Mutants of *Phytophthora infestans* resistant to dimethomorph fungicide

S.F. Bagirova¹, An Zsan Li¹, A.V. Dolgova¹, S.N. Elansky¹, D.S. Shaw² & Yu. T. Dyakov¹

¹Moscow State University, Moscow, 119899, Russia,
Fax: +7 095 939–3970, E–mail: dyakov@1.mycol.bio.msu.ru.

²School of Biol. North Wales University, Bangor, Gwynedd, LL57 2UW UK,
Fax: (0248) 370–731, E–mail: d.s.shaw@bangor.ac.uk.

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 10⁻² in their first step—mutagenesis and 6.4 x 10⁻⁴ — 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, tentic low—germinated sporangia, and were instable (on media without DMM they reverted to sensitivity and normal growth). The anomalous segregation in the F1 hybrids was analysed regarding to mating type inheritance, linkage between mating type and dimR loci, and death of resistant hybrids.

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

INTRODUCTION

Dimethomorph (DMM) is a novel fungicide against oomycetous plant pathogens from family Peronosporaceae and genus *Phytophthora*. It has antisporeulation activity (Albert et al., 1988; Cohen et al., 1995). DMM possibly affects cell wall formation and prevents zoospores incystment (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 susceptible 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 populations (Albert et al., 1988; Kadish et al., 1990; Kuhn et al., 1991; Chabane et al., 1993; Derevjagina et al., 1997; Gisì, Cohen, 1996; Alboure et al., 1998).

2. DMM is a systemic fungicide with prolonged protective 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 surface (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 standard 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).

**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 similar media without DMM (with dilution, for isolation of resistant mutants) and similar media without 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 agar. For determination of sporulation activity on the border of a colony agar blocks were taken, sporangia was 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°C.

**Oospores production.** Mating between resistant mutants and wild strain K 27 was used for oospores generation. Agar blocks, containing mature oospor es, 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 oospor es (hybrid or not hybrid): PCR were per—

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.

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 \times 10^{-2}. 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; Derevyagina 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 \times 10^{-8} from first mutation to second against 6.27 \times 10^{-7} from first mutation to second after NMU treatments can be dominant or semi–dominant and must be checked in hybrid progeny. Resistant mutations after NMU treatments can be dominant or semi–dominant and must be checked in hybrid progeny. 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 \times 10^{-8} from first mutation to second against 6.27 \times 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>
<thead>
<tr>
<th>Strains</th>
<th>Properties in vitro</th>
<th>Properties in planta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lethal concentration</td>
<td>Colony diameter</td>
</tr>
<tr>
<td></td>
<td>(mg/l)</td>
<td>after 10 days of growth (mm)</td>
</tr>
<tr>
<td>VNIIF</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>Dm3</td>
<td>4</td>
<td>65</td>
</tr>
<tr>
<td>Dm1–1</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td>Dm3–1</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

TABLE 1

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.](image)

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*

<table>
<thead>
<tr>
<th>Strains</th>
<th>Phenotype</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (Dm3–1)</td>
<td>A1 vcg1 dimR</td>
<td>1</td>
</tr>
<tr>
<td>P2 (K27)</td>
<td>A2 vcg2 dimS</td>
<td>1</td>
</tr>
<tr>
<td>F1</td>
<td>A2 vcg2 dimS</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>A1 vcg1 dimR</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A1 vcg2 dimS</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A2 2nc dimS</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>A1 2nc dimR</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sf 2c dimS</td>
<td>2</td>
</tr>
</tbody>
</table>

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.

dimR, dimS, dimSR – 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 than 50% of control).

TABLE 3

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Colony diameter on the 10th days of growth (mm)</th>
<th>Lethal DMM concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>44</td>
<td>8</td>
</tr>
<tr>
<td>P2</td>
<td>88</td>
<td>2</td>
</tr>
<tr>
<td>F1</td>
<td>N52</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>N72</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>N42</td>
<td>8</td>
</tr>
</tbody>
</table>

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.

dimR, dimS, dimSR – 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 than 50% of control).

Fig 2. Sporangia of sensitive wild type (A) and resistant to Dm mutant (b).
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 monoosporic cultures has been analyzed (Table 4).

Revertants sensitive to metalaxyl gave, in F1 segregation, A1 : A2 = 3 : 1. This segregation has also been examined 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 dominant 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 vegetative growth, and sexual and asexual sporulations. The resistance 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 financially supported by Royal Society (UK) and RFBS (grant № 00–15–97808).

**LITERATURE CITED**


