The inheritance of drug resistance and compatibility type in *Phytophthora drechsleri*

By IKRAM A. KHAKI and D. S. SHAW

*School of Plant Biology, University College of North Wales,
Bangor, North Wales*

SUMMARY

Methods have been developed for the selection of mutants resistant to *p*-fluorophenylalanine, chloramphenicol and actidione. Resistance to *p*-fluorophenylalanine and to chloramphenicol was inherited as if determined in each case by a single dominant allele present in diploid somatic nuclei. Inheritance of mating type was anomalous in crosses involving drug-resistant mutants. Presumptive mating-type heterokaryons were generated in a few per cent of sexual progeny from a wild-type cross.

1. INTRODUCTION

The Oomycetes, to which *Phytophthora* belongs, includes pathogenic fungi causing blights, downy mildews, root, stem and fruit rots as well as a large assemblage of saprophytic water moulds. These fungi are oogamous with aseptate hyphae: asexual reproduction is mostly by zoospores which are biflagellate and usually uninucleate.

Cytological evidence of the last decade (Sansome, 1963, 1965, 1966) has strongly indicated that in species of *Pythium*, *Phytophthora* and *Achlya* meiosis occurs in the gametangia and not, as was previously believed, in the zygote. These observations have now been confirmed in members of the Saprolegniaceae, Leptomitaceae, Pythiaceae and Peronosporaceae (Galindo & Zentmyer, 1967; Barksdale, 1968; Howard & Moore, 1970; Huguenin & Boccas, 1970; Flanagan, 1970; Win-Tin, 1972; Sansome & Brasier, 1973). Vegetative hyphae and zoospores of these fungi must then contain diploid or conceivably polyploid nuclei. The DNA content of vegetative and gametangial nuclei of *Saprolegnia terrestris* and *Apodachlya brachynema* has been estimated using a microspectrophotometric method (Bryant & Howard, 1969; Howard & Bryant, 1971). These studies clearly showed that in both organisms gametangial nuclei had a 4C (pre-divisional) or 1C (post-divisional) DNA content and that vegetative nuclei were 2C. Genetical evidence is required to complement the above findings so that an unequivocal definition of the type of life-cycle may be made.

Most previous work on the genetics of these fungi has been carried out with species of *Phytophthora*. The inheritance of naturally occurring characters such as the colour and morphology of the colony, compatibility type and virulence

reaction on a series of host plants with different resistance genes were examined in earlier studies reviewed by Erwin *et al.* (1963) and Gallegly (1968, 1970). Castro, Zentmyer & Belser (1968) followed by Timmer *et al.* (1970) selected stable auxotrophic mutants which they analysed genetically. As these workers have pointed out, interpretation of their results has been aggravated by the low-percentage germination of zygotes (mostly $< 10\%$). Although their data has been used to support the hypothesis that somatic nuclei are haploid they are inconclusive and could equally be taken to indicate that somatic nuclei are diploid. More data on the inheritance of stable nuclear markers are obviously required to define the level of ploidy.

Since some single gene mutations to drug resistance are known to be dominant or semi-dominant in other fungi (e.g. *Aspergillus nidulans* Warr & Roper, 1965) we presumed that a search for drug resistance might be rewarding in a fungus which, on cytological evidence, is diploid. In a preliminary study (Shaw & Elliott, 1968), stable streptomycin resistance and dependence in the homothallic species *P. cactorum* occurred spontaneously at a high frequency ($c.\ 10^{-4}$). Progeny did not segregate when mutants were selfed. Timmer *et al.* (1970) also found that mutation to streptomycin resistance occurred spontaneously at a high frequency in *P. capsici*. Although resistant and sensitive progeny were recovered from crosses of wild-type and resistant mutants, somatic segregation also took place during vegetative growth of resistant progeny. Certain mutations to streptomycin resistance and dependence in *Chlamydomonas reinhardi* occurred at high frequency and were inherited extra-chromasomally (Sagar, 1954). These characters may also be determined by extra-chromosomal factors in *Phytophthora*.

Isolates of heterothallic species of *Phytophthora* are known to be bisexual but self-sterile (e.g. Ashby, 1922; Gadd, 1924; Galindo & Gallegly, 1960). They can be assigned to either of two compatibility types, A^1 or A^2, on the basis of their mating reaction with tester isolates of *P. infestans* (Savage *et al.* 1968). In *P. infestans*, Galindo & Gallegly (1960) have shown that while most isolates produce functional antheridia and oogonia, so that matings of A^1 with A^2 allow reciprocal crossing, some isolates function more as males and others more as females. The number of antheridia and oogonia contributed by each parent in a cross therefore depends upon their relative sexualities.

We report below on the inheritance of drug resistance in *Phytophthora drechsleri* and provide further data on the inheritance of compatibility types. A preliminary short paper on some of these findings has already been published (Shaw & Khaki, 1971).

2. MATERIALS AND METHODS

Compatible isolates 6500 and 6503 of *P. drechsleri* from Mexican pepper (*Capsicum* spp.) and mutants derived from these have been used throughout this study. Vegetative mycelium was grown on a standard medium agar (SMA) or in a standard medium broth (SMB) based on sucrose and asparagine (Shaw & Elliott, 1968). When colony counts were required, zoospores were plated on SMA

containing 0.5% L-sorbose which had no effect on viability but allowed each zoospore to develop into a small, compact and distinct colony. Cultures were incubated at 30 °C unless otherwise stated.

Asexual reproduction was induced by growing a vegetative mycelium on pea-meal agar and transferring the mycelial mat to H_2O agar (Shaw & Elliott, 1968). When large numbers of zoospores were required for mutant selection, the following procedure was used. A thick mycelial mat grown on the surface of pea broth (300 g frozen garden peas blended in 1 l H_2O) was washed free of nutrients in distilled H_2O and fragmented coursely in a Waring blender. Large penicillin flasks ($23 \times 19 \times 6.5$ cm) of H_2O agar (1% Oxoid No. 3 agar) were inoculated with hyphal fragments and incubated at 24 °C for 4 days, after which zoosporangia had formed abundantly. Zoospores were released by flooding cultures with distilled H_2O, chilling at 15 °C for 1 h and incubating at 22 °C for a further $\frac{1}{2}$ h. These large cultures each yielded $c.$ 10^8 zoospores.

Matings were made by placing hyphal inocula of two compatible isolates 1 cm apart on plates of SMA containing 1% oat extract (Shaw & Elliott, 1968) and incubating at room temperature (18–22 °C) with alternating diffuse daylight and darkness (Galindo & Zentmyer, 1967). Oospores formed along a line where the parental mycelia had come into contact. A sample of mature oospores from cultures 14–21 days old was cut out in a block of agar, blended in distilled water firstly in an MSE homogenizer fitted with a 10 ml vortex beaker then in a glass tissue grinder (Quickfit BC 15/150) which destroyed most hyphal fragments. The oospore suspension was washed four times in distilled H_2O, filtered through coarse filter paper to remove remaining hyphae and plated out on H_2O agar at a density of $c.$ 100 per plate. After incubation for 4 days at room temperature with alternating diffuse daylight and darkness, single oospores, some of which had already produced a germ-tube, were transferred on blocks of agar to single plates of SMA and incubated for a further period of 7 days. The percentage of oospores able to germinate and form colonies was then determined. It should be noted that the blended oospore suspension from most crosses contained low frequencies ($< 5\%$) of damaged, empty or obviously inviable oospores which were not transferred to SMA and therefore were not included in percentage germination data. Germination calculated on this basis and using this method was often as high as 100% (see Table 3).

Stocks of all cultures were kept on slopes of oatmeal agar (30 g oatmeal/l distilled H_2O) and were subcultured every 3 months. Drug-resistant mutants were maintained on oatmeal agar supplemented with concentrations of drug completely inhibitory to wild-type.

3. RESULTS AND DISCUSSION

(i) *The sensitivity of 6500 and 6503 to a selection of drugs*

A range of concentrations of commonly used microbial inhibitors were added to SMA and to SMB after autoclaving. The efficiency of each drug in inhibiting linear extension of hyphae from hyphal inocula on plates of SMA and inhibiting

colony establishment from zoospores on plates of SMA and in flasks of SMB was assessed. The sensitivity of both 6500 and 6503 to the range of drugs was very similar. A summary of the results for colony establishment of 6503 is given in Table 1. Actidione, *p*-fluorophenylalanine (FPA), chloramphenicol and tetracyclin efficiently and consistently inhibited zoospores germination and vegetative growth in all tests at relatively low concentrations. Mutations to resistance to each of these four drugs is known to arise by single gene mutation in other micro-organisms (Wilkie & Lee, 1965; Reeve, 1968; Reeve & Suttie, 1968; Sinha, 1967). These drugs were therefore used in the experiments which follow to select for resistance.

Table 1. *Effect of various drugs on colony establishment of 6503 from zoospores in liquid medium*

Drug	Minimum concentration suppressing colony establishment (μg/ml)
Actidione	10
Chloramphenicol	100
p-Fluorophenylalanine	100
Tetracyclin	100
Iodoacetic acid	100
Acriflavin	> 100
Emitine	> 100
Erythromycin	> 100
Sulphanilamide	> 100
Streptomycin	300

(ii) *The sensitivity of 6503 to mutagenic agents*

Zoospore suspensions, 10^6/ml distilled H_2O, were irradiated with ultraviolet light (UV) from a Hanovia lamp (serial no. S/f11/F1102) for 1–350 sec at a distance of 50 cm, and were plated on SMA with L-sorbose in the dark. Colony-forming ability was scored over a period of 8 days because of the pronounced asynchrony of the developing sporlings following irradiation. The plateau in the middle of the kill curve, shown in Fig. 1, is thought to be due to a small proportion (c. 1 %) of binucleate zoospores which were more resistant to killing but eventually showed approximately the same rate of exponential decay as the uninucleate spores. A dose of 5 min of UV gave an inactivation of 99 % of zoospores, and was found to induce mutations to FPA resistance and possibly to tetracyclin resistance (see Table 2).

The sensitivity of zoospores to inactivation by ethylmethane sulphonate (EMS) was examined. In preliminary experiments zoospores were suspended in 0·1 M citrate or 0·1 M phosphate buffer solutions before treatment but the spores were rapidly killed and the use of buffers was discontinued. Zoospores (10^6/ml distilled H_2O) were shaken with various concentrations of EMS for 15 min, washed three times in distilled water and plated on SMA with L-sorbose. Their ability to form colonies was recorded after 72 h and was compared with a control. A treatment

using $0.3\,\mu$g/ml EMS for 15 min gave approximately 50 % survival and was used in screening experiments in which mutants resistant to actidione and FPA were recovered (see Table 2).
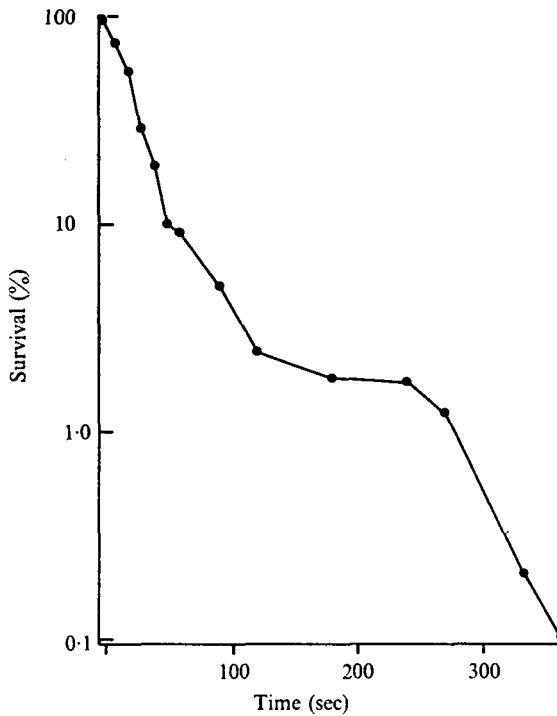

Fig. 1. Inhibition of colony-forming ability of zoospores of 6503 by ultra-violet light.

Table 2. *Numbers of colonies recovered from drugged medium*
per 10^{10} zoospores of 6503

Treatment	Actidione	FPA	Resistant to chloramphenicol	Tetracyclin
UV	0	52	0	6
EMS	29	34	0	0
NTG	0	0	6	0
None	0	3	0	0

Finally the action of N-methyl-N-nitroso-N^1-nitroguanidine (NTG) on zoospore suspensions was examined. NTG was added to zoospore suspensions (10^6/ml distilled H_2O) at various concentrations. After 10 min, spores were washed three times in distilled H_2O and plated on SMA with L-sorbose. Colony establishment was recorded after incubation for 72 h. Mutants resistant to chloramphenicol were isolated from treatments of zoospores with $0.1\,\mu$g/ml NTG for 10 min, which gave approximately 75 % survival (Table 2).

6

(iii) *The selection of drug-resistant mutants*

One mutant (FA 4) was isolated from a fast-growing sector of 6500 on a plate containing 100 μg/ml FPA. All other mutants were derived from single zoospores of 6503. In a series of experiments to select resistant mutants, populations of zoospores, usually treated with a mutagen, were incubated in SMB at a concentration of 10^5 spores/ml for 12 h at 22 °C to allow the spores to germinate and express any resistant phenotype. Inhibitory doses of drug were then added and incubation was continued for 7 days. Any colonies developing during this period were rescued and transferred to plates of SMA containing the same concentration of the drug. Table 2 shows the number of drug-resistant isolates rescued from a series of screening experiments. Single zoospore isolates of presumptive mutants were tested for increased resistance, for the stability of their resistance following culturing on drug-free SMA, for their retention of reproductive capacity and for cross-resistance. Several suitable mutants resistant to FPA or chloramphenicol, but not cross-resistant, were chosen for genetic analysis. Isolates recovered from SMB containing tetracyclin were discarded because of their loss of resistance on transfer to solid medium. Isolates resistant to 10 μg/ml actidione were stored for analysis at a later date.

(iv) *The inheritance of resistance to* p-*fluorophenylalanine and chloramphenicol*

Resistance was assayed by measuring linear growth from hyphal inocula on SMA containing different concentrations of the drug and comparing with a control. Wild-types 6500 and 6503 were completely inhibited by concentrations of either drug in excess of 100 μg/ml whereas FPA- and chloramphenicol-resistant mutants were indifferent to concentrations of 100 μg/ml FPA and chloramphenicol respectively. Two mutants resistant to FPA (FA 1 and FA 4) and three resistant to chloramphenicol (C 1, C 2 and C 3) were mated to 6500 or 6503 and the progenies tested for resistance.

Progeny from matings involving FA 1, C 1, C 2 and C 3 were resistant to 100 μg/ml of the respective drug. Although progeny from the cross involving FA 4 were all inhibited by 100 μg/ml they were fully resistant to 30 μg/ml. These were said to have intermediate resistance (Table 3). An F_1 of a suitable mating type from each cross was then backcrossed to its resistant parent and another F_1 was backcrossed to the sensitive wild-type parent. Two F_1's were also intercrossed. Two F_2 progeny of the C 1 × 6500 mating were further backcrossed to 6500. Table 3 shows the frequencies of the different phenotypes in progenies of these crosses and includes an estimate of the viability of the oospores from each cross. Oospores from parental matings tended to have a high viability whereas those from backcrosses and F_2's tended to give a lower percentage germination. Backcross and F_2 progeny of FA 1, C 1 and C 2 showed a similar pattern of resistant/sensitive phenotypes. Backcrosses to the resistant parent yielded wholly resistant progeny whereas backcrosses to the sensitive parent segregated to yield wholly resistant progeny and progeny of wild-type sensitivity in a ratio which in

Table 3. *Crosses between resistant mutants, sensitive wild-types and their progeny*

Mutant	Derived from	Resistant to (μg/ml)	Origin	Mating	Progeny R:S or R:I:S*	Expected ratio if diploid	Compatibility type of progeny				Germination of oospores %
							A^1	A^2	Neuter	A^1A^2	
FA 1	6503	100 FPA	Spontaneous (zoospore)	× 6500	125:0	1:0	5	52	1	4	100
				F_1 × 6500	68:57	1:1					100
				F_1 × FA 1	65:0	1:0					100
				F_1 × F_1	35:11	3:1					—
FA 4	6500	100 FPA	Spontaneous (sector)	× 6503	0:57:0	1:0	0	48	9	0	100
				F_1 × 6503	0:14:20	1:1					90
				F_1 × FA 4	0:37:11	3:1					100
				F_1 × F_1	0:38:26	9:7					100
C 1	6503	100 chloramphenicol	NTG	× 6500	66:0	1:0	14	2	0	0	51
				F_1 × 6500	60:48	1:1					76
				F_1 × C 1	59:0	1:0					47
				F_1 × F_1	51:17	3:1					58
				F_2(1) × 6500	25:19	1:1					44
				F_2(2) × 6500	18:25	1:1					41
C 2	6503	100 chloramphenicol	NTG	× 6500	71:0	1:0	72	2	4	0	97
				F_1 × 6500	43:32	1:1					75
				F_1 × C 2	60:0	1:0					60
				F_1 × F_1	40:13	3:1					53
C 3	6503	100 chloramphenicol	NTG	× 6500	57:0	1:0	31	0	3	0	57
				F_1 × 6500	—	—					—
				F_1 × C 3	32:0	1:0					50

* R = resistant, S = sensitive, I = intermediate resistance.

6-2

each case was not significantly different from 1:1. Segregation also occurred in the F_2 progeny, resistant:sensitive phenotypes having a good fit to a 3:1 ratio. Both backcrosses of the F_2 isolates from the C 1 matings segregated to give resistant and sensitive progeny, the proportions of which did not deviate significantly from 1:1. Matings with C 3 progeny could not be completed due to the absence of A² mating types from the F_1; however, the phenotypes in the progenies which were analysed were identical to those in similar matings of C 1 and C 2.

Clearly the pattern of inheritance of drug resistance described here is difficult to account for in a haploid organism but is typical of that expected in a monohybrid cross of a diploid organism. We believe that resistance to FPA in FA 1 and to chloramphenicol in C 1 and C 2 is determined by single dominant alleles, that F_1's were heterozygous and thus segregated in test-cross and F_2, and that resistant parents were homozygous. The mutant strains could have become homozygous most easily if the resistance allele were semi-dominant so that any homozygous nuclei arising from the original heterozygous nuclei would be selected. Homozygous nuclei could have been generated during storage of stock cultures, by further spontaneous mutation or by a process similar to the mitotic crossing-over which occurs in diploid lines of higher fungi. The tendency for oospore viability to decline with inbreeding is also an expected result in an outbreeding diploid organism but not in a haploid organism.

The results of matings with the spontaneous mutant FA 4 do not conform to the above pattern (Table 3). The absence of fully resistant progeny from the backcross to the resistant parent and in the F_2 exclude explanations based on polygenic control of resistance unless this is due to a cytoplasmic suppressor, in which case resistance could be determined by dominant alleles at two loci giving 1:1 and 3:1 ratios in backcrosses and a 9:7 ratio in the F_2.

The above genetical data together with those recently produced on the inheritance of a quantitative character in *P. syringae* (Boccas, 1972) and on the inheritance of methionine requirement in *P. cactorum* (Elliott & MacIntyre, 1973) are fully consistent with the cytological and microchemical data. This leads us to support the conclusion of Sansome (1963) that the Oomycetes have a life-cycle in which meiosis is followed immediately by karyogamy and therefore have diploid somatic nuclei.

(v) *The inheritance of compatibility type*

The inheritance of mating type in *Phytophthora* spp. has been reviewed by Gallegly (1970). Wild-type crosses have yielded a variety of proportions of A¹:A² in the F_1 ranging from equal proportions, through a predominance of A¹, to F_1's of entirely A¹ phenotype. Timmer *et al.* (1970) also found a variable predominance of A¹ in progenies of matings of auxotrophic mutants no matter whether the mutant parent was of A¹ or A² compatibility type.

In our crosses we have scored the compatibility types of F_1 progeny (Table 3) and have found that A¹ phenotypes tended to predominate in the wild-type mating. In crosses with four mutants of compatibility type A² (FA 1, C 1, C 2 and C 3) the low proportion of A² phenotypes was most marked. An unexpected

result was the absence of A^1 compatibility types in progeny of the cross of FA 4, the only mutant derived from A^1 wild-type 6500. The high percentages of germination recorded rule out the possibility that the infrequent class of oospore had a low viability. Isolates not able to form oospores with either wild-type parent were present at low frequencies in some progenies and were classified as neuter phenotypes. Matings between FA 4 and C 1, C 2 or C 3, expected to be fertile, were in fact sterile. This last result indicates that these drug-resistant mutants had a defective mating reaction which could be complemented by the wild-type and offers an interpretation of the predominance of the compatibility type of the wild-type parent in the progenies. If oogonial development were blocked in the mutant strains they would function mainly as males in crosses of wild-type, they would be sterile on intercrossing, and progeny would receive maternally inherited characters from the wild-type parent. The regular Mendelian behaviour of the progenies with respect to drug resistance appears to rule out the possibility that the predominance of one compatibility type is due to selfing of one of the parents. Selfing has been shown to occur when A^2 cultures of several *Phytophthora* spp. including *P. drechsleri* are stimulated by *Trichoderma viride* (Brasier, 1971) and has been invoked to explain unexpected ratios in progeny of crosses with *P. drechsleri* (Sansome, 1970). However, a full pedigree analysis is required before any conclusions on compatibility type determination can be reached. Reciprocal matings should be attempted with marked strains to identify any possible cytoplasmic control of compatibility type.

The wild-type mating yielded a few per cent of A^1A^2 phenotypes (Table 4) which were sexually compatible with both A^1 and A^2 parents. We think it probable that A^1A^2 cultures are compatibility type heterokaryons for the following reasons: mycelial plug inocula from single oospore colonies formed colonies on oat-extract agar which were self-fertile; these colonies gave rise to sterile sectors of A^1 or A^2 phenotypes during vegetative growth; single zoospore isolates of A^1A^2 cultures were either A^1 or A^2 phenotypes; single hyphal tips, although giving mainly A^1 and A^2 segregants, rarely yielded the A^1A^2 phenotype. A heterokaryon test (Jinks, 1954) could distinguish between a heterokaryon and a heteroplasmic condition and thus between nuclear and cytoplasmic control of mating type.

Niederhauser (1961), Laviola (1969) and Castro & Zentmyer (1969) have all obtained self-fertile cultures from single oospores formed in crosses of wild-type *Phytophthora* spp. In each case cultures appeared to be stable monokaryons as single zoospores propagated the self-fertile phenotype. Neiderhauser, however, pointed out that some isolates yielded high frequencies of bi- and multi-nucleate zoospores which could have propagated a heterokaryon. In *P. infestans* Laviola (1968) has shown that occasionally the germ sporangium from a single germinated oospore may contain zoospores giving rise to A^1 and zoospores giving rise to A^2 phenotypes. Such oospores if grown into colonies would probably have produced A^1A^2 phenotypes like those we describe. B. Boccas (personal communication) has recently found that zoospores from A^1A^2 phenotypes of *P. palmivora* segregate to give A^1 and A^2 phenotypes. Oospores giving rise to two phenotypes could have

resulted from the dual fertilization of a binucleate oogonium. Rare oogonia containing two oospheres, and restitution nuclei indicating failure of both meiotic divisions, have recently been observed in the *P. infestans* material used by Laviola (1968), and invoked as an explanation of his genetical results (Sansome & Brasier, 1973). Compatibility-type heterokaryons are well known in *Phycomyces blakesleeanus* (Blakeslee, 1906) and in *Neurospora sitophila*, where secondary homothallism is due to the formation of ascospores heterokaryotic for compatibility type (Sansome, 1946).

Table 4. *Compatibility type of progeny of a wild-type cross*

Parents	Germination of oospores (%)	Progeny			
		A^1	A^2	Neuter	A^1A^2
6500×6503	70	67	41	0	4

It is not yet clear to what extent heterokaryons of diploid nuclei and heteroplasmons are involved in the variation observed in natural isolates. Fusions between vegetative hyphae have been reported (Leach & Rich, 1969) but other workers have failed to confirm this. Attempts are now being made to synthesize heterokaryons using marked strains, to assess their stability and to perform heterokaryon tests to define the relative roles of cytoplasmic and nuclear factors in the determination of natural variation.

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