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Occurrence in Italy and characteristics of lettuce downy mildew (*Bremia lactucae*) resistant to phenylamide fungicides

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Abstract

Control of lettuce downy mildew (*Bremia lactucae*) with phenylamide fungicides has failed in some intensive lettuce-producing areas in Northern Italy since Spring 1993. Before then, these chemicals and particularly metalaxyl, provided the best disease control. The sensitivity of *Bremia lactucae* isolates collected in such areas to metalaxyl was evaluated in the laboratory. These strains grew and sporulated profusely on lettuce seedlings treated with 100 and 200 ppm of metalaxyl, whereas sensitive control strains were completely inhibited when treated with fungicide concentrations ranging from 0.5–1 ppm. Thus in Italy occurrence of resistance to phenylamide fungicides in *Bremia lactucae* has also been demonstrated, as in almost all the countries where these chemicals were previously authorised. Subsequently, a demonstration of occurrence of resistance was made and the virulence pattern of several strains (resistant and sensitive to metalaxyl) was characterised using differential NL series containing the 13 DM resistant genes or R factors. The results suggest the occurrence of a new pathotype in Italy different from all the 16 NL *Bremia lactucae* races studied.

Introduction

Downy mildew (*Bremia lactucae*) is a constant risk for lettuce crops, in both the glasshouse and field, particularly with cool and rainy weather conditions. In Northern Italy, these are the typical climatic conditions during spring and autumn, and therefore epidemic outbreaks of the disease are common in these seasons. Outbreaks of the disease on crops close to maturity adversely affects quality, causing great economic damage due to the need to remove diseased leaves at harvest and, in extreme circumstances, leads to complete loss of marketable yield.

In the early 1990s, following the extension of the registration of phenylamide fungicides (oxadixyl and subsequently metalaxyl both in mixture with copper) for use on lettuce, an effective control of the disease was achieved. Several field trials carried out in Emilia Romagna region (NE Italy) during 1990–93 confirmed the satisfactory control of downy mildew with two applications of metalaxyl or oxadixyl during the growing season [6, 7]. In 1993, however, a reduced efficacy of these chemicals in controlling the disease was observed in the intensive lettuce-growing area of Rimini (east part of Emilia-Romagna). This situation was confirmed subsequently by experimental field tests carried out in autumn 1993 and spring 1994. In the Bologna area (central part of Emilia-Romagna), however, phenylamide fungicides still retained their efficacy in the field (Figure 1) [4]. In the winter 1994 failure of control was also observed in some greenhouse lettuce crop in the Piedmont region (NW Italy - Cuneo area).

To establish whether the reduced efficacy was caused by the possible resistance of *Bremia lactucae* to phenylamides, as already reported in many other countries [1, 11, 17, 20, 21, 23], in 1994–95 samples collected from these three areas were tested for sensitivity to metalaxyl in comparison with strains from

untreated home gardens. Preliminary results showed a decrease of sensitivity to phenylamide fungicides [5].

Control of *B. lactucae* has been largely based on the use of resistant cultivars. To date, 13 single dominant resistance genes (*Dm*) in the host and their complementary virulence genes in the pathogen have been described [8, 9, 15, 19]. An established lettuce tester set (differential series), containing the 13 Dm resistant genes or R factors, has been used to determine the virulence pattern of the isolates [18]. From 1995 to 1996, sensitivity to metalaxyl was continuously monitored and some of the strains were characterised for the virulence pattern.

Table 1. Differential series

Cultivar	Genes	Cultivar	Genes
Cobham Green	Dm 0	UCDM 10	Dm 10
Lednicky	Dm 1	Capitan	Dm 11
UCDM 2	Dm 2	Hilde	Dm 12
Dandy	Dm 3	Pennlake	Dm 13
Line R4T57D	Dm 4	UCDM 14	Dm 14
Valmaine	Dm 5/8	PIVT 1309	Dm 15
Sabine	Dm 6	LSE/18	Dm 16
Mesa 659	Dm 7/13	Mariska	R 18

Materials and methods

Origin and characteristics of the isolates

Five isolates were tested for sensitivity to metalaxyl in 1994-95. Three of these isolates were collected from intensive lettuce-growing area in different parts of North Italy. Two samples (isolates 1 and 2) came from a field in the Rimini area (Emilia-Romagna - NE Italy) and from a lettuce green-house in the Cuneo area (Piedmont - NW Italy), where phenylamide fungicides were used but failed to control downy mildew. Isolate 3, came from a farm in the Bologna area (Emilia-Romagna - NE Italy) where phenylamide fungicides were used to control downy mildew with effective disease control. The other two isolates (4 and 5) were collected in untreated home gardens and were considered as 'controls'. Seven more additional isolates were tested for their sensitivity to metalaxyl in 1995–96. Five of these isolates (isolates 6, 7, 8, 9, 10) came from intensive lettuce-growing fields in the Bologna and Rimini areas. The other two (isolates 11 and 12) were collected in untreated home gardens in the same areas. Samples were collected at each site from the same lettuce variety.

Sensitivity tests to phenylamides

The sensitivity to metalaxyl of the different isolates was determined according to procedure described previously by Leroux et al. [17]. Lettuce seeds (11–12) of the sensitive cultivar Regina di Maggio were planted in closed glass-pots containing a mix of peat and sand and incubated at 18–20 °C. Lettuce seedlings with fully expanded cotyledons (about 1 week after sowing) were sprayed to runoff with different concentrations of metalaxyl (Ridomil 25% wp) and allowed to dry, before closing the glass-pot again. Seedlings were inoculated 24 h later with the sporangial suspension (10–20 $\times$ 10^4sporangia per ml) and placed in a growth room at 15 °C for the first 24 h to support the infectious process and then at 20 °C (12 h photoperiod) for the incubation period [22]. Approximately 8–10 days after inoculation, the percentage of cotyledons bearing spores was calculated and sporulation severity was assessed using a 0-3 scale, where 0 = no sporulation; 1, 2 and 3 = light, medium and heavy sporulation. Sensitivity tests were repeated at least twice and results were expressed as the mean; the metalaxyl concentrations used ranged from 0 to 200 ppm in 6–11 fold dilutions.

In 1995/6, only the 100 and 200 ppm concentrations were used for routine tests of field isolates suspected to be resistant to metalaxyl.

Determination of virulence pattern

During 1996, the virulence pattern in a host differential set was determined for several isolates according to the procedure described by Milchelmore and Crute [18]. Lettuce seeds (11–12) of the differential series were sown on filter paper in closed glass-pots or on a single bench; when lettuce seedlings had fully expanded cotyledons (about 1 week after sowing), they were inoculated with the sporangial suspension (10–20 $\times$ 10^4sporangia per ml) and placed in a growth room at 15 °C for the first 24 h and then at 20 °C (12 h photoperiod). The seedlings were examined for the presence of asexual sporulation, approximately 7–12 days after inoculation.

As it had been demonstrated that the pathotype of single-conidial lines was almost identical to that of their progenitor isolates taken from individual plants [16], the isolates tested were collected from single lesions or plants.

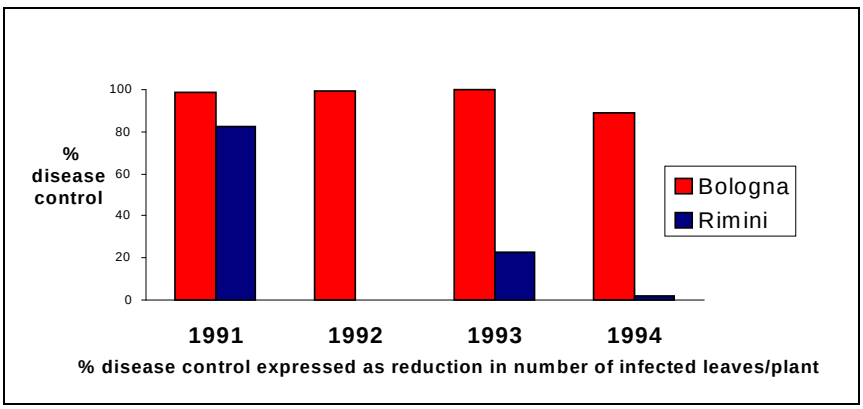

Figure 1. Efficacy of phenylamide fungicides in the field against *Bremia lactucae* over 4 years at two sites of Emilia-Romagna.

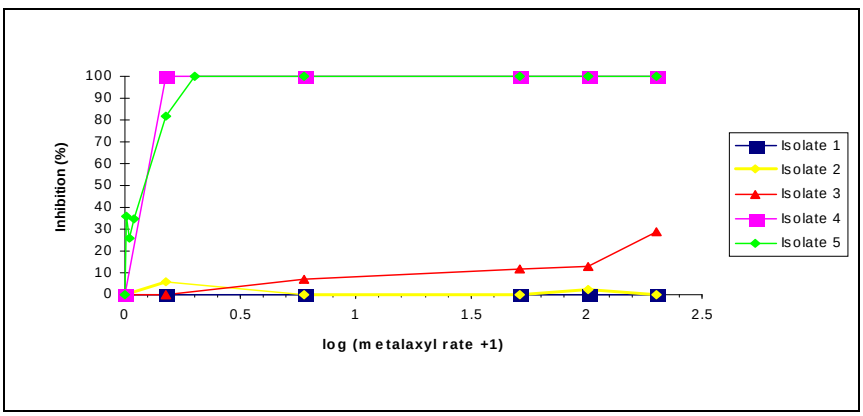

Figure 2. 1994–95 - Metalaxyl sensitivity in *Bremia lactucae*: inhibition (%) of sporulation on cotyledons cv. Regina di Maggio.

Isolates coming from phenylamide treated lettuce fields were used to inoculate cotyledons of cultivar Cobham Green sprayed with 100 ppm of metalaxyl. The inoculum was recollected and tested for pathotype over the differential series (Table 1).

Results

Metalaxyl sensitivity

In 1994/5, isolates 1 and 2 (Rimini and Cuneo areas) grew and sporulated profusely in lettuce seedlings treated with 100 and 200 ppm of metalaxyl. Sporulation of isolate 3 (Bologna area) was only partially inhibited by 100-200 ppm of metalaxyl. Isolates 4 and 5, considered as sensitive control strains, were completely inhibited when treated with fungicide concentrations ranging from 0.5-1 ppm (Table 2 and Figure 2). From Figure 2, it is possible to estimate an ED_{50} for control strains ranging from 0.1 to 0.5 ppm, while the ED_{50} for the other strains was > 200 ppm and the resistance factor (RF) determined was 400-4000x. These results confirmed therefore the presence of strains resistant to metalaxyl.

In 1995/6, seven new isolates from intensive lettuce-growing fields in the area of Bologna and Rimini were tested for sensitivity to metalaxyl (Table 3 and Figure 3). Isolates 6, 7 and 8, collected from lettuce crops where heavy outbreaks of the disease appeared despite phenylamide treatments and therefore were suspected to be resistant, grew and sporulated profusely on lettuce seedlings treated with 100 and 200 ppm of metalaxyl. Isolate 9, from the Rimini area, and 10, from the Bologna area, were both collected from organic farms using copper treatments for lettuce downy mildew control. Isolate 9 showed an intermediate behaviour; light symptoms of the disease were noticed on a few cotyledons treated with 50 ppm of metalaxyl. Isolate 10, like the isolates 11 and 12 collected in home gardens (no fungicide treatments),

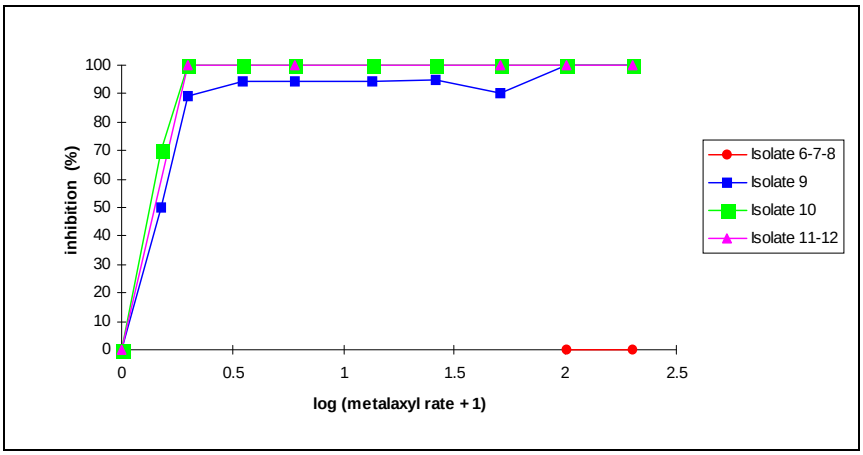

Figure 3. 1995–96 - metalaxyl sensitivity in *Bremia lactucae*: inhibition (%) of sporulation of different isolates collected in some intensive lettuce-producing areas of North - Italy.

Table 2. 1994–95 - Metalaxyl sensitivity in *Bremia lactucae*: incidence and severity of disease on cotyledons cv. Regina di Maggio

Metalaxyl concentration (ppm)	Isolate 1		Isolate 2		Isolate 3		Isolate 4		Isolate 5	
	Incidence*	Severity **	Incidence*	Severity**	Incidence*	Severity**	Incidence*	Severity**	Incidence*	Severity**
0	100	3	85	3	68	3	90	3	100	3
0.01	–	–	–	–	–	–	–	–	64	3
0.05	–	–	–	–	–	–	–	–	74	2
0.1	–	–	–	–	–	–	–	–	65	2
0.5	100	3	80	3	69	3	0	0	18	1
1	–	–	–	–	–	–	0	0	0	0
5	100	3	100	3	63	3	0	0	0	0
50	100	3	100	3	60	3	0	0	0	0
100	100	3	83	3	57	2	0	0	0	0
200	100	3	88	3	48	2	0	0	0	0

* Percentage of infected cotyledons calculated from 20 cotyledons. Test repeated minimum at least twice.
** Based on a 0-3 scale: 0 = no visible symptoms; 1,2 and 3 = light, medium and heavy sporulation.

showed a sensitivity similar to the control strains and so can be considered sensitive to metalaxyl.

Determination of virulence pattern

The pathotype of 8 isolates was determined: 3 were resistant to phenylamides (isolates 6, 7, 8), 1 was moderately sensitive (isolate 9) and 4 were sensitive (isolates 5, 10, 11 and 12). All isolates tested showed the same pathotype regardless of geographic origin and phenylamide sensitivity. Symptoms of disease were detected on all cvs except Mariska, indicating the absence of virulence towards the R18 factor. A comparison of the virulence pattern of the isolates tested with the Netherlands pathotypes [2, 3], used as a *Bremia lactucae* resistance reference system by seed companies, showed that our pathotype appeared to be different from all the 16 NL races; our virulence pattern was similar but not identical to that of the 16th race, because it also showed virulence on UCDM 14 (DM 14) and PIVT 1309 (DM 15) (Table 4).

Since our isolates were not generated from single-conidial lines but obtained from single lesions additional checks were undertaken in order to exclude the presence of a mixture of different NL strains. The following procedure was used. Inoculum from the genotypes UCDM 2 (Dm2) or Dandy (Dm3) were collected and put on LSE/18: if spores are produced, the isolate can not be NL 1 to 15. With this same inoculum genotype PIVT 1309 (Dm 15) must also be infected. If the results are negative (no sporulation) the original inoculum has to be indeed a mixture of probably an NL

Table 3. 1995–96 - metalaxyl sensitivity in *Bremia lactucae*: incidence and severity of disease on cotyledons cv. Regina di Maggio of different isolates collected in some intensive lettuce-producing areas of North - Italy

Metalaxyl concentration (ppm)	Isolates 6, 7, 8		Isolate 9		Isolate 10		Isolates 11, 12	
	Incidence∗	Severity ∗∗	Incidence∗	Severity∗∗	Incidence∗	Severity∗∗	Incidence∗	Severity∗∗
0	–	–	100	3	100	3	100	3
0.5	–	–	50	3	30	3	–	–
1	–	–	11	2	0	0	0	0
2.5	–	–	5.5	2	0	0	–	–
5	–	–	5.5	3	0	0	0	0
12.5	–	–	5.5	3	0	0	–	–
25	–	–	5	1	0	0	–	–
50	–	–	10	2	0	0	0	0
100	100	3	0	0	0	0	0	0
200	100	3	0	0	0	0	0	0

∗ Percentage of infected cotyledons calculated from 20 cotyledons. Test repeated at least twice.
∗∗ Based on a 0-3 scale: 0 = no visible symptoms; 1, 2 and 3 = light, medium and heavy sporulation.

11/12 like strain plus a NL 16 like strain. If positive (presence of spores) that will suggest a new pathotype. The positive results obtained with this procedure suggest the occurrence of a new pathotype.

Discussion

As reported in many other countries, the occurrence in Italy of resistance to phenylamide fungicides in *Bremia lactucae* clearly exists. Most of the isolates tested were shown to be highly resistant to metalaxyl, thus providing an explanation for the failure of disease control with phenylamide fungicides frequently observed in the field. In rare cases the sensitivity monitored was not directly correlated with product performance in the field: isolates resistant or partially resistant in laboratory tests to metalaxyl were detected, even in fields where phenylamide fungicides continue to provide a good control of lettuce downy mildew (i.e. isolate 3). The sensitivity test probably overestimated the degree of resistance present within the pathogen population in the field.

From our tests on host differentials, a new *Bremia lactucae* pathotype seems to be present in Italy that differs from all of the 16 races classified in the Netherlands. Since the Italian pathotype overcomes all the known genetic resistance factors except R18, regardless of sensitivity to phenylamides, an integrated strategy of genetic resistance with phenylamide fungicide treatments, like that proposed by Crute et al. for United Kingdom, can not be proposed in our region [9, 11]. In that case the pathotype insensitive to metalaxyl was avirulent on cultivars carrying Dm 11, and the pathotypes of *B. lactucae* virulent on cultivars carrying Dm 11, although not infrequent, were all phenylamide sensitive. Consequently, good downy mildew control was re-established by combined use of fungicides containing metalaxyl with cultivars carrying the Dm 11 allele located in the most important commercial lettuce cultivars in the UK at that time. It will be theoretically possible to adopt a similar integrated strategy in our region combining the use of phenylamide fungicide with cultivars carrying R18. However, this strategy might be too risky and select a new pathotype resistant to R18 and at the same time resistant to metalaxyl? Therefore, although protectant fungicides (i.e. copper) do not achieve complete disease control, we suggest their preventive use in combination with cultivars carrying R18, hoping that if a new pathotype resistant to R18 develops, it will be sensitive to metalaxyl. The advice of reducing the inoculum potential in order to safeguard the R 18 factor for a longer time, is also extremely important because effective and registered fungicides against *Bremia lactucae* are not currently available [4].

In the future, sensitivity monitoring will be applied to isolates from different geographic areas to establish whether *Bremia lactucae* is evolving with and without phenylamides control (resistance is permanent or not). Furthermore, it would be very interesting to demonstrate the probable cross-resistance and multiple resistance between the different active ingredients, whether they belong to the phenylamides group or not.

Table 4. Differential lettuce cultivars used to determine phenotypic virulence of Italian *Bremia lactucae* isolates, compared with the phenotypes performance of NL pathotypes [2, 3]

Differential cultivars	Dm gene or R factor	NL Pathotypes														Italian pathotype
		1	2	3	4	5	6	7	10	11	12	13	14	15	16	
Cobham Green	R ?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lednicky	Dm 1	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
UCDM 2	Dm 2	+	+	-	+	-	+	+	+	-	-	-	+	+	+	+
Dandy	Dm 3	-	+	-	-	+	-	+	+	-	-	+	+	+	+	+
Line R4T57D	Dm 4	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Valmaine	Dm 5/8	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+
Sabine	Dm 6	-	+	+	(-)	-	(-)	+	+	+	+	(-)	+	(-)	+	+
Mesa 659	Dm 7/13	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+
UCDM 10	Dm 10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Capitan	Dm 11	-	-	-	-	-	+	-	-	(-)	+	+	+	+	+	+
Hilde	R 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pennlake	Dm 13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
UCDM 14	Dm 14	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+
PIVT 1309	Dm 15	-	-	+	-	+	-	-	(-)	+	+	(-)	-	-	-	+
LSE/18	Dm 16	-	-	+	-	(-)	-	-	-	+	+	-	-	-	+	+
Mariska	R 18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = compatible reaction, profuse sporulation.
- = incompatible reaction, no sporulation.
(-) = incompatible reaction, sparse sporulation associated with necrosis.

In this way, it will be possible to explain the assumption that a change in the target site is an important mechanism involved in the resistance to phenylamide fungicides [12, 13, 14]. At the same time, determination of pathotype will be continued to evaluate its selective advantage in *Bremia lactucae* population.

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