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Sensitivity of *Phytophthora infestans* to flumorph: *in vitro* determination of baseline sensitivity and the risk of resistance

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The sensitivity of 127 *Phytophthora infestans* isolates to flumorph was determined in 2003 and 2004. The isolates originated from two geographical regions and showed similar levels of sensitivity in both years. Baseline sensitivities were distributed as a unimodal curve with EC_{50} values for growth of mycelia ranging from 0·1016 to 0·3228 μg mL^{-1}, with a mean of 0·1813 (± 0·0405) μg mL^{-1}. There was no cross-resistance between flumorph and metalaxyl. Laboratory studies were conducted to evaluate the risk of *P. infestans* developing resistance to flumorph. Mutants resistant to metalaxyl or flumorph were obtained by treating mycelium of wild-type isolates with ultraviolet radiation. Metalaxyl-resistant mutants were obtained with a high frequency and exhibited resistance factor values (EC_{50} resistant/EC_{50} sensitive phenotypes) of more than 100, while flumorph-resistant mutants were obtained at much lower frequencies and had very small resistance factors (1·5–3·2). There was cross-resistance between flumorph and dimethomorph, but not with azoxystrobin or cymoxanil. Most flumorph-resistant mutants showed decreases in hyphal growth *in vitro* and in sporulation both *in vitro* and on detached leaf tissues. These studies suggested that the risk of resistance developing was much lower for flumorph than metalaxyl. However, as *P. infestans* is a high-risk pathogen, appropriate precautions against resistance development should be taken.

Keywords: azoxystrobin, cymoxanil, dimethomorph, fungicide resistance, metalaxyl, potato late blight

Introduction

Flumorph is the ISO-proposed common name of the systemic fungicide 4-[3-(3,4-dimethoxyphenyl)-3-(4-fluorophenyl)-1-oxo-2-propenyl] morpholine (SYP-L190). It has a similar structure to dimethomorph (Fig. 1), and both compounds are active against oomycetes, e.g. *Peronospora* and *Phytophthora* spp. As a cinnamic acid derivative developed by the Shenyang Research Institute of Chemical Industry of China in 1994, flumorph has been patented in China (ZL.96115551.5), the USA (US6020332) and Europe (0860438B1) (Liu *et al*., 2000). It has been registered for controlling *Phytophthora infestans* on potato and tomato, *P. capsici* on pepper, *Pseudoperonospora cubensis* on cucumber and *Plasmopara viticola* on grapevine in China. The mechanism of its biological action is currently the subject of intense investigation. Little is known about whether it has a similar mode of action to, or cross-resistance with, established oomycete-specific products, such as cymoxanil, dimethomorph and metalaxyl.

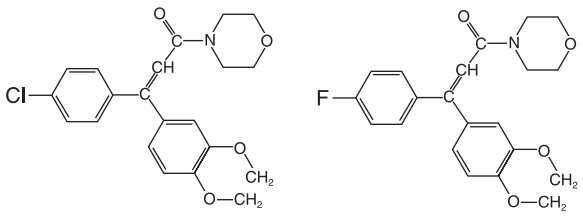

Figure 1 Chemical structures of dimethomorph (left) and flumorph (right).

Outbreaks of potato late blight caused by *Phytophthora infestans* were first reported in China in the 1950s. Following the introduction of several resistant potato cultivars in the late 1960s to early 1970s, disease epidemics were successfully suppressed. However, since the 1980s,

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potato late blight has increased in its severity, causing severe economic damage (Song *et al*., 1996). Selection and use of resistant varieties, and application of fungicides are two ways to manage this disease, but loss of effectiveness of host resistance and development of fungicide resistance have compromised control strategies. Although metalaxyl and related phenylamide fungicides have provided excellent control of the disease in the past, the efficacy of these fungicides has decreased as a result of the emergence of resistant strains of *P. infestans* in China (Zhang *et al*., 2001). Flumorph offers an alternative to phenylamide fungicides for the control of potato late blight.

Although flumorph has been marketed in China for about 6 years, it is still in the early stages of use on potato. Until now, no information on the baseline sensitivity and resistance of *P. infestans* to flumorph has been available. The objectives of this study were as follows: (i) to determine the *in vitro* response profiles of *P. infestans* field populations to flumorph, and establish the baseline sensitivity; (ii) to induce laboratory-resistant mutants of *P. infestans* to flumorph and study their biological characteristics; and (iii) to carry out a preliminary evaluation of the risk of *P. infestans* developing resistance to flumorph, using metalaxyl, a representative of phenylamide fungicides, as a reference.

Materials and methods

Media

Rye A agar (RA) (Caten & Jinks, 1968) was used routinely to culture and test for fungicide sensitivity of *P. infestans* isolates. A selective medium was made by adding antibiotics (20 μg rifampicin mL^{-1}; 200 μg ampicillin mL^{-1}) and fungicides (50 μg PCNB mL^{-1}; 10 μg carbendazim mL^{-1}) to autoclaved, cooled (50°C) RA medium, and used to isolate *P. infestans* from blighted potato tissue and maintain cultures. All plates for each of the experiments were prepared from the same batch of medium in order to reduce variability.

Origin and collection of isolates

In August 2003 and 2004, 127 samples were collected at the end of the potato-growing season from Inner Mongolia and Heilongjiang Province of China where flumorph or dimethomorph had not been used. Blight-affected leaves were collected and tissue from a single lesion was cut from the margin of affected foliage and inserted into a small cut-open potato tuber. The cut tuber was sealed with adhesive tape and put into a plastic bag for transfer to the laboratory. Each lesion was randomly sampled from different fields separated by at least 1 km. Tubers inoculated with leaf lesions were cultured at 19°C for 3 days in the laboratory. When white mycelia could be seen along the cut areas, it was transferred to selective RA and incubated at 18–20°C for 5–7 days whereupon subcultures were transferred to RA slants in small bottles and stored at 10–12°C.

Fungicides

Technical grade flumorph, metalaxyl, dimethomorph and cymoxanil were kindly provided by the Shenyang Research Institute of Chemical Industry, Agrolex P. Ltd, Jiangsu Frey Agrochemicals Co. and Jiangsu Xinyi Agrochemicals Co., respectively. Azoxystrobin SC (25%) was purchased from Syngenta (China). The fungicides were dissolved in methanol, except azoxystrobin, which was dissolved in sterile distilled H_2O, to prepare the stock solutions and stored at 4°C in the dark to maintain and preserve fungicide activity. The stock solutions were added to molten RA, when they were cooled to approximately 50°C.

Fungicide sensitivity test

Flumorph baseline-sensitivity assay in vitro

Assessment of the inhibition of hyphal growth was determined *in vitro* by transferring plugs (5 mm in diameter) of mycelium from the leading edge of an actively growing colony to a series of RA plates amended with 0·05, 0·1, 0·2, 0·3, 0·4 and 0·5 μg flumorph mL^{-1}. In all cases (including nonamended control plates), the final methanol concentrations were the same. Each isolate was tested in triplicate and incubated at 19°C for 10 days in darkness with four replicates. Mean colony diameter (minus the diameter of the inoculation plug) was measured for each treatment and expressed as percentage growth inhibition. The median effective concentration value (EC_{50}) for each isolate was calculated by regressing percentage growth inhibition against the log of fungicide concentration.

Metalaxyl-resistance test and cross-resistance

For each isolate, metalaxyl sensitivity was determined by analysing the cross-resistance between metalaxyl and flumorph. Metalaxyl was added to RA medium to yield a final active ingredient concentration of 10 μg mL^{-1}. Each isolate was transferred to three Petri dish plates of metalaxyl-amended agar and three nonamended control plates using mycelial plugs cut from the outer zones of actively growing cultures aged 7–10 days. Colony diameters were measured after plates were incubated at 19°C for 10 days in darkness. The sensitivities of isolates to metalaxyl were designated as MS (sensitive), MI (intermediate) and MR (resistant), according to the criteria of Shattock (1988).

Induction of resistant phenotypes

Seven-day-old colonies of three isolates (N0305, H0304 and Phy09), growing on RA, were placed 20 cm from a 254-nm UV light source and exposed for 45 s, after which mycelial plugs (2 mm in diameter) were excised randomly from whole irradiated colonies and placed inverted on RA in Petri dishes containing metalaxyl (10 μg mL^{-1}) or flumorph (1, 2, 5 or 10 μg mL^{-1}). One hundred plugs were used for each isolate at every concentration of fungicides. After incubation at 19°C for 30–45 days in darkness, resistant phenotypes were identified on 10 μg mL^{-1}

metalaxyl or 1 μg mL^{-1} flumorph. For the stable flumorph-resistant mutants, single-zoospore isolates were established. The level of fungicide resistance, namely the resistance factor, is equal to EC_{50} of the resistance phenotype/EC_{50} of the sensitive wild-type parental isolate.

Characterization of flumorph-resistant mutants

Cross-resistance

Resistant mutants and their sensitive parental isolates were subcultured on RA for 7 days at 19°C in darkness, after which 5-mm-diameter mycelial agar plugs were transferred from the margins of the colonies onto a series of concentrations of metalaxyl-, dimethomorph-, cymoxanil- or azoxystrobin-modified RA media in 90 mm Petri dishes, and incubated at 19°C. Colony diameters were measured after 10 days and percentage inhibition relative to control and EC_{50} values for both resistant mutants and wild-type parental isolate calculated as described above. The sensitivity of mutants to flumorph and to the other four fungicides were compared and cross-resistance was analysed using correlation analysis (Suty & Stenzel, 1999).

Hyphal growth and sporulation in vitro

Hyphal growth rate and sporangial production *in vitro* of parental isolates and mutant strains were compared on fungicide-free RA plates, with four replicates in each case. After incubation at 19°C for 10 days in darkness, the mean colony diameter (minus the diameter of the inoculation plug) was measured. Meanwhile, the sporulation of each strain was assayed according to the previously described method for quantification of sporangia (Caten & Jinks, 1968). Ten of the plugs (2·5 mm in diameter) were harvested approximately 2 mm from the colony margin and 10 approximately 2 mm from the edge of the initial inoculum plug. All 20 plugs were placed into 15 mL plastic tubes with 10 mL of sterile water and agitated for 15 s to dislodge the sporangia, and the sporangial suspension was quantified with a haemocytometer.

Pathogenicity and sporulation in vivo

Fully expanded leaflets of similar age were excised from glasshouse-grown potato plants (cv. Shepody) and surface-sterilized in 0·5% sodium hypochlorite for 1 min. Leaflets were rinsed three times in sterile distilled water, allowed to dry, and 30-mm-diameter leaf discs were cut with a sterilized cork borer. Leaf discs were randomly selected and placed on filter paper in 90 mm Petri dishes, with 4 mL of sterile water. Colonized mycelial plugs (2·5 mm in diameter) of parental and mutant cultures were inoculated onto the abaxial surface of 30 leaf discs for each isolate. Inoculated discs were incubated at 19°C (12 h photoperiod) for 96 h, and the percentage incidence of late blight calculated from leaf disks with and without symptoms. Each of the three sets of 10 discs was placed in a 15 mL centrifuge tube containing 10 mL dH_2O and agitated for 15 s on a vortex. The sporangia released were quantified with a haemocytometer. The mean number of sporangia per leaf disc was calculated.

Stability of resistance

One flumorph-resistant mutant was selected for each of these three parental isolates and 30 single zoospore isolates established as mycelial cultures. Each of the latter was transferred to RA containing 1·0 μg flumorph mL^{-1}. In addition, each of the mutant phenotypes was transferred five times onto new RA plates with no fungicide or stored at 10°C for 3 months to determine whether the resistance was stable.

Results

Baseline sensitivity to flumorph

A total of 127 *P. infestans* isolates were tested for their sensitivities to flumorph. The sensitivities of isolates collected from different places in the same year and isolates sampled in 2003 and 2004 from the same province were compared (Table 1). There was no evidence of geographical variation in the sensitivity of *P. infestans* to flumorph, and the sensitivities of isolates remained unchanged in both years.

The frequency distribution of the EC_{50} values for 127 isolates was a unimodal curve (Fig. 2), ranging from 0·1016 to 0·3228 μg mL^{-1}. The mean EC_{50} value was 0.1813 (± 0·0405) μg mL^{-1}, representing a range-of-variation factor of 3·2. It also suggested that there was no resistant subpopulation among the isolates used in the study. Thus, these sensitivity data could be used as a baseline for observing the shift of sensitivity in *P. infestans* populations to flumorph.

Resistance to metalaxyl in field isolates

Three different sensitivity phenotypes (MS, MI and MR) to metalaxyl were identified among 127 *P. infestans* isolates. A third of these were sensitive, while most isolates

Table 1 Baseline sensitivity data for field isolates of *Phytophthora infestans* on flumorph-amended agar

Year	Province	Number of isolates	EC_{50} values (μg mL^{-1})		
			Range	Mean[a]	SD
2003	Inner Mongolia	29	0·1016–0·3228	0·1839 a	0·0487
2003	Heilongjiang	29	0·1114–0·2976	0·1782 a	0·0399
2004	Heilongjiang	69	0·1054–0·3012	0·1814 a	0·0375

[a]Mean values followed by the same letter were not significantly different with Duncan's test at $P = 0·05$.

Table 2 Sensitivity of metalaxyl-sensitive, -intermediate and -resistant phenotypes of *Phytophthora infestans* to flumorph *in vitro*

Metalaxyl sensitivity	Number of isolates	Frequency (%)	EC_{50} values (μg mL^{-1}) for flumorph		
			Range	Mean	SD
MS	41	32.3	0·1016–0·3228	0·1913	0·0487
MI	11	8.7	0·1114–0·2976	0·2014	0·0390
MR	75	59.0	0·1054–0·3012	0·1728	0·0335

Table 3 Effect of flumorph on the mycelial growth of wild-type parental isolates of *Phytophthora infestans* and flumorph-resistant mutants

Isolate	Regression equation $y = bx + a$	Correlation coefficient (r)	EC_{50} value (μg mL^{-1})[a]	Resistance factor[b]
N0305	$y = 7{\cdot}3073x + 2{\cdot}5485$	0·9990	0·1243	–
F.N0305.1	$y = 1{\cdot}6239x + 5{\cdot}6406$	0·9809	0·4032	3·2
F.N0305.2	$y = 1{\cdot}8782x + 5{\cdot}8879$	0·9762	0·3367	2·7
F.N0305.3	$y = 3{\cdot}1187x + 6{\cdot}3574$	0·9644	0·3671	3·0
F.N0305.4	$y = 1{\cdot}0942x + 5{\cdot}6998$	0·9855	0·2293	1·8
H0304	$y = 7{\cdot}3234x + 2{\cdot}7058$	0·9596	0·1385	–
F.H0304.1	$y = 4{\cdot}0936x + 6{\cdot}7553$	0·9995	0·3726	2·7
F.H0304.2	$y = 2{\cdot}1471x + 5{\cdot}8601$	0·9812	0·3976	2·9
F.H0304.3	$y = 2{\cdot}7166x + 6{\cdot}1562$	0·9738	0·3753	2·7
Phy09	$y = 6{\cdot}8215x + 2{\cdot}6994$	0·9465	0·2114	–
F.Phy09.1	$y = 1{\cdot}9178x + 5{\cdot}4881$	0·8791	0·5565	2·6
F.Phy09.2	$y = 2{\cdot}3079x + 5{\cdot}8757$	0·9741	0·4174	2·0
F.Phy09.3	$y = 1{\cdot}7325x + 5{\cdot}8790$	0·9930	0·3109	1·5

[a]Concentration to reduce growth by 50%.
[b]Resistance factor = EC_{50} resistant mutants/EC_{50} sensitive parent.

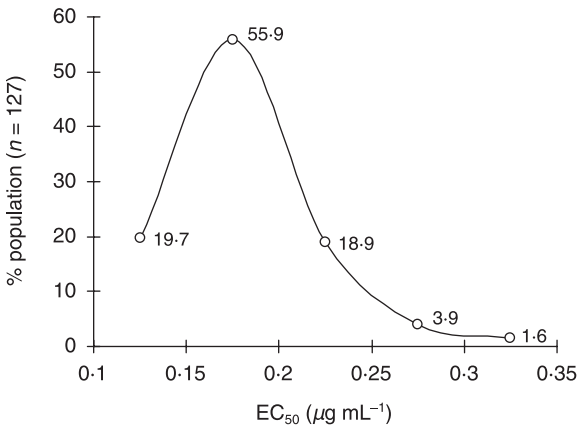

Figure 2 *In vitro* sensitivity of 127 field isolates of *Phytophthora infestans* to flumorph.

were resistant to metalaxyl with only 11 of intermediate phenotype (Table 2). To investigate cross-resistance between metalaxyl and flumorph, the sensitivities to flumorph of isolates with different levels of sensitivity to metalaxyl were compared. Flumorph had an equal effect on both metalaxyl-resistant and -sensitive isolates (Table 2), indicating an absence of cross-resistance between these two fungicides.

Development of resistance to flumorph and metalaxyl *in vitro*

Among three strains used to induce resistant mutants to flumorph, only N0305 was sensitive to metalaxyl. Metalaxyl-resistant mutants of N0305 were obtained with a frequency of 30% among UV-irradiated mycelial plugs on RA plates amended with 10 μg metalaxyl mL^{-1}. Ten of these mutants were tested further for their resistance to the phenylamide. The resistance factors all exceeded 100. In parallel experiments, 10 flumorph-resistant mutants were generated, with a mutation frequency of 4, 3 and 3%, respectively, for strains N0305, H0304 and Phy09 (Table 3). Although these mutants could grow on RA media containing 1 μg flumorph mL^{-1}, while their original parental isolates could not, changes in sensitivity to flumorph were very small, with resistance factors ranging from 1·5 to 3·2. A comparison of the sensitivity profiles of these resistant phenotypes with their original parents (Table 3) indicated that *P. infestans* easily developed resistance to metalaxyl more frequently than to flumorph after ultraviolet irradiation of mycelium.

Characteristics of flumorph-resistant mutants

Cross-resistance

Comparison of the sensitivities of 10 flumorph-resistant mutants together with their wild parental strains showed a high correlation between sensitivities to flumorph and dimethomorph (r = 0·8487), but not between flumorph and azoxystrobin (r = 0·0469) or flumorph and cymoxanil (r = 0·0703) (Fig. 3), indicating positive cross-resistance in *P. infestans* between flumorph and dimethomorph only.

Hyphal growth and sporulation in vitro

The *in vitro* growth rates of most of the seven flumorph-resistant mutants were significantly (P = 0·05) lower

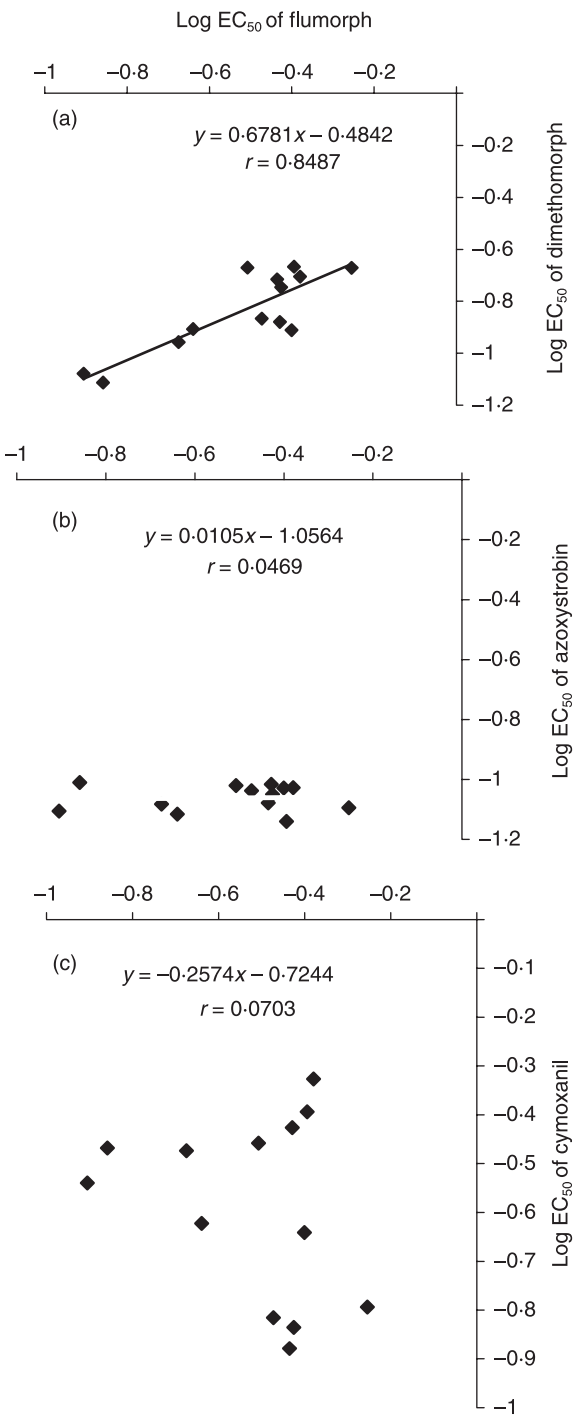

Figure 3 Correlation between flumorph-resistant mutants and their parental strains and the fungicides dimethomorph (a), azoxystrobin (b) and cymoxanil (c).

than those of their wild-type sensitive parents and only F.N0305.3 was similar to its parent N0305. *In vitro* production of sporangia on RA of all flumorph-resistant mutants was substantially lower than that of the wild strains (Table 4).

Pathogenicity and sporulation in vivo

Compared with wild-type parental isolates, the incidence of mycelial growth on plug-inoculated leaf discs of flumorph-resistant mutant strains was not significantly different, but sporulation of the mutants was significantly reduced ($P = 0{\cdot}05$) (Table 4).

Stability

Thirty single zoospore progeny of each of three representative resistant mutant isolates, F.N0305.1, F.H0304.1 and F.Phy09.1, all grew on RA with 1 μg flumorph mL^{-1}, indicating stability of resistance to the fungicide. Also, these three representative mutants maintained their resistance through five transfers on RA over 2 months and when stored on RA slants at 10°C for 3 months.

Discussion

A total of 127 single-lesion field isolates of *P. infestans* were tested for their sensitivities to flumorph in this study. Their EC_{50} values showed a narrow range of distribution from 0·1016 to 0·3228 for the most- and least-sensitive isolates. Isolates were sampled from two geographical origins and showed similar sensitivity levels over 2 years. Flumorph was equally effective on both metalaxyl-resistant and wild-sensitive pathogens which are widespread in late-blight populations worldwide (Gisi & Cohen, 1996). The resistance factor for flumorph was far lower than that for metalaxyl, and phenotypes resistant to the latter were more easily recovered following UV-mutagenesis of *in vitro* cultures. These results are similar to those from a study of the laboratory resistance to dimethomorph in *P. infestans* and other *Phytophthora* spp. (Chabane & Leroux, 1993; Young *et al*., 2001; Stein & Kirk, 2004).

Flumorph-resistant mutants were cross-resistant to flumorph's structural analogue dimethomorph. At present, it might be assumed that because they have a similar chemical structure, they act by the same mechanism. The biochemical mode of action of dimethomorph is suggested to be related to a disruption of cell wall formation, specifically the organization rather than the synthesis of wall components (Albert *et al*., 1988; Kuhn *et al*., 1991). Difficulty in developing high levels of resistance was also observed with dimethomorph in *P. infestans* (Bagirova *et al*., 2001; Young *et al*., 2001; Stein & Kirk, 2004). Although variation in the sensitivity of *P. infestans* to dimethomorph under field conditions has been noted (Dereviagina *et al*., 1999), there has been no conclusive evidence of disease control failure due to emergence of resistant isolates. The absence of cross-resistance between flumorph and metalaxyl, azoxystrobin or cymoxanil indicates different modes of action.

Most resistant mutants showed decreases in hyphal growth rate and in sporulation both *in vitro* and in sporulation-detached leaf tissues. These results and the laboratory studies and the experiences in practical use of dimethomorph to control potato late blight in past decades suggest there is a low risk of *P. infestans* developing resistance to flumorph in the field. Nevertheless,

Table 4 Hyphal growth (colony diameter) and sporulation (no. of sporangia) on rye A agar, and incidence of symptom development and sporulation on potato leaf discs for wild-type parental isolates of *Phytophthora infestans* and flumorph-resistant mutants derived by UV mutagenesis

	In vitro on RA media		*In vivo* on leaf discs	
Strain	Hyphal growth (mm)	Sporulation (per mycelial plug)	Incidence of symptoms (%)	Sporulation (per leaf disc)
N0305	86·5 a[a]	265·3 a	100·0	381·7 a
F.N0305.1	38·0 d	2·8 b	100·0	41·0 c
F.N0305.2	83·8 b	2·3 b	96·7	75·0 b
F.N0305.3	87·5 a	7·3 b	100·0	33·7 c
F.N0305.4	69·5 c	8·0 b	96·7	41·3 c
H0304	59·0 a	181·8 a	96·7	298·0 a
F.H0304.1	31·4 d	12·8 b	93·3	102·7 b
F.H0304.2	52·8 b	9·0 b	96·7	86·0 bc
F.H0304.3	34·1 c	2·0 b	100	58·3 bc
Phy09	51·6 a	230·3 a	100	264·0 a
F.Phy09.1	40·5 b	6·0 b	96·7	28·3 b
F.Phy09.2	40·5 b	6·3 b	96·7	38·0 b
F.Phy09.3	39·1 bc	4·3 b	93·3	56·3 b

[a]Figures for each parental isolate and its flumorph-resistant mutants followed by the same letter within a column were not significantly different with Duncan's test at $P = 0{\cdot}05$.

oomycetes in general, and *P. infestans* in particular, are high-risk pathogens (Brent & Hollomon, 1998) and highly pathogenic flumorph-resistant phenotypes may arise and, for this reason, antiresistance strategies should be considered. Indeed, flumorph has only been used commercially in mixtures with the protectant fungicide zineb. Mixture with other compounds, such as azoxystrobin or cymoxanil examined here, or use in alternating product spray programmes might also be considered.

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