Mutants of *Phytophthora infestans* resistant to dimethomorph fungicide

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ABSTRACT

After two–step mutagenesis with nitrosomethyl urea the resistant to dimethomorph (DMM) mutants of *Phytophthora infestans* were obtained. The frequencies of mutations were low – 6,27 x 10^{-7} in their first step–mutagenesis and 6,4 x 10^{-8} – in the second. Lethal concentrations of DMM were increased from 2 mg/l to 8 mg/l. Fitness of mutants *in vitro* and *in planta* was low. Most of F1 hybrids between resistant to DMM and sensitive strains were phenotypic similar to sensitive parent. The rare resistant hybrid strains have decreasing fitness, teratic low–germinated sporangia, and were instable (on media without DMM they reverted to sensitivity and normal growth). The anomalous segregation in the F1 hyrids was analysed regarding to mating type inheritance, linkage between mating type and dim^R loci, and death of resistant hybrids.

Key words: Phytophthora infestans, dimethomorph, resistance, hybridization.

Багирова С. Ф., Ань Цзань Ли, Долгова А. В., Еланский С. Н., Шоу Д. С., Дьяков Ю. Т. Мутанты *Phytophthora infestans,* резистентные к фунгициду диметоморфу.

Ступенчатым мутагенезом под действием нитрозометилмочевины получены мутанты *Phytophthora infestans*, резистентные к диметоморфу ДММ. Частота мутаций была низкой – от 6,27 x 10^{-7} до 6,4 x 10^{-8}. Летальная концентрация (ДММ) у мутантов составила 8 мг/л против 2 мг/л у исходного штамма. Скорость роста на искусственной среде и агрессивность мутантов были понижены. Большинство гибридов F1 между резистентным и чувствительным штаммами были фенотипически сходны с чувствительным родителем. Редкие резистентные гибриды имели низкую приспособленность, уродливые, слабо прорастающие спорангии, и были нестабильными (на среде без ДММ восстанавливали чувствительность и нормальный рост). Причины отклонения от ожидаемого расщепления проанализированы с точки зрения наследования типа спаривания, возможного сцепления между локусами спаривания и резистентности к ДММ и гибели резистентных гибридов.

INTRODUCTION

Dimethomorph (DMM) is a novel fungicide against oomycetous plant pathogens from family Peronosporaceae and genus *Phytophthora.* It has antisporulation activity (Albert et al., 1988; Cohen et al., 1995). DMM possibly affects cell wall formation and prevents zoospores incyst–ment (Albert et al., 1988; Kuhn et al., 1991; Thomas et al., 1992). Positive properties of DMM are listed below:

1. DMM is equally toxic for isolates resistant and sus–ceptible to phenylamide fungicides (Derevjagina et al., 1999). There is no cross resistance between DMM and phenylamides, that have lost their effectiveness after widespread appearance of resistant strains in field popu–lations (Albert et al., 1988; Kadish et al., 1990; Kuhn et al., 1991; Chabane et al., 1993; Derevjagina et al., 1997; Gisi, Cohen, 1996; Alboure et al., 1998).

2. DMM is a systemic fungicide with prolonged pro–tective activity after treatment. It can move inside the plant only with xylem transport and it leads to a weak systemic effect; the fungicide cannot move from treated leaves to untreated and from abaxial to adaxial leaf sur–face (Cohen et al., 1995; Derevjagina et al., 1997).

3. DMM is active in very low concentrations (Bissbart, Schlosser, 1991; Kuhn et al., 1991).

4. It was found that the risk of appearance of strains resistant to DMM in field populations is much less than with phenylamides. Attempts to find resistant strains in field populations of *Plasmopara viticola, Phytophthora cactorum* and *P. infestans* using stan–dard methods were unsuccessful (Staub, Sozzi, 1984; Blankenagel, Semmer, 1990; Derevjagina et al., 1999). Attempts to detect resistant mutants after mutagenic treatment of molds were also unsuccessful (Bissbart, Schlosser, 1991). However, Chabane at al. (1993) and Chabane et al. (1995), using UF–exposure, obtained

resistant clones of *P. parasitica* that retained their resistance.

In our experiments using a quantitative method of monitoring resistance (Dyakov et al., 2000) resistant strains of *P. infestans* were isolated from field populations treated and untreated with DMM (Derevjagina et al., 1995, 1999). After being kept on agarized media without DMM isolates loose their resistant properties. They were possibly ousted by sensitive revertants having greater fitness.

The aims of the present work were to obtain and analyse resistant mutants from sensitive *P. infestans* strains.

MATERIALS AND METHODS

Source of isolates. There were two isolates used in this work: VNIIF (A1 mating type, resistant to metalaxyl, sensitive to DMM, isolated from potato) and K27 (A2 mating type, sensitive to metalaxyl and DMM, isolated from potato).

Test for mating type and compatibility groups. Mating type was determined by growing the sample isolates together with tester isolates of known mating type in a Petri dish containing oat–meal agar. Each sample isolate was grown together with each of known mating types (A1 and A2) on a separate plate. Each plate was assessed for oospore formation 10 to 15 days after test started. The isolates that formed oospores on plates with the known A1 mating type were registered as A2. Those isolates that formed oospores with the known A2 mating type were registered as A1. Those isolates that formed oospores with both known mating types were considered to be self–fertile.

The vegetative compatibility groups (vcg) were determined by analysing the boundary zone between strains grown on the oat–meal agar (Gorbunova et al., 1989). 1S1 (A2 vcg2) and B5 (A1 vcg1) strains were used as control.

Resistance test. The reaction of *P. infestans* to the phenylamide fungicide metalaxyl was evaluated by transferring each isolate to oat–meal agar medium treated with 1, 10 and 100 mg/l of active ingredient of metalaxyl. Four plugs, 3–6 mm in diameter, were placed equidistant on each plate. The radial growth from each plug was recorded at 5 and 10 days after inoculation. The relative growth of each isolate growing in fungicide treated media was obtained by dividing the radial growth of the isolate growing in metalaxyl treated media by the radial growth of the same isolate growing on media without fungicide. The isolates were recorded as sensitive (S) when the relative radial growth at 10 mg of metalaxyl per liter was less than 10%. They were recorded as semiresistant (SR) when the relative radial growth at 10 mg metalaxyl per liter was greater then 10% but less then 40%, and as resistant when the isolate growth was more than 40% at 10 mg/l and growth continued at the highest concentration of 100 mg metalaxyl per liter.

Resistance to DMM was tested similarly to that to metalaxyl. Resistant isolates can grow on oat–meal agar with 2 mg/l DMM, sensitive ones cannot grow on this medium, and semiresistant ones grow slowly on it; with colony diameter less then 50% of the control.

Aggressiveness testing. A quantitative method was used for agressiveness testing (Dyakov et al., 2000). Leaves of tomato (Talalikhin cv., no resistance to *P. infestans* genes) were placed in moist chambers (S=800 cm^2 , wet filter paper on the base) adaxial side up. The total area of the moist chamber was covered by leaves, that were treated by spraying with DMM (50 mg/m) or water (control). Twelve hours later the leaves were inoculated by spraying with cystospore suspension (1 ml of suspension containing 1000 spores per one chamber). The wet chambers were incubated for 3 days and leaves were viewed against the light and the number of late blight lesions was recorded. Aggressiveness or fitness (effectiveness of cloning, EC) was determined as the ratio between number of lesions on infected leaflets and number of cystospores in inoculum. Resistance to DMM on plants was determined as the ratio between number of lesions in leaves treated with DMM and in those treated with water.

Mutagenesis. All experiments were conducted with *P. infestans* isolate VNIIF. The mycelium was cultivated on oat–meal agar (130 g of oat grains in 1 l of water heated to boiling, filtered through cheese–cloth, 15 g of agar–agar added before autoclaving 30 min, 1 atm.), or potato–salt liquid media (Gaertneur, 1959). Cystospore suspensions were treated for 18–20 hours with 0.005% N–nitrosomethyl urea (NMU). Spores were washed from the mutagen by double centrifugations in sterile distilled water and were spread on the oat–meal agar media with DMM (without dilution, for isolation of resistant mutants) and similar media without DMM (with dilution, for counting the survival of spores). Speed of linear growth of mutants was measured on oat–meal agarized media. For determination of sporulation activity on the border of a colony agar blocks were taken, sporangia were washed out with distilled water and counted in a hemocytometer. Amounts of zoospores were calculated based on counting of empty sporangia after exposure on 3–5^{0}C.

Oospores production. Mating between resistant mutants and wild strain K 27 was used for oospores generation. Agar blocks, containing mature oospores, were homogenized in distilled water, centrifuged at 2000 rpm, and placed in sterile *Trichoderma viride* supernatant. *T. viride* was grown in liquid media containing *P. infestans* mycelium. After 10 min. of exposure oospores were washed by centrifuging 3 times and placed on Petri dishes with 1.2% agar. Growing oospores were placed to oat–meal agar under the light microscope.

Determination of hybrids. Partenogenic oospores can be formed by *P. infestans* together with hybrid oospores. The RAPD method was used for determination of the structure of oospores (hybrid or not hybrid): PCR were per–

formed as described by Judelson et al., (1995) modified by Maufrand et al. (1995) in 25 using 2 ng of template DNA and 10–mers primers from Operon Technologies: Y11–AGACGATGGG; Y04–GGCTGCAATG. Products were separated on 1% agarose gels in TBE (89 mM Tris, 89 mM H_3B0_3, 2mM Na_2–EDTA) and stained with ethidium bromide.

RESULTS AND DISCUSSION

Obtaining of DMM resistant mutants and their properties. Growth of wild type mycelium (strain VNIIF) was much reduced on media having DMM concentration 2 mg/l (w/v). Selection of resistant mutants was conducted with concentration 3 mg/l. The frequency of resistant mutations was 6,27 x 10^{-7}. This is similar to the frequency of resistance mutations to several antibiotics. The frequency of resistance mutations to phenylamides was about 10^{-5} (Dolgova, Dyakov, 1986; Derevjagina et al., 1993).

The mutants obtained did not have increased level of resistance to metalaxyl. They did not differ from the wild type in colony morphology and rate of growth on oat–meal agar without fungicide. The lethal concentration of DMM for mutants was 4 to 6 mg/l instead of 2 mg/l in wild type; LD_{50} increased from 0.3 mg/l for wild type to 1,4–2,0 for mutants.

The low selectivity of obtained mutants lead us to the assumption that resistance to DMM is of "penicillin" not "streptomycin" type (Schnitzer, Grunberg, 1957). Modification of one gene connected with resistance leads to a small increase of resistance level. Resistance is polygenic and can be increased by several treatments with mutagen. To test this assumption we made a second treatment with NMU of the resistant mutants obtained earlier. The DMM concentration on selective media was increased from 3 to 6 mg/l. Several mutants have been obtained: Dm1–1 from Dm1, Dm3–1 from Dm3. Lethal concentration was increased from 4 to 8 mg/l. The frequency of mutations was lower: 1,67 and 6,4 x 10^{-8} – from first mutation to second against 6,27 x 10^{-7}– from wild type. One possible explanation is that resistance to DMM is controlled by several additive loci. Each successive mutation decreases the target size and leads to decreasing of mutation frequencies. Double mutants, in comparison with single and wild type, had a lower rate of growth *in vitro* and lower aggressiveness (table 1).

As shown in Table 1, fitness of resistant mutants is very low. EC of mutant Dm1–1 was only 26% of wild type, and EC of mutant Dm3–1 was about 1%. The percentage of resistant clones of mutants was 10 times higher than for wild type. Increase of resistance *in planta* leads to decrease of fitness.

Resistance to DMM appears to be polygenic and additive. Mutations in single genes slightly increase the lethal dose of fungicide, and increase of resistance in double mutants is associated with decreasing fitness both *in vitro* and *in planta*. Strains resistant to DMM occurred in field populations with very low frequency; DMM – fungicide incurs a low risk of resistant strains appearing (Dekker, 1988).

TABLE 1

Growth rates aggressiveness and dimethomorph resistance of *P.infestans* mutants

Strains	Properties *in vitro*		Properties *in planta*		
	Lethal concentration (mg/l)	Colony diameter after 10 days of growth (mm)	EC on tomato leaves treated with water	EC on tomato leaves treated with DMM	Per cent of resistant clones
VNIIF	2	60	0.552	0.018	3.3
Dm3	4	65	–	–	–
Dm1–1	8	–	0.145	0.056	38.6
Dm3–1	8	44	0.007	0.003	42.9

– not determined

Hybrids between resistant to DMM and sensitive strains. 107 monoosporic strains were isolated in pure culture after mating of double mutant DM3–1 with sensitive wild strain K27. All of them had hybrid origin confirmed by RAPD data (Fig. 1). Some properties of parental strains and hybrids are shown in Table 2.

Fig. 1. RAPD–patterns of the strains of *P. infestans.*
M – marker, P1 and P2 – parent strains, the rests – hybrids.

Several considerations have to be discussed before interpreted these data.

1. The possibility of non–hybrid origin of oospores and progeny similar to the sensitive parent. This is refuted by data from RAPD analyzes.

2. High lethality of hybrids resistant to DMM. This can influence on the randomization of selection. Some data support this factor. *P. infestans* has diploid nuclei. Resistance mutations after NMU treatments can be dominant or semi–dominant and must be checked in hybrid progeny. Resistant offsprings had lower growth in comparison with sensitive ones (Table 3). Growth of another resistant offspring can be too slow to check and isolate in pure culture.

Together with slow growth on agarized media mutants resistant to DMM had abnormal morphology: weak de–

TABLE 2

Characteristics of parent strains and F1 hybrids of *P.infestans*

Strains	Phenotype	Number of isolates
P1 (Dm3–1)	A1 vcg1 dim^R	1
P2 (K27)	A2 vcg2 dim^S	1
F1	A2 vcg2 dim^S	89
	A1 vcg1 dim^{SR}	1
	A1 vcg2 dim^S	1
	A2 2nc dim^S	6
	A1 2nc dim^{SR}	1
	Sf 2c dim^S	2

A1, A2 – mating types; Sf – self–fertility.

vcg1, vcg2 – groups of vegetative incompatibility; 2hc – non–compatible with testers of both groups; 2c – compatible with both groups.

dim^R, dim^S, dim^{SR} – resistant (can grow on oat–meal agar with 7 mg/l of DMM), sensitive (cannot grow on this medium) and semiresistant (slow growth on this medium; colony diameter less then 50% of control).

TABLE 3

Rate of vegetative growth and resistance to DMM of parent strains and hybrids of *P.infestans*

Strains		Colony diameter on the 10th days of growth (mm)	Lethal DMM concentration (mg/l)
P1	(Dm3–1)	44	8
P2	(K27)	88	2
F1	N52	83	2
	N72	84	2
	N4	72	8
	N42	76	8

veloped substrate mycelium, enormous branching, loops at hyphal ends. Sporangia have atypical for *P.infestans* form – very long with enormous tip (Fig. 2). Sporangia production by mutants started 3 days later than by wild type. Average length of wild type sporangium (VNIIF) – 41.25 mkm, resistant mutant Dm3–1 – 72.64 mkm, size of papilla 2–4 and 8–16 mkm respectively. The numbers of sporangia formed by resistant mutants and by wild strain were similar but on the resistant strain the sporangia were fixed more strongly on hyphal branches. The main part of the sporangia of resistant strains did not germinate. A small part of the germinated sporangia formed a small hypha with another sporangium at its end (microcyclic life span). Germination with hyphae occurred only on revertants that lose resistance, not resistant mutants.

The morphological anomalies mentioned above are typical for fungi having defects in the chemical structure of the cell wall (see Peberdy, 1990). There are data indicating that DMM disturbs cell wall biosynthesis or cell wall – target of DMM action (Kuhn et al., 1991). Modification of the target in resistant strains can cause a decrease of their viability.

During the cultivation of resistant mutants on DMM–free media fast growing sectors with sensitive mycelium appeared. It appears that in selected nuclei reversion to sensitivity took place. Later, sensitive fast growing mycelium displaced the slowly growing resistant type and the isolate became sensitive. Revertants restore the high rate of colony growth (Fig. 3) and normal sporangia germination. Possibly because of frequent reversion we were unable to store field isolates resistant to DMM (Derevjagina et al., 1995).

3. A2 mating type prevailed in the progeny although both of the offsprings resistant to DMM have A1 mating type. It leads to the supposition that the DMM resistance gene is linked with the mating type locus.

There is no generally accepted point of view on the mating type inheritance. Different authors introduce a wide range of data about F1 – from 1:0 to 0:1, but 1:1 – 1:2 segregation prevails (Timmer et al., 1970; Khaki, Shaw, 1974: Judelson, 1996). One of the widespread hypotheses is that mating type is controlled by one two–allelic gene; A1 isolates are homozygous on recessive allele (*aa*) but A2 are heterozygous (*Aa*) (Shaw, 1983). Under this control in hybrid progeny, even in the case of inde–

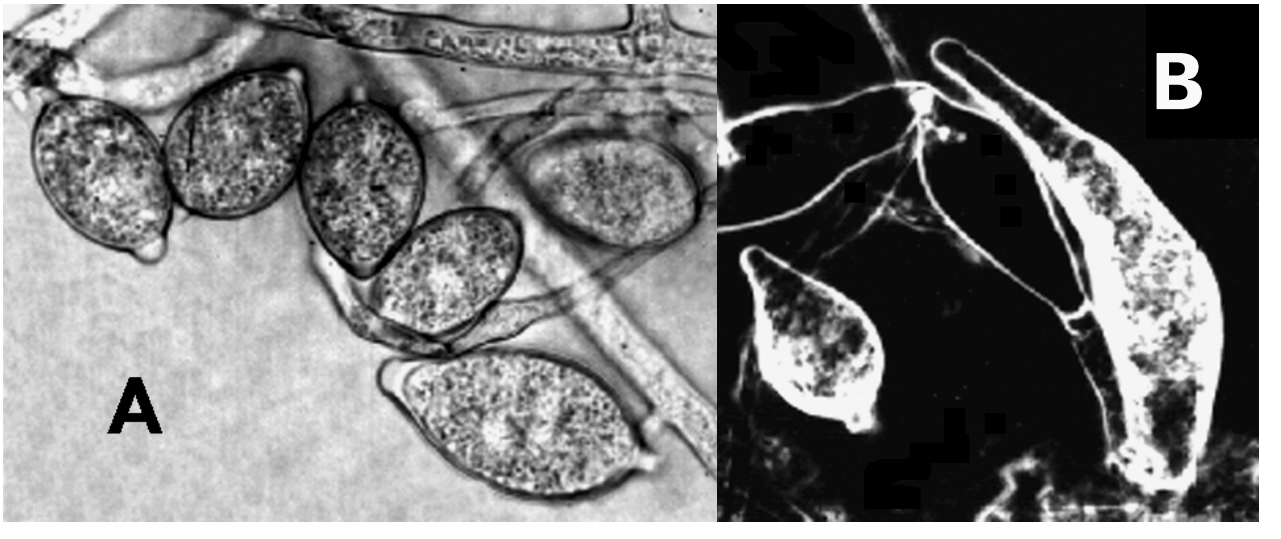

Fig 2. Sporangia of sensitive wild type (A) and resistant to Dm mutant (b).

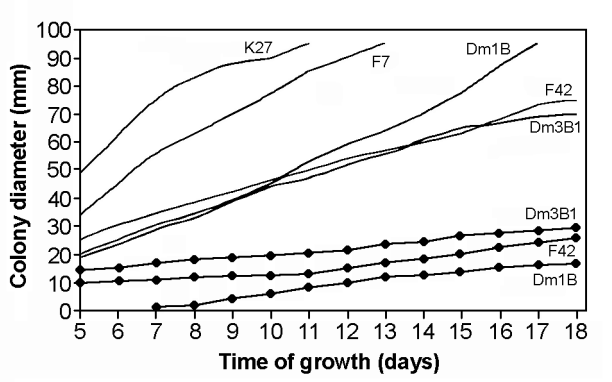

Fig 3. Growth of resistant to Dm mutants (Dm3b1, F42 and Dm 1b), formerly resistant but now sensitive revertant (F7), and sensitive wild strain (K27) on oat meal agar. Shown with (lines with circles) and without Dm.

TABLE 4

Phenotypes of parents and progeny of *P.infestans*

Strains	Phenotypes	Number of progenies
P1 (dim revertant)	A1 vcg1 met^R	
P2	A2 vcg2 met^S	
F1	A1 vcg1 met^R	14
	A1 vcg2 met^R	4
	A1 vcg2 met^{SR}	1
	A2 vcg2 met^R	4
	A2 vcg2 met^{SR}	2

met^R – can grow in oat–meal agar with 100 mg/l of metalaxyl; met^{SR} – on oat–meal agar with 10 mg/l metalaxyl; met^S – with 1 mg/l metalaxyl. Segregations:

– met^R : met^{SR} = 7 : 1;

– A1 : A2 = 3 : 1;

– vcg1 : vcg2 = 9 : 7.

pendent inheritance of mating type and resistance there can occur (A1 DmR : A2 DmS : A1 DmS : A2 DmR = 1:1:1:1). If on the contrary, heterozygotes (*Aa*) will have A1 and homozygotes (*aa*) – A2 then resistant progeny of two diploid parents [VNIIF – DmS *a*/DmR *A* (DmR A1) and K27 – DmS *a*/DmS *a* (DmS A2)] will have A1and all sensitive progeny – A2. According to data of Judelson et al. (1995) DNA markers closely or absolutely linked to the determinants of the mating type in *P.infestans* demonstrated that the mating type involves a single locus that is heterozygous in the A1 parent with A1 dominant: *Aa* x *aa.* Deviation from 1:1 can be caused by lethality of DMM resistant offsprings or non–participation of resistant gametangia in fertilization.

For the better understanding of connections between mating type and resistance to DMM, mating of sensitive revertant and sensitive wild isolate has been effected. Germinated oospores were isolated and obtained cultures were tested for resistance to metalaxyl, mating type and vegetative incompatibility. A total of 25 monooosporic cultures has been analyzed (Table 4).

Revertants sensitive to metalaxyl gave, in F1 segrega–tion, A1 : A2 = 3 : 1. This segregation has also been exam–ined in other research (Shattock, 1988). Deviation from this segregation in previous mating experiments was possibly caused by death of a part of the resistant isolates and linkage between loci of mating type and DMM resistance.

Segregation of hybrids supports the idea of the domi–nant character of metalaxyl resistance (Dolgova, Dyakov, 1986: Shattock, 1988) and a supposition about binding between types of vegetative compatibility and mating types and the possibility of their division in hybrids.

CONCLUSIONS

Genetic control of DMM resistance in *P. infestans* is additive.

DMM resistant mutations are very unstable. A high frequency of reverse mutations is observed.

There is no DMM and phenylamides cross–resistance.

DMM resistant mutations exert influence on vegeta–tive growth, and sexual and asexual sporulations. The re–sistance is not transmitted to offsprings and may affect the mating type segregation patterns.

DMM resistant mutants are characterized by very low aggressiveness.

The risk of dissemination of the mutants in naturally occurring late blight epidemy is insignificant.

ACKNOWLEDGEMENTS

This research work was financialy supported by Royal Society (UK) and RFBS (grant № 00–15–97808).

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