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Dimethomorph and metalaxyl sensitivity in somatic hybrids of *Phytophthora parasitica* obtained by protoplast fusion

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Summary. Protoplasts were successfully isolated from wild-type and mutant strains of *Phytophthora nicotianae* var. *parasitica* using Novozym 234. Putative somatic hybrids were recovered following protoplast fusions from the first time to dimethomorph resistant strain P 310 (Dim^r) or metalaxyl P 26 (Met^r) by selection on agar amended with dimethomorph and metalaxyl. Fusion products from this cross were resistant to dimethomorph and metalaxyl. Zoospore progeny from the fusion products retained this phenotype, suggesting that nuclear fusion had taken place.

Introduction

Phytophthora species are far from ideal candidates for genetical work because of the low recovery of sexual progeny and their type of sexual reproduction from oospores (Shaw, 1983). Resistance to drugs and fungicides may however be dominant or semidominant (Long and Keen, 1977; Chang, 1990; Shattock, 1988; Crute, 1987). Hyphal heterokaryon formation occurs naturally when two hyphae of different genotype fuse and the nuclei of the two hyphae are mixed; it is considered to be rare in *Phytophthora* (Long, 1977; Shaw, 1988). Recently in *P. megasperma* f. sp. *glycinea* (Layton, 1988) and in *P. capsici* (Lucas, 1990) resistant heterokaryons to drugs have been obtained by protoplast fusion. Isolates of *Phytophthora nicotiana* var. *parasitica* with resistance to dimethomorph and metalaxyl were utilised in the first place because they were stable and retained pathogenicity to tobacco leaves. The present paper reports the results of attempts to introduce an additional marker, resistance to the fungicide dimethomorph, by UV irradiation.

Techniques for isolating protoplasts from *P. parasitica* were refined, and putative somatic hybrids isolated following protoplast fusion and selection on fungicide-amended media. Data on the resistance of the putative hybrids and their zoospore progeny are presented.

Materials and methods

Fungus isolates. The parental isolates of *P. parasitica* from which the mutants used in this study originated were P 310 of A1 mating type isolated in Australian from tobacco and P 26 of A2 mating type; they were kindly provided by Nicole MAIA (INRA, Station of Phytopathology, Antibes, France). The P 310 isolate was naturally resistant to streptomycin.

The metalaxyl resistant mutant P 26 (Met^r) and the dimethomorph resistant mutant P 310

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(Dim^r) were derived from P 26 and P 310 by UV mutagenesis as previously described (Chabane, 1993). All isolates were respectively maintained on V8C agar (2% V8 juice, 3% $CaCo_3$, clarified by centrifugation) without fungicide at 24°C in the dark.

Fungicide sensitivity. Routine screening of isolates for sensitivity was done using V8C agar amended with 0.1, 1, 10 and 100 µg ml^{-1} metalaxyl (technical grade, Ciba-Geigy), or 0.8, 2, 5 and 12.5 µg ml^{-1} dimethomorph, or streptomycin 300 µg ml^{-1}.

Protoplast isolation. Plugs cut from colonies on V8C agar were incubated in dishes containing V8C broth for 48 h at 25°C. The V8C broth was removed then by pipette and after a rinse in distilled water replaced by 10 ml of 0.35 M $CaCl_2$, pH 6.2 sterile solution. After 1 h, enzyme solution was then added.

Initial experiments were based on the procedure of Lucas *et al.* (1990) in which 1 ml of Cellulase CP (20 mg ml^{-1}) in 1 M mannitol and 7mM $MgSO_4$ solution were utilised. In later experiments Novozym 234 (Sigma) was substituted for Cellulase CP and also tested at 5 mg ml^{-1}. Finally, Novozym 234 was utilised at 10 mg ml^{-1}. Digestion proceeded for 1-3 h and protoplast release was monitored microscopically. Protoplasts were harvested by filtering through two layers of sterile tissue to remove mycelial debris, and centrifuged at 3000 *g* for 3 min. The enzyme supernatant was discarded and the protoplasts resuspended in 5 ml of 0.1 M $CaCl_2$ and 0.4 M mannitol, pH 6.2 solution. Yields were determined by haemocytometer count and viability was assessed by plating 0.1 ml of the protoplast suspension on regeneration agar medium (V8 broth containing 0.1 M $CaCl_2$, 4 M mannitol, and 1.25% w/v agar). Plates were examined microscopically after 16 h. Dilutions of protoplast suspensions were prepared in $CaCl_2$/mannitol, plated on regeneration agar, and colonies counted after 48 h.

Protoplast fusion. Fusions were done between mutant P 26 (Met^r) and the mutant P 310 (Dim^r), using suspensions containing 1-3 x 10^6 protoplasts ml^{-1}. A 0.5 ml suspension of each isolate was added and mixed in a sterile Eppendorf tube; control tubes containing 1 ml of either isolate were also prepared. Protoplasts were pelleted by centrifugation at 3000 *g* for 3 min. The supernatant was discarded and replaced by 0.5 ml fusion mixture (40% PEG 8000, 50 mM $CaCl_2$, 20 mM Tris pH 7.4). Fusions were conducted for 20 min and the suspension then mixed which molten regeneration agar in a Petri dish and incubated for 16 h to allow regeneration of cell walls. Plates were then overpoured with selection medium (V8C agar containing 25 µg ml^{-1} metalaxyl and 10 µg ml^{-1} dimethomorph). Colonies growing to the surface of the agar after one week were subcultured onto V8C agar prior to evaluation.

Evaluation of fusion products. Putative heterokaryon colonies recovered following fusion were tested for dose response to metalaxyl, dimethomorph and streptomycin as described above under "fungicide and drug sensitivity".

Sporulation and zoospore production. Zoospore progenies from selected heterokaryons were also tested. Putative hybrids were grown on V8C agar for 4 days in darkness at 25°C and then transferred to an illuminated incubator for a further 3 days. Zoospores were released in chilled, sterile distilled water and counted by haemocytometer.

Results

Protoplast isolation. Viable protoplasts were successfully isolated from young mycelium using Novozym 234 with 0.35 M $CaCl_2$ as osmoticum. Release took place over 1-3 h, giving yields of 1-3 x 10^6 protoplasts per ml. Regeneration rates were 12-15% in the experimental system used.

Heterokaryon formation. Protoplast fusion was attempted between P 26 (Met^r) and P 310 (Dim^r) with selection of fusion products after overnight regeneration by overpouring with agar amended with metalaxyl and dimethomorph. No colonies grew through the selection medium on control plates containing fused protoplasts from either parent. The resistant parental strains used do not sectors when they are regenerated from protoplasts. Twenty putative heterocaryons able to grow in the presence of both fungicides were obtained from four experiments. On fusion plates from the met^r x dim^r cross, some strongly growing colonies (4-10 per plate) were recovered in experiments 1,

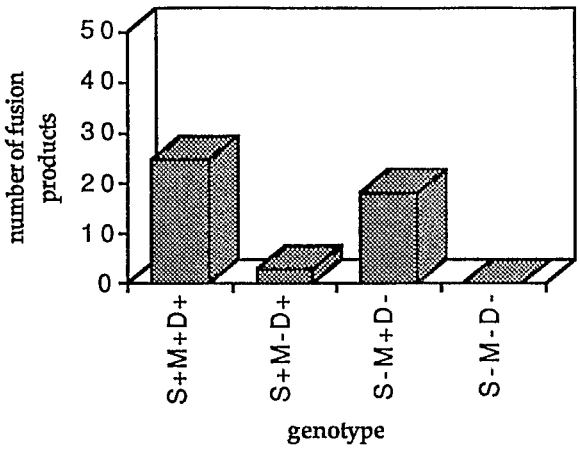

Fig. 1 - Sensitivity of putative hybrids recovered from four crosses to streptomycin (S), metalaxyl (M), dimethomorph (D).

The number of heterokaryons in each experiment is summarized in Table I. The metalaxyl resistant parental phenotype is represented more than the dimethomorph resistant phenotype.

First generation of cloning. The putative recombinant (n° 10) from experiment 4 was cultured under conditions promoting sporangium formation and zoospores were induced. Zoospore production yield from heterokaryons was lower (approximately 1 x 10^3 ml^{-1}) than from the resistant parents (>1 x 10^5 ml^{-1}). Single zoospore progenies were recovered and tested for response to fungicides and streptomycin. Among the 40 progenies screened, 31 possessed the same resistance phenotype as

TABLE I. - The heterokaryon formation for crosses between metalaxyl-resistant isolate and dimethomorph-resistant isolate of *P. parasitica* in each experiment.

N° assay	Heterokaryon formation	Phenotype			
		[S^+, M^+, D^+]	[S^+, M^-, D^+]	[S^-, M^+, D^-]	[S^-, M^-, D^-]
1	13	4	1	8	0
2	9	3	0	6	0
3	14	12	2	0	0
4	10	6	0	4	0
total	46	25	3	18	0

2, 3 and 4. Fortysix putative hybrids from the four experiments were obtained. The heterokaryons often had irregular colony margins and some colonies were sectored.

Resistant phenotype of fusion products. The 46 putative hybrids from experiments 1, 2, 3 and 4 were tested for growth on media supplemented with a range of concentrations of dimethomorph, metalaxyl or streptomycin. Results indicated that the isolates fell into three categories (Figure 1):

Type 1 : 25 were resistant to dimethomorph, metalaxyl and streptomycin antibiotic [S^+, M^+, D^+].
Type 2 : 18 were metalaxyl resistant [S^-, M^+, D^-].
Type 3 : 3 were dimethomorph and streptomycin resistant [S^+, M^-, D^+].

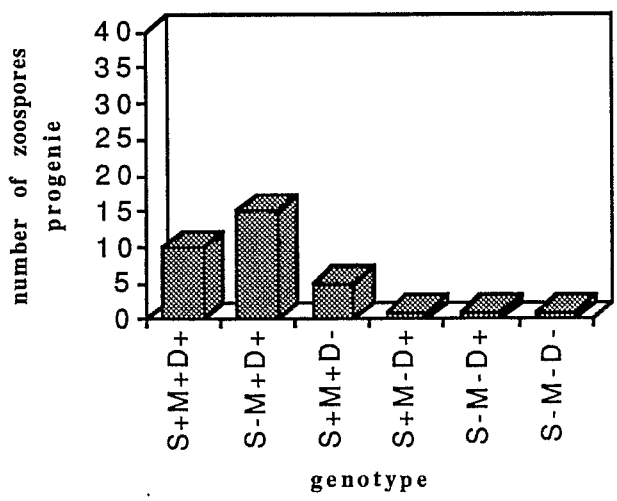

Fig. 2 - First generation of monozoospore cloning derived from the heterokaryon n° 10 [S^+, M^+, D^+].

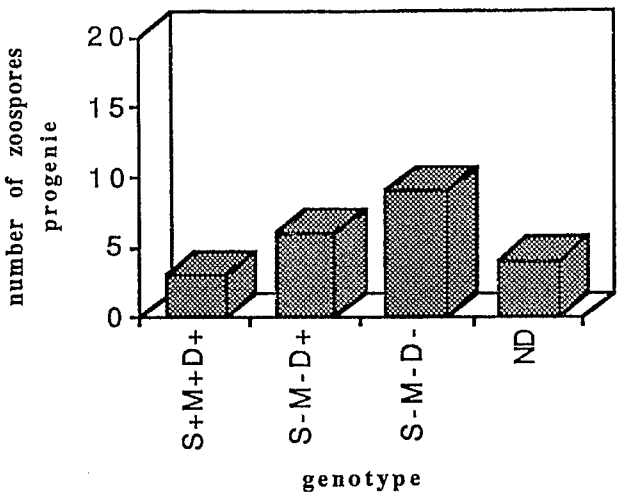

Fig. 3 - Zoospore segregation of the second generation derived from the parental monozoospore strain n° 1 [S+, M+, D+].

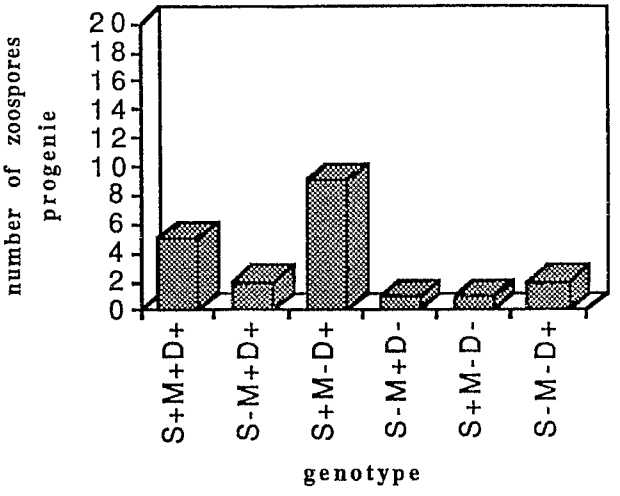

Fig. 4 - Zoospore segregation of the second generation derived from the parental monozoospore strain n° 22 [S+, M+, D+].

the putative hybrids from which they originated, including 9 with an intermediate sensitivity to metalaxyl and 6 to dimethomorph. 12 putative hybrids were resistant to both fungicides and to streptomycin. The results are presented in Fig. 2.

Second generation of cloning. A second generation of monozoospore strains was obtained from the monozoospore strains 1 and 22 [S+, M+, D+] and 5 and 40 [S-, M+, D+]. The segregations obtained are showed in Figures 3, 4, 5, 6. The segregation pattern in this second generation of oospores changed with time. From the parental strain n. 1 (Figure 3), only one recombinant was metalaxyl, dimethomorph and streptomycin resistant (Type 1). 45% of the zoospores progenies were sensitive to fungicides and antibiotic. 30% maintened resistance to dimethomorph. With zoospore hybrid n. 22 (Figure 4), 25% had the [S+, M+, D+] phenotype (Type 1), 37% [S-, M+, D+] but 42%, 11% were respectively [S+, M-, D+] (Type 3) and [S-, M-,

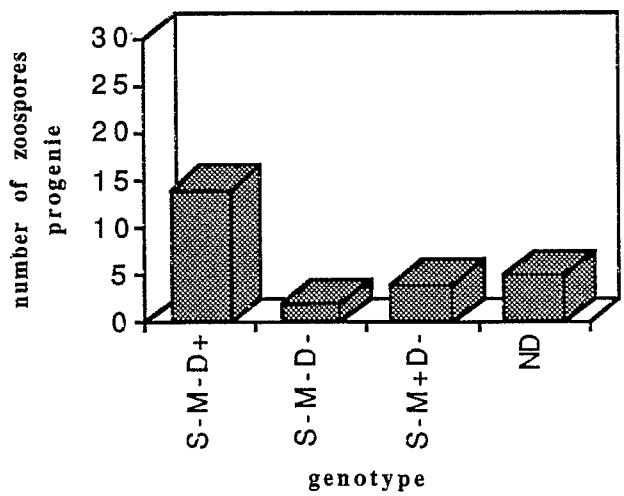

Fig. 5 - Zoospore segregation of the second generation derived from the parental monozoospore strain n° 5 [S-, M+, D+].

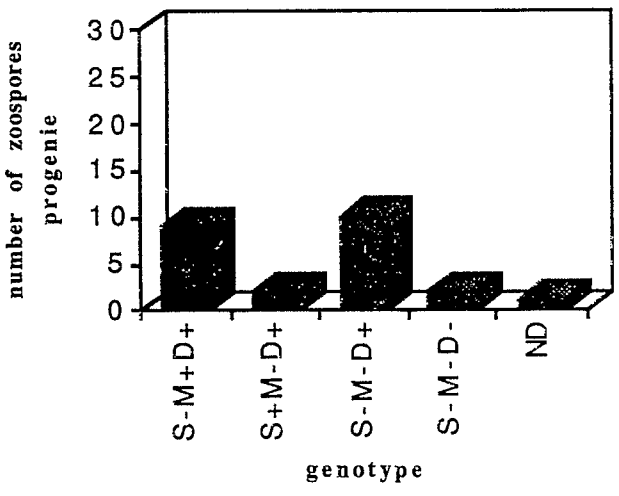

Fig. 6 - Zoospore segregation of the second generation derived from the parental monozoospore strain n° 40 [S-, M+, D+].

D^+]. Only one was sensitive to both fungicides and antibiotic.

From zoospore hybrid n. 5 and n. 40 with the [S^-, M^+, D^+] phenotype, zoospore progenies are presented in Figures 5 and 6. The phenotype of most zoospore progeny differed from that of the parental strain.

Discussion

Many techniques have been described for isolating protoplasts from *Phytophthora* spp. (Bartnicki-Garcia and Lipman, 1976; Pesti and Ferenczy, 1979). Recently, Layton and Kuhn (1988) obtained improved yields of viable protoplasts from germinated zoospores of *P. megasperma* f. sp. *glycinea* using driselase. Novozym 234, has, however, been successfully used to produce viable protoplasts from *P. parasitica* and *P. infestans* (Jahnke *et al.*, 1987; Campbell *et al.*, 1989). Most rapid release was observed with Novozym and the viability of the isolated protoplasts was not reduced as observed in *P. capsici* (Lucas *et al.*, 1990). Novozym 234 at 10 mg ml^{-1} gave good yields of protoplasts from actively-growing hyphae of comparable viability.

We have established a method for protoplast production which provides satisfactory yields of regenerable protoplasts. The development of methods for protoplast production in *Phytophthora* is important, because protoplast formation is the first step in transformation. Protoplasts from drug-resistant mutants were fused and mycelium expressing multiple drug resistance was obtained. Zoospores from these heterokaryotic mycelia were tested for resistance to fungicides and streptomycin in order to determine the nuclear composition of the heterokaryon. Layton and Kuhn (1988) used resistance to the amino acid analogue p-fluorotryptophan (FTP), in combination with metalaxyl resistance, to select for protoplast-derived heterokaryons in *P. megasperma* f. sp. *glycinea*. Lucas *et al.* (1990) used resistance to the same drugs and to phosphorous acid to select fusion products in *P. capsici*.

This is the first time that fusion products have been obtained from mutant resistant to the novel fungicide dimethomorph (Chabane, 1993).

In the present study, putative heterokaryons were recovered following fusions between protoplasts from strains of *P. parasitica* either resistant to metalaxyl P 26 (Met^r) or dimethomorph P 310 (Dim^r) by selection on a medium amended with metalaxyl and dimethomorph.

Hyphal anastomosis (Stephenson, 1974) permitting spontaneous heterokaryon formation is thought to be rare or absent in *Phytophthora* spp. but some authors have isolated recombinant phenotypes from mixtures of strains carrying different genetic markers. Shattock and Shaw (1976) obtained double-drug resistant isolates by mixing zoospores or co-culturing streptomycin and chloramphenicol resistant strains of *P. infestans*. Long and Keen (1977) with *P. megasperma* f. sp. *glycinea*, isolated possible heterokaryons using fluorophenylalanine and cycloheximide resistant strains. In the present study, attempts were made to select heterokaryons from mixed cultures using mycelial plugs. In our experiments resistant heterokaryons to both fungicides and streptomycin have been recovered, the heterokaryons are recognizable because of their irregular morphology. This phenomenon has been reported by Burnett (1975). The origin of the resistant phenotypes to the two drugs was not spontaneous mutation, as it was with *P. megasperma* f. sp. *glycinea* (Layton *et al.*, 1988), because in our preliminary test, the risk is low. Hulbert and Michelmore (1988) have recently provide convincing molecular evidence for natural somatic hybridization in an Oomycete *Bremia lactucae*, based on RFLP analysis. Putative heterokaryons recovered by fusion protoplasts in the presence of PEG and calcium chloride, regenerating overnight in V8-agar, and they were selected by overpouring agar amended with metalaxyl and dimethomorph. Most of the colonies had a slower growth than the parent strains, and displayed combined resistance to metalaxyl and dimethomorph. Among fusion products which had been regenerated on a medium containing both drugs, we recovered parental phenotypes resistant to metalaxyl or to dimetomorph and recombinants resistant to both fungicides. We notice that in the different tests, the parental type metalaxyl-resistant is more represented that the dimethomorph-resistant. Most zoospore colonies derived from f 10 retained the same multiple resistance phenotype, with an RL>100 to metalaxyl. The metalaxyl resistance in both *Phytophthora* (Shattock, 1988) and *Bremia* (Crute, 1987) is governed by a single nuclear locus with incomplete dominance, but our experiment suggests complete dominance. In sexual crosses between metalaxyl-sensitive and resistant isolates of *P. parasitica*, single oospore F1 progeny showed complete dominance to the fungicide (Chang and Ko, 1990). The discrepancy may be due to

difference in isolates used as related by Chang. Streptomycin resistance is intermediate (50%) between that of the dimethomorph-resistant (88%) and the sensitive strain P 26 (0%). This intermediate resistance could be the consequence of parental cytoplasmic mixing. The variability observed in the offspring is explained by the dissociation of the heterokaryotic or heteroplasmic state. In the monozoospore mononuclear offsprings we noticed a disjunction of the resistance characters. This disjunction demonstrates the existence of events of recombination at nuclear level: by mitotic recombination or a chromosome loss. After karyogamy, the ploidy level increases. In the *Phytophthora*, which is usually diploid, we pass go from 2n to 4n, then during the successive division it can be arrive a chromosom loss wich could be accompaned by loss of one or two resistance markers (Talbot *et al.*, 1988). The aneuploid state leads also to viability loss as we observed in our 2nd clonage generation. The same phenomenon was observed in our experiment in the second generation of progeny. The stability of the zoospore colonies for resistance to the two fungicides suggests that karyogamy had taken place following somatic fusion. Effectively, in our experiment about 50% of the zoospore colonies tested, produced double-drug resistance. The selection procedure used might have permitted hybrid selection rather than heterokaryons. Layton and Kuhn (1988), were unable to form heterokaryons between *P. megasperma* f. sp. *glycinea* mutants and a single isolate of *P. megasperma* f. sp. *medicaginis* resistant to metalaxyl.

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