporulated treated seedlings remained stable throughout the 12 maintenance cycles. Thus it may be concluded that metalaxyl resistance in *P. halstedii* was not stable in time in lack of fungicide.

Competitiveness of ApR2 isolate (Figure 3)

Whatever the ratio A:ApR2 inoculum, the rate of sporulated treated seedlings decreased during maintenance cycles. From the third cycle, no seedlings prealably infected by 20A:1ApR2 and 30A:1ApR2 population showed symptoms. However, during the fifth cycle, there was still respectively 46 % (8% to 83%) and 30% (17% to 42%) of sporulated seedlings after primary infection with 4A :1ApR2 and 10A :1ApR2 populations. So ApR2 isolate was maintained in a downy mildew population containing up to 10 times more metalaxyl sensitive zoosporangia than resistant.

Figure 1. Effect of metalaxyl on the number of zoosporangia (10^5) produced by a metalaxyl sensitive (A) and a metalaxyl insensitive (ApR2) isolates of *Plasmopara halstedii*. Values labelled by the same letter are not significantly different at P=0.05.

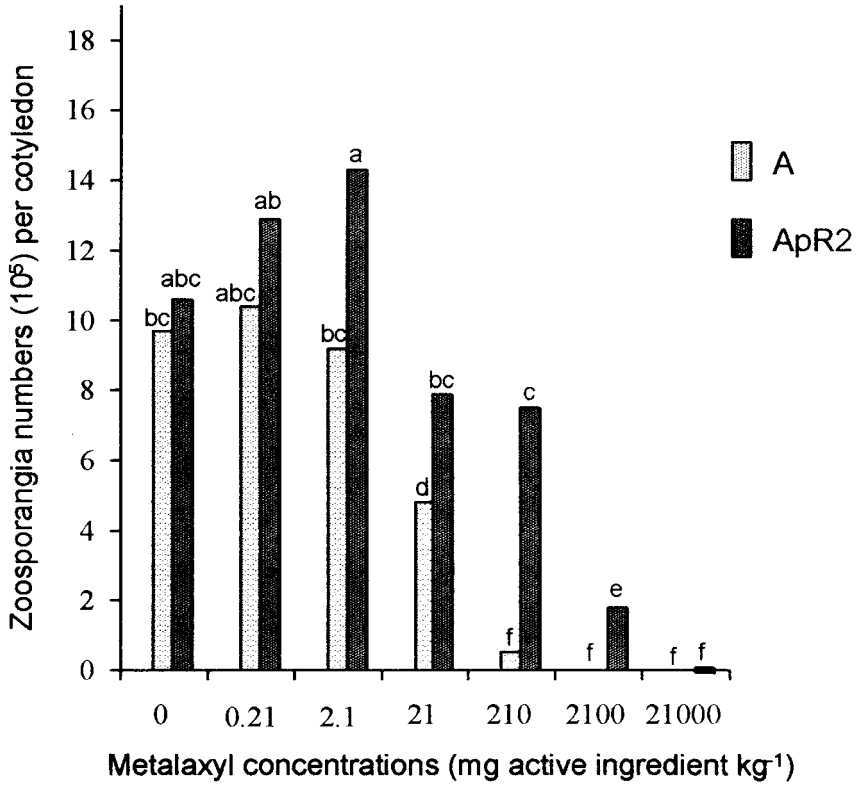


Figure 2. Variations in percentage of Peredovik seedlings treated with 2100 mg metalaxyl kg^{-1} after primary infections of ApR2 isolate of *P. halstedii* according to number of maintenance cycles (maintenance on no treated seedlings △ ; maintenance on seedlings treated with 2100 mg metalaxyl kg^{-1} ○). The vertical lines represents the standard deviation of the means of 10 experiments per cycle.

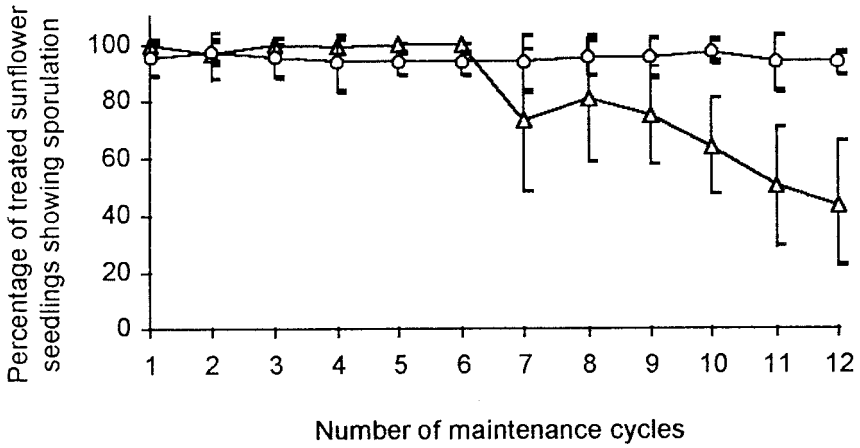
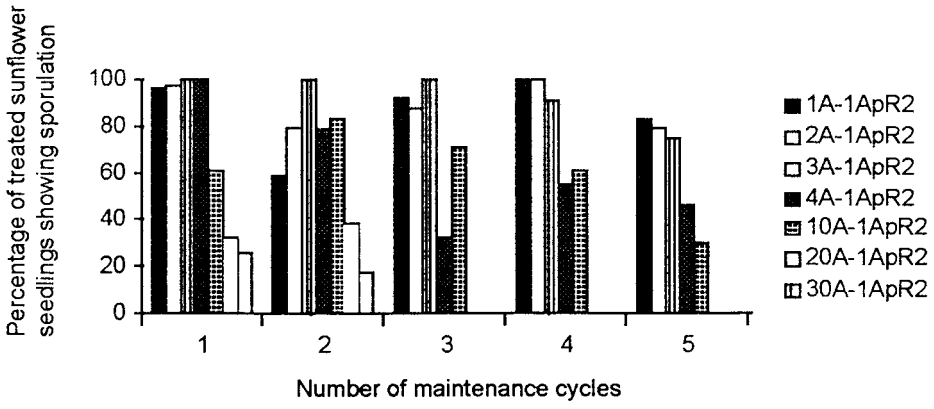


Figure 3. Variation in percentage of Peredovik seedlings treated with 2 100 mg metalaxyl kg^{-1} seed, showing sporulations after primary infections of A and ApR2 isolates of *P. halstedii* in mixture according to number of maintenance cycles.



DISCUSSION

Resistance to metalaxyl has been described in a many oomycetes (Katan and Bashi, Staub and Sozzi, 1981, Holmes and Channon, 1984, Pappas, 1985, Leroux *et al.*, 1988). Until recently, for *P. halstedii*, only metalaxyl tolerance for 1 to 2 ppm had been reported (Viranyi *et al.*, 1992) whereas the registered labelled rate is 2100 ppm. In this study, the isolates showed a high metalaxyl resistance level similar to other metalaxyl resistant oomycete isolates (Klein, 1994). All isolates tested here were not affected by metalaxyl concentrations 10 times greater than the registered rate, so it is impossible to imagine their control by use of high metalaxyl concentration. Since there was no increase in resistance factor after eleven ApR2 maintenance cycles on seeds treated with the registered rate of metalaxyl and this resistance factor was the same in some isolates collected in 1996, it cannot be considered that maintenance cycles caused any selection for resistance.

The metalaxyl resistant isolates used in this study showed variability which has also been noted in some other oomycetes. Staub and Sozzi (1981) reported *Plasmopara viticola* strains showing a resistance level ranging between 100 and 1000. In *Pseudoperonospora humuli* isolates, the resistance level ranged between 60 and more than 600 (Klein, 1994).

Studies on ApR2 isolates showed that sunflower hybrids susceptible to race A were infected indiscriminately, by this isolate. Whereas, hybrids resistant to race A were resistant to ApR2 as well. Mechanisms of metalaxyl resistance in *P. halstedii* thus do not involve sunflower, but will be only the fungus. According to Davidse and Van Den Berg-Velthius (1989), metalaxyl may act by interfering with the RNA polymerase-I-template complex of sensitive *Phytophthora spp* inhibiting ribosomal RNA synthesis. Resistance could thus be the result of a mutation event which leads to a change at the binding site. However, metalaxyl resistance in ApR2 was unstable in time. This phenomenon is also found in some isolates of *Phytophthora citricola* (Joseph and Coffey, 1984), *Phytophthora infestans* (Davidse *et al.*, 1983) and *P. viticola* (Irhir, 1987). But this isolate didn't became completely sensitive to metalaxyl. According to Irhir and Clerjeau (1988), this resistance may come from a phenotypical adaptation with sublethal metalaxyl doses.

Further, acquisition of resistance was not accompanied by any loss of ApR2 agressivity. In fact, although the difference was not statistically significant, we observed an absolute value of ApR2 sporulation capacity greater than that shown by the sensitive isolate. This metalaxyl resistance was not linked to a decrease of ApR2 competitiveness, because this isolate was able to remain in populations where its concentration was up to 10 times lower than that of the sensitive isolate. This maintenance of fitness was described in *P. infestans* (Kadish and Cohen, 1988, Holmes and Channon, 1984), *P.*

citricola (Joseph and Coffey, 1984) and *Bremia lactucae* (Leroux *et al.*, 1988), which indicates that appearance of metalaxyl resistance is not necessarily linked to a decrease of pathogen fitness.
These results concerned only the ApR2 isolate and it would be interesting to determine whether the other metalaxyl resistant isolates showed the same characteristics. Since their appearance in 1995, the increase of metalaxyl resistant isolates number suggests that they had at least similar fitness to that of the metalaxyl sensitive isolates or possibly even greater. If all metalaxyl resistant *P. halstedii* isolates were similar to ApR2, it was very probable that these isolates will extend in the field. For the moment, loss of metalaxyl efficacy has been noted only in a few fields, but if this phenomen increases, use of cultivars resistant to all French downy mildew races will be the only means to control this pathogen.

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