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Selection for growth-rate during asexual and sexual propagation in *Phytophthora cactorum*

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SUMMARY

Selection for high and low growth-rate was carried out during eight generations of asexual propagation by zoospores and seven generations of sexual reproduction by oospores. The fungus has previously been shown to be diploid during its vegetative phase. In the zoospore lines there was no significant variation and no response to selection, except for the occasional appearance of fast-growing sectors. A high line was established from such a sector; in its sexual progeny the inheritance of growth-rate was non-Mendelian. Propagation through self-fertilized oospores released very considerable genetic variation, and both high and low lines responded to selection. At first the variation within families, and the response to selection, increased with succeeding generations, despite the intense inbreeding. In later generations the high line became less variable, and the progeny oospore cultures resembled the fast-growing sectors. It is concluded that growth-rate is controlled by a polygenic system and by cytoplasmic determinants, a mutant form of which is responsible for the fast-sectoring phenotype.

1. INTRODUCTION

Despite their importance as plant pathogens, we know little about the genetics of any species of Phytophthora. In a previous paper (Elliott & MacIntyre, 1973) we demonstrated the suitability of *Phytophthora cactorum* for genetical work. This species is homothallic, and can be readily propagated asexually by zoospores and sexually by oospores, both of which are generally uninucleate. We provided evidence in support of the view (Sansome, 1961, 1965; Sansome & Brasier, 1973) that the vegetative hyphae have diploid nuclei, with meiosis occurring in the gametangia immediately prior to nuclear fusion, by showing that treatment of asexual spores with a mutagen could result in heterozygosis for recessive mutations which segregated in Mendelian fashion in selfed progenies. In the present paper we compare the variation released by a single wild-type strain of *P. cactorum* during selection for high and low growth-rate over eight asexual generations (propagation by zoospores) and seven sexual generations (propagation by oospores produced by self-fertilization).

2. METHODS

(i) *Strain*

The strain used (P94) was obtained from Dr G. W. F. Sewell, East Malling Research Station, who had isolated it from apple rootstock 18 months prior to the commencement of the experiments.

(ii) *Media*

(*a*) *Minimal medium*. Sucrose, 10·0 g; L-asparagine, 1·0 g; K_2HPO_4, 0·5 g; $MgSO_4.7H_2O$, 0·25 g; calcium glycerophosphate, 0·25 g; NaCl, 0·12 g; thiamine hydrochloride, 1·0 mg; trace element solution, 1·0 ml; water, 1 l., solidified with 1% Oxoid agar no. 3. The trace-element solution contained $Na_2B_4O_7.10H_2O$, 88 mg; $CuSO_4.5H_2O$, 393 mg; $Fe_2(SO_4)_3.9H_2O$, 910 mg; $MnCl_2.4H_2O$, 72 mg; $Na_2MoO_4.2H_2O$, 50 mg; $ZnSO_4.7H_2O$, 4403 mg; ethylenediamine tetra acetic acid, disodium salt, 5 g; water 1 l.

(*b*) *Pea extract medium* (*PM*). Equal volumes of double-strength minimal medium and of pea extract, the latter made by bringing 300 g frozen garden peas to the boil in 1 l. water and filtering through muslin; solidified with 1% agar.

(*c*) *Pea meal agar*. 300 g frozen garden peas blended with 1 l. water; 1% agar.

(*d*) *Medium for producing oospores* was PM to which had been added 1% (v/v) oat extract, made by extracting oatmeal with light petroleum in a Soxhlet apparatus and removing the petroleum under vacuum.

(iii) *Zoospore progenies*

Zoosporangia were produced by cutting strips (5 × 0·5 cm) from the mycelial felt of 4-day-old cultures on pea meal agar, placing three such strips in 9 cm Petri dishes containing 1% agar (Oxoid agar No. 3), and incubating them in the dark at 25 °C for 3 days. Zoospores were released from the sporangia formed on these strip cultures by flooding with 5·0 ml distilled water at 15 °C and incubating at 15 °C for 30 min; 2·5 ml of 6% sucrose solution at 15 °C was then added to each culture, and after 15 min a further 2·5 ml 6% sucrose was added. The cultures were removed to room temperature and the zoospores drawn off and diluted as necessary in 3% sucrose (Shaw & Elliott, 1968). Zoospore progenies were obtained by plating the suspensions on pea extract medium.

(iv) *Oospore progenies*

Oospores were obtained from 9 cm Petri dish-cultures after 3 weeks' incubation at 25 °C in the dark. One to three such cultures were blended with *c.* 100 ml water in an Atomix (Measuring and Scientific Equipment, Ltd.) for 10 min at half speed. The suspension was centrifuged at *c.* 2000 *g* for 10 min, and the spore pellet washed by resuspension in water and centrifugation five times. The washed spores were plated on 1% agar. Individual oospores were picked up with a needle and transferred to fresh Petri dishes of 1% agar, and incubated at room temperature

under continuous light from a bank of 'Gro-Lux' fluorescent tubes 20 cm above the dishes. After 10 days germinated spores were transferred to pea extract medium (Elliott & MacIntyre, 1973).

(v) *Measurement of growth*

Linear growth-rate was measured at 25 °C in 9 cm Petri dishes containing 16 ml minimal medium, which were inoculated centrally with a plug 4 mm in diameter cut from a culture on pea extract medium. The greatest and least diameters of all the colonies of a batch were measured when the fastest growing culture was some 50 mm across, and the mean radial increase per day for each was calculated.

(vi) *Selection procedure*

The individuals chosen to be parents of each succeeding generation were the fastest or slowest of a random sample of 20 single spore cultures, zoospores or oospores, as the case may be, whose growth-rate was measured in duplicate. Each generation through zoospores occupied about 3 weeks and through oospores about 7 weeks, and so these lines were maintained independently. However, each generation of the fast and slow zoospore lines were grown concurrently, the 40 replicated cultures being randomized together during incubation for the measurement of growth-rate; the oospore lines were treated similarly. The first generation of zoospores is denoted Z1 and the successive generations ZH2, ZH3...ZH8 and ZL2, ZL3...ZL8 for the high and low lines respectively. The first generation of oospores was called O1 and successive generations OH2–OH7 and OL2–OL7.

3. RESULTS

(i) *Selection for growth-rate with zoospore propagation*

The results of eight generations of high and low selection for growth-rate in zoospore progenies are illustrated in Fig. 1.

During the growth-rate determinations, it was observed that a small number of colonies formed sectors with increased growth-rate. Such colonies belonged to families ZH2, ZL3, ZL4 and ZL8. Inoculum from a fast-growing sector in one of the replicates of family ZH2 was selected to produce zoospores for the next generation of the high line. Subsequently the high line maintained a growth-rate approximately twice as fast as that of the low line and did not undergo any further change. This 'fast variant' differed from wild type in a number of characters besides growth-rate. It did not form sectors, and was more fertile. (Oospore counts were performed on the wild type and the fast variant as described by Elliott (1968), and counts significantly higher at the 5% level were obtained for the fast variant.) There was considerable environmental variation between generations, but as the high and low families of each generation were measured together, we can measure the response to selection by the difference between the high and low family means (Falconer, 1960, pp. 198–200). There was no significant change

in the difference between the means for generations 3, 4, 5, 7 and 8 (Fig. 4). Wild-type growth-rate was retained by members of the low line over the eight generations, and apart from the establishment of the fast variant there was no detectable response to selection.

Fig.1. Frequency distribution of growth-rate during eight generations of selection for high and low growth-rate during propagation by zoospores. Each unit area of the histograms represents the mean growth-rate of two replicate measurements. The individuals selected as parents for the succeeding generation are unblocked.

In an analysis of variance of growth-rate for each family, the mean square between zoospores was not significantly greater than that for replicates in most families. This agrees with the apparent lack of response to selection in generations following the establishment of the fast variant. Variation between individuals of the first generation was significant at the 0·1% level. Boccas (1972), with *Phytophthora syringae*, also found greater variation in the first generation of zoospore propagation than in the second, and interpreted his results in terms of

segregation of the components of a heterokaryon. In the present instance the difference between the fastest- and slowest-growing zoospores was not inherited (Fig. 1). Highly significant variation was again detected in family ZL6 which contained zoospores of two types, some being similar to the wild type while others were similar to the fast variant. The faster-growing colonies may have resulted from the segregation of determinants at zoospore formation or may have been due to the formation of a fast-growing sector during the preparation of the strip cultures. Little importance is attached to the other instances in which variation was detected (ZH4 (significant at the 1% level) and ZH6 (5% level)), and it is concluded that zoospore propagation does not typically release variation except for the occasional production of sectors with increased growth-rate.

(ii) *Genetic analysis of the fast variant*

Evidence concerning the genetical change involved in the production of the fast variant was sought by observing its pattern of inheritance upon selfing. Twenty single oospore cultures (S_1) were derived from the fastest-growing colony of family ZH6 and, for comparison, the slowest colony of family ZL6. A further generation (S_2) was obtained by selfing 5 of the 20 single oospore cultures of ZH6-S_1. A single growth-rate measurement was obtained from each oospore culture, and these values are shown in Fig. 2. It is seen that the S_1 oospore family derived from the zoospore of family ZL6 was composed of individuals having growth-rates similar to that of the wild type, while the S_1 offspring of the fast variant of ZH6 segregated 18:2 for fast vs. slow (wild-type) growth-rate. This approximates to the 3:1 ratio expected if the fast variant was heterozygous for a dominant mutant allele. However three of these fast-growing S_1 individuals produced, on selfing, uniformly fast S_2 progenies, and the two slow-growing S_1 individuals gave S_2s which comprised mainly fast growers but also some slow growers, and these slow growers frequently sectored for increased growth-rate and after several mass hyphal transfers became indistinguishable from the fast variant.

Clearly this pattern of inheritance does not admit an explanation of the sectoring based on mutation, or any other simple theory implicating nuclear genes, e.g. mitotic crossing over with the wild-type heterozygous for a recessive gene for fast growth. It would be possible for the sectors to have resulted from non-disjunction in aneuploid nuclei, analogous to that in *Aspergillus nidulans* (Käfer, 1960; Nga & Roper, 1969), but this would require the original isolate to have been aneuploid, and the frequency of sectoring of wild type was far lower than has been observed in aneuploids. Alternatively, the fast variant may be under the control of cytoplasmic determinants. The wild type could have been heteroplasmic at the onset, or become so during the early stages of the selection experiment through the spontaneous mutation of cytoplasmic determinants. Segregation of mutant determinants at hyphal tip formation could account for the occasional production of fast-growing sectors in wild-type colonies.

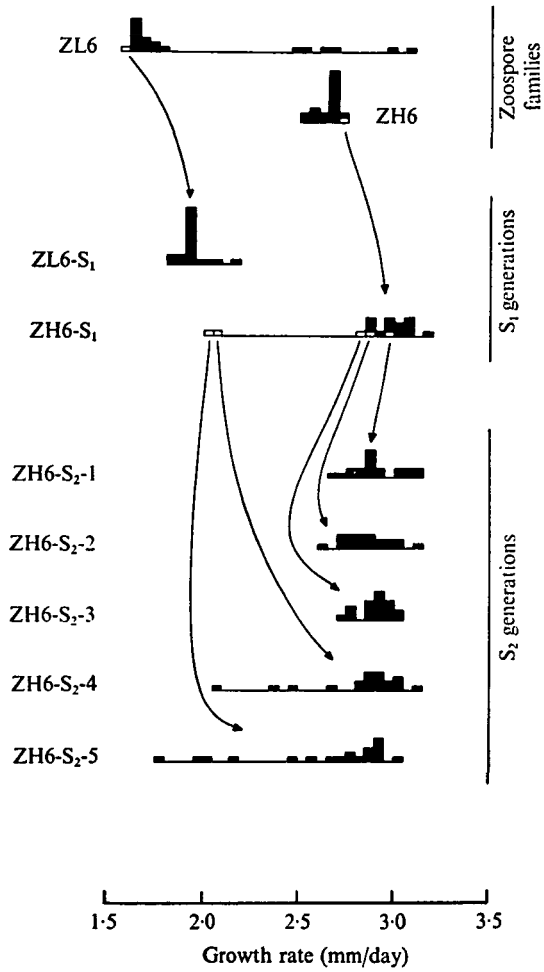

Fig. 2. Frequency distribution of growth-rate in families of oospores produced during two generations of sexual reproduction by selfing the fastest-growing member of zoospore family ZH6 and during one generation of selfing of the slowest-growing member of zoospore family ZL6.

(iii) *Selection for growth-rate in oospore progenies*

The results of seven generations of selection for fast and slow growth-rate in oospores progenies are shown in Fig. 3. Selection was for the extreme phenotypes except for families OL5 and OL6 from which the second and third slowest individuals respectively were selected: the more extreme members of these families produced no oospores on PM + oat extract.

Comparing Fig. 1 for zoospore progenies with Fig. 3 for oospore progenies, it is clear that oospore propagation released far greater variation for growth-rate than did zoospore propagation. The analysis of variance showed that the variation between oospores was significant at the 0.1% level in every instance.

The first generation of zoospores indicated that the original isolate, P94, may

have been heterokaryotic for differences in growth-rate. If so, the first generation of oospores could similarly have been influenced by the segregation of the components of the heterokaryon. However, this source of variation would not be in operation for subsequent generations, as the oospores of *P. cactorum* are uninucleate (Blackwell, 1943).

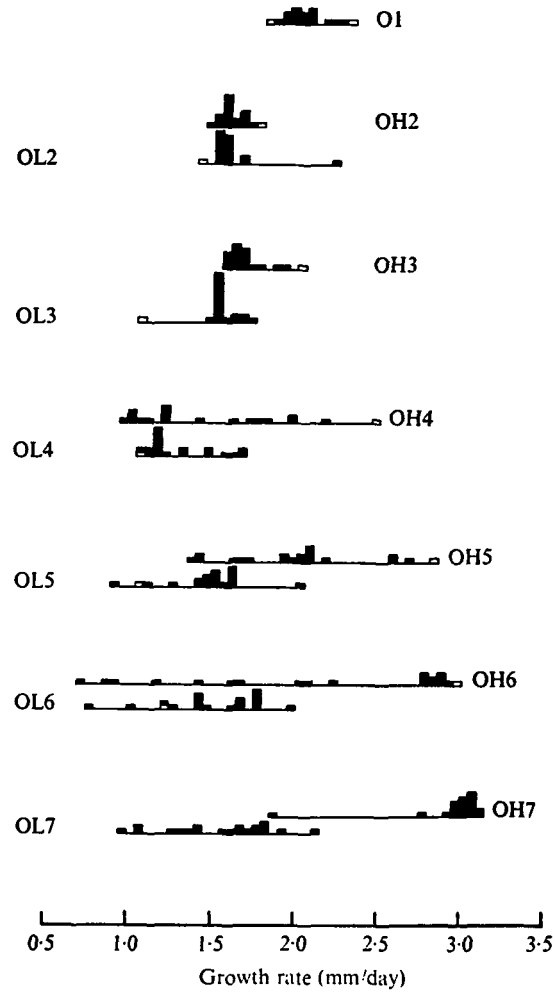

Fig. 3. Frequency distribution of growth-rate during seven generations of selection for high and low growth-rate during propagation by oospores.

Table 1 gives the estimates of variance between oospore cultures ($\hat{\sigma}_b^2$) and error ($\hat{\sigma}_e^2$) for the high and low selection lines for each generation of selection. For the low line, $\hat{\sigma}_b^2$ gradually increased over the seven generations of selection while $\hat{\sigma}_e^2$ remained relatively constant throughout; $\hat{\sigma}_b^2$ for the high line gradually increased up to generation six, but for the seventh a low value was obtained. Similarly $\hat{\sigma}_e^2$ for the high line reached a peak midway through the course of the experiment. The increase in $\hat{\sigma}_b^2$ accompanying selection in both high and low lines is unexpected;

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Table 1. *Estimates of error variance* ($\hat{\sigma}_e^2$) *and the variance between oospores* ($\hat{\sigma}_b^2$) *for growth-rate in each generation of the selection lines, and the response to selection*

Generation	$\hat{\sigma}_e^2$		$\hat{\sigma}_b^2$	
1	0·0072		0·0336	
	Low	High	Low	High
2	0·0046	0·0016	0·0581	0·0098
3	0·0002	0·0018	0·0286	0·0211
4	0·0044	0·0107	0·0776	0·4072
5	0·0009	0·0515	0·1105	0·3120
6	0·0042	0·0175	0·1746	1·2303
7	0·0124	0·0075	0·2950	0·1537

The estimates are derived from the analysis of variance of growth-rate for each generation. The mean squares have the following expectations: between oospores, $\sigma_e^2 + 2\sigma_b^2$; replicate cultures, σ_e^2, when there are two replicates of all the oospores.

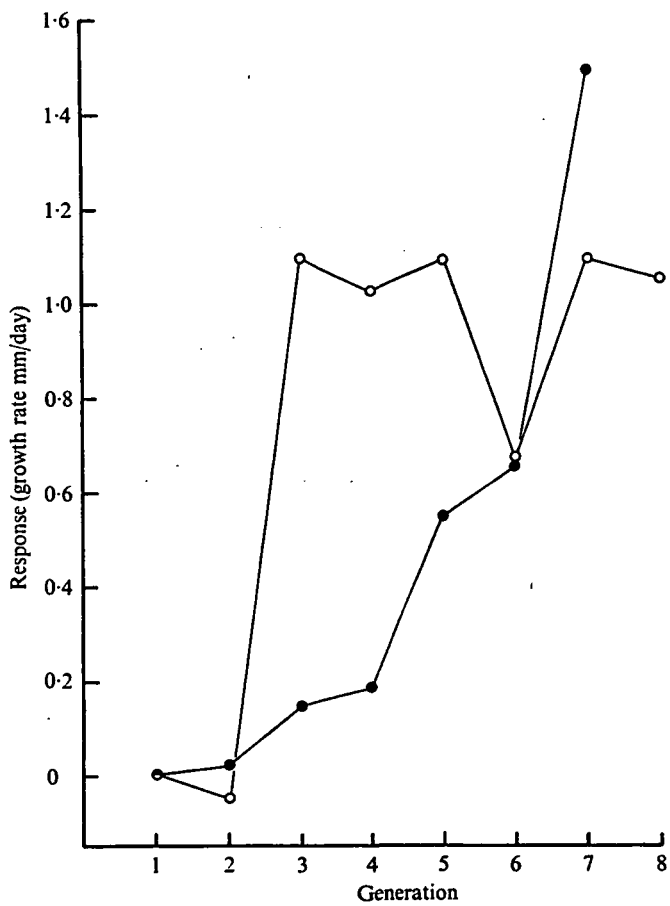

Fig. 4. Response (difference between mean growth-rate of high and low families) at successive generations of asexual propagation by zoospores (○—○) and sexual propagation by oospores (●—●).

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one would predict that it should decrease with increasing homozygosis on inbreeding.

The response to selection, measured as the difference between high and low family means, is shown in Fig. 4. In succeeding generations the response increased, gradually at first, but more steeply later. Increasing response with selection differs from the predicted response curve, and indeed from the pattern of response found in most other organisms (Sismanidis, 1942; Mather & Harrison, 1949; Clayton, Morris & Robertson, 1957; Pateman, 1959; Connolly & Simchen, 1968; Papa, 1971). Typically the response has been found to decline with inbreeding and selection.

In the later generations of selection for increased growth-rate, single oospore cultures were found which were indistinguishable from the fast variant obtained in the zoospore lines. Such cultures included 19 individuals from family OH7, 10 individuals from OH6 and a few of the faster-growing colonies of family OH5. Other colonies of these progenies produced sectors resembling the fast variant at a far higher frequency than did the wild type. The form of the distribution of families OH7 and OH6 (Fig. 3) was similar to the growth-rate distribution of the single oospore cultures derived by selfing the fast variant, ZH6-S_1 (Fig. 2). The parent of family OH7, like the fast variant, produced more oospores than the wild type but in this instance the difference was not significant.

(iv) *The pattern of segregation on selfing before and after a period of storage*

The appearance in later generations of the high selection line of oospore cultures with characters similar to the fast variant implied that segregation in these families was not purely the result of the recombination of nuclear genes. Evidence on this point was obtained by adopting the approach used by Shaw & Elliott (1968) to investigate a mutant morphological character, presumed to be under extrachromosomal control, in another strain of *Phytophthora cactorum*. They found that more mutant segregants appeared among zoospore progenies of mycelium stored for a period under liquid paraffin than were obtained before storage. In the present instance, differences between oospore progenies obtained from the same parent on different occasions would suggest that the character under consideration was not controlled by nuclear genes. Second progenies were therefore obtained from the parents of families OH4, OH5 and OH6, the cultures having meantime been stored under paraffin at room temperature on minimal medium. There was no marked difference in the distribution of growth-rates for families OH4 and OH5 on the two occasions, but family OH6 had a much lower variance on the second occasion than on the first, and the cultures obtained on the second occasion were all similar in growth-rate and morphology to the fast variant.

(v) *Segregation in zoospore progenies of single oospore cultures*

To test whether the cultures used as parents for each of the oospore progenies were heteroplasmic, zoospore progenies were obtained from 10 of the 13 single oospore cultures used as parents during the selection experiment. Inoculum was taken from the bottles in which the parents had been stored under paraffin,

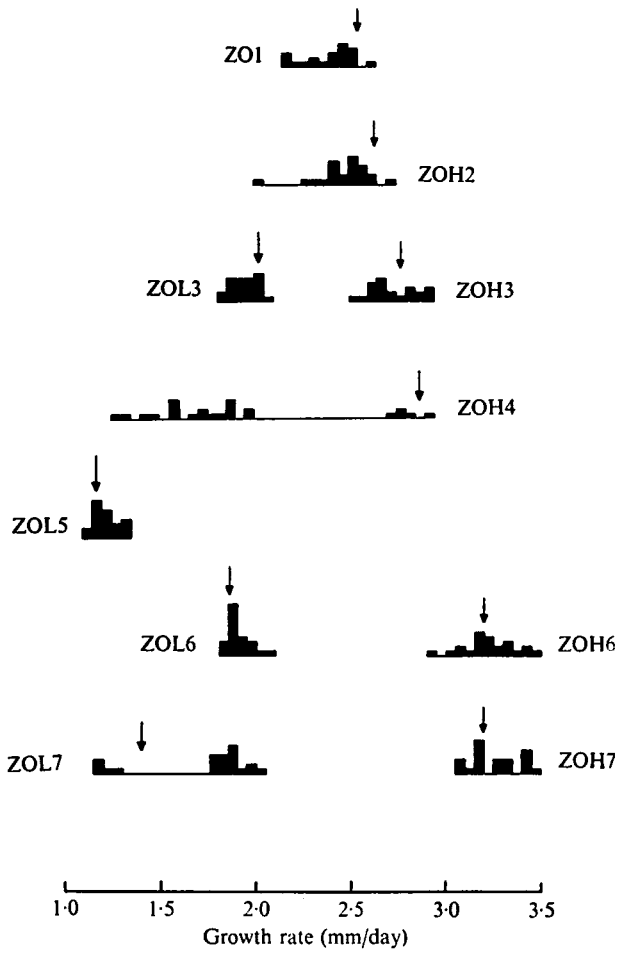

Fig. 5. Frequency distribution of growth-rate in families of zoospores produced by certain parents of the oospore selection lines. The parent value is indicated by the arrows.

and from each a sample of 24 single zoospore cultures was obtained. The zoospore progenies were termed ZOL2, ZOH2, ZOL3, etc. to correspond with oospore progenies OL2, OH2, etc. derived from the same parents. The growth-rates of the 24 individuals of the 10 progenies and their parents were determined at the same time. The values obtained are illustrated in Fig. 5; the arrows mark the parental growth-rates. A continuous distribution was obtained for eight progenies, while

two, ZOL7 and ZOH4, segregated for a clear-cut difference in growth-rate. Fast- and slow-growing colonies of family ZOL7 were morphologically similar, but the segregants of family ZOH4 differed, the fast-growing colonies producing compact mycelium while slow-growing cultures produced diffuse mycelium. A further generation of zoospore cultures was obtained from individuals of families ZOH4 and ZOL7. The second-generation progenies were easily classified as fast- or slow-growing (Table 2). With the persistence of the segregation in some of the second-generation families, the possibility that the parents were heterokaryotic, rather than heteroplasmic, may be rejected.

Table 2. *Phenotypes of second-generation zoospore cultures produced from six individuals of zoospore family ZOL7 and five individuals of family ZOH4*

Family	First generation phenotype	Second-generation phenotypes: numbers of colonies	
		Slow	Fast
ZOL7	Slow	31	0
		30	0
		30	3
	Fast	0	33
		0	35
		1	34
ZOH4	Slow	16	18
		37	9
		33	3
	Fast	1	30
		0	33

Table 3. *Estimates of variation between oospores of family OH4(2), between lines independently maintained but derived from the same oospore, and between replicate measurements, during four 'generations' of mass hyphal tip propagation*

'Generation'	Oospore variance $\hat{\sigma}_b^2$	Line variance $\hat{\sigma}_l^2$	Error variance $\hat{\sigma}_e^2$
1	0·5019	0·0140	0·0140
2	0·3631	0·0265	0·0316
3	0·2653	0·1553	0·0215
4	0·3151	0·3313	0·0070

From the growth-rate determinations R1, R2 and R3 for each generation (see text), the quantities $S = \mathrm{R2}+\mathrm{R3}+2\mathrm{R1}$, $T = \mathrm{R2}+\mathrm{R3}-2\mathrm{R1}$ and $U = \mathrm{R2}-\mathrm{R3}$ were calculated. The analysis of variance was

	Mean square	Expected mean square
Between oospores	$\frac{1}{10}\ \Sigma\ (S-\bar{S})^2$	$6\sigma_e^2+8\sigma_l^2+16\sigma_b^2$
Between lines	$\frac{1}{20}\ \Sigma\ T^2$	$6\sigma_e^2+8\sigma_l^2$
Error	$\frac{1}{20}\ \Sigma\ U^2$	$2\sigma_e^2$

there being 20 oospores in the experiment.

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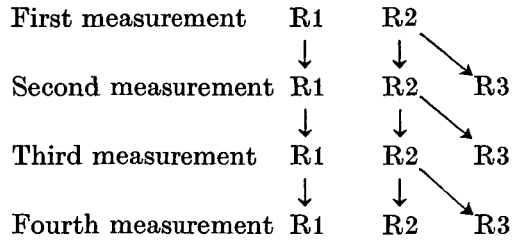
(vi) *Phenotypic change during mass hyphal propagation*

The colonies of the second oospore family OH4 (OH4(2)) could be classified as having diffuse or compact mycelium. While the former generally grew more slowly than the latter, the distributions of growth-rates overlapped and formed a continuous distribution; this was in contrast to the situation in the zoospore family ZOH4 from the same parent mycelium, in which a clear-cut segregation occurred. Most of the colonies of the oospore family OH4(2) produced sectors, generally faster-growing, but some slower-growing.

Mycelium from the 20 single oospores of OH4(2) was propagated by mass hyphal transfer. The two replicates of each oospore culture used in the initial growth-rate determinations (R1 and R2) were subcultured on to minimal medium by taking a plug 4 mm diameter from the edge of the measured colony; one subculture was taken from R1 and two from R2, and the growth-rate of these three colonies measured. This was repeated to give four sets of growth-rate measurements as follows:



The variation between lines from the one oospore, i.e. the difference between R1 and the mean of R2 and R3, increased over these four 'generations', becoming equal to the variation between different oospores (Table 3). This result suggests that a substantial part of the variation between oospores is due to cytoplasmic factors.

4. DISCUSSION

The selection experiments described above illustrate the considerable potential for variation in *Phytophthora cactorum*, especially when propagated by oospores. In comparison with oospore propagation, zoospore propagation did not result in the release of continuous variation, and selection for extremes of growth-rate produced a response only because of the occasional production of discrete sectors. This differs from results with *Phytophthora infestans* (Caten & Jinks, 1968; Caten, 1970, 1971), where zoospore progenies exhibited continuous variation for growth-rate and pathogenicity, and showed a gradual response to selection for these characters.

Inoculum taken from a fast-growing sector was used to establish the fast variant. The pattern of inheritance over two generations of selfing of the fast variant was not that expected of nuclear genes in a diploid organism, and the explanation preferred was that the fast variant resulted from the segregation of mutant

cytoplasmic determinants at hyphal tip formation. Information on the nature of the variation could also have been obtained by comparison of the effects on the sectoring of different kinds of mutagens, e.g. ultraviolet light and acriflavin, but such experiments were not carried out.

Contrasting with zoospore propagation, oospore propagation released a considerable range of continuous growth-rate variation. Boccas (1972) also observed a continuous growth-rate distribution for oospore progenies of *Phytophthora syringae* which was greater in range than the variation released upon zoospore propagation. This evidence was presented to support a theory for diploidy for the homothallic *P. syringae*, assuming that the variation between oospore cultures resulted largely from the recombination of nuclear genes for which the wild type was heterozygous. While the first few generations in our selection experiments suggested polygenic control of growth-rate, later generations indicated that a considerable proportion of the variation between cultures resulted from a mechanism other than the recombination of nuclear genes. Thus: (1) the response to selection was initially small but increased in later generations; (2) the variation between oospore cultures gradually increased despite inbreeding and selection; and (3) in later generations of the high line, single oospore cultures appeared which were phenotypically similar to the fast variant. Additionally, (4) the fast-growing oospore cultures of the high line, like the fast variant, were more fertile than the wild type; (5) after a period of storage, the parent of family OH6 lost the ability to form segregating oospore progenies; (6) in two cases, segregation for growth-rate and morphological characters occurred in two successive generations of zoospores derived from single oospores, and (7) lines independently propagated by mass hyphal transfer from single oospore cultures of OH4(2) diverged.

The discontinuous variation for growth-rate released in zoospore progenies implies that a cytoplasmic entity was segregating which exists in two forms, wild type and mutant, and that each zoospore contained only one of these entities (cf. Shaw & Elliott, 1968). If zoospores contained only one such entity, one may suppose that where segregation occurred over several zoospore generations, as in ZOH4 and ZOL7, the particular genetic background had made the determinants especially unstable. The continuous distribution in the oospore families could have been due to the segregation of a number of nuclear genes affecting growth-rate, or to segregation of cytoplasmic entities of which each oospore contained several (growth-rate being related to the proportion of mutant determinants it contained), or to both possibilities. The phenotypic change on mass hyphal transfer requires change in the population of cytoplasmic determinants, and these determinants could have arisen either from a single unstable one, or from several of two kinds, which were present in the single oospore from which the lines arose. It is not possible with the data available to distinguish these various possibilities. We do not yet have adequate nuclear markers to perform a 'heterokaryon test' (Arlett, Grindle & Jinks, 1962). However, we think it more likely that the oospores contain a number of the cytoplasmic entities.

The most curious feature of the selection experiments, that is the increasing variability and increasing response with succeeding generations despite the intense inbreeding, could be due to selection for the mutant cytoplasmic determinants. Since the fast variant was more fertile than the wild type, parts of the mycelium possessing an above-average proportion of the mutant determinants would contribute more oospores to the next generation than mycelium with fewer mutant determinants; also the oospores selected for high growth-rate would contain a higher proportion of them. Thus the proportion of mutant entities in the population would increase with inbreeding. Starting with mycelium with few mutant determinants, the increasing proportion of the mutant form would be accompanied by an increase in the variance of growth-rate between oospore cultures, and this variance would be greatest when wild type and mutant determinants were present in equal numbers in the cytoplasm of the parent. Further increase in the proportion of mutant determinants would result in a decline in variance between oospore cultures and an increase in the proportion of fast-growing segregants in each generation. The effects of back selection in such a system would be interesting, but they have not been investigated.

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