Variation in Virulence in Successive Single-Zoospore Propagations of *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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Variation in virulence was studied in successive single-zoospore propagations involving isolates of races 3 and 6 of *Phytophthora megasperma* f. sp. *glycinea*. Following zoospore inoculation of etiolated hypocotyls of soybean cultivars Wayne, Harosoy, and nine other cultivars each carrying a different *Rps* gene for resistance to *P. megasperma* f. sp. *glycinea*, the gain and loss of virulence was observed in successive single-zoospore propagations and in mass mycelial transfers in one race 3 lineage. Less variability was observed following mass mycelial transfers than following single-zoospore propagations. Isolates of races 1 and 3, lacking virulence on all 11 soybean cultivars, retained the ability to infect and elicit glyceollin production; this demonstrated that loss of virulence following single-zoospore propagation was not necessarily associated with loss of pathogenicity.

The physiology of the interaction of soybeans *Glycine max* (L.) Merr. with *Phytophthora megasperma* Drechs. f. sp. *glycinea* (Hildeb.) Kuan and Erwin (*Pmg*) has been the subject of extensive study in recent years (e.g., 18). There are several cultivars that each carry a single major gene for resistance (1–4,6,19,27) and corresponding physiological races of the pathogen have been identified (15,16,20,21,25,26,28,29,33). It has been assumed that the interaction is an example of a gene-for-gene system (8,9,17). Studies of the genetics of the pathogen, however, have met with limited success (22–24) and mechanisms by which physiological races arise remain poorly understood. Recently, the number of such races identified has been greatly expanded by screening isolates from field soil and by the use of additional differential cultivars (14). An understanding of the genetic basis of variation in virulence is needed both in the study of the physiology of the host-pathogen interaction and for control of the pathogen in the field.

Several authors have observed variability in virulence among single-zoospore isolates derived from a common ancestor (13,14,22). In this paper, additional observations of this phenomenon are reported.

MATERIALS AND METHODS

Based on preliminary tests with races 1–6 of *Pmg*, isolates of races 1, 3, and 6 were chosen for further study; our isolate of race 6 gave expected responses, whereas isolates of races 1 and 3 were atypically avirulent on all cultivars tested. Isolates of races 1, 3, and 6 were derived from those used by Haas and Buzzell (12). The isolates of races 1 and 3 had been maintained in culture on V-8 juice agar for several years by making mass mycelial transfers every 6–8 wk. The isolate of race 6 had been maintained similarly, but it was reisolated from infected host material and checked for virulence at least once a year. During the course of the study, isolates were grown in petri dishes on V-8 juice agar and routinely subcultured by mycelial transfer (5-mm-diameter plugs) at weekly intervals. Zoospores were obtained from cultures grown for 5–6 days according to procedures described previously (33). For single-zoospore isolations, cysts were germinated on water agar and transferred to V-8 juice agar.

Eleven soybean cultivars or lines were used. Nine of these carried a different identified *Rps* gene for resistance to *Pmg*. These were as follows: Harosoy 63 (*Rps*$_1$), OX681 (*Rps*$_1^b$), OX682 (*Rps*$_1^c$), OX678 (*Rps*$_1^k$), L7O-6494 (*Rps*$_2$), OX676 (*Rps*$_3$), PRX27-108-2 (*Rps*$_4$), L62-904 (*Rps*$_5$), and Altona (*Rps*$_6$). The two remaining cultivars were Harosoy, which has an unidentified *Rps* gene giving resistance to races 12, 16, 18, and 19 (16), and Wayne which is susceptible to all available races, 1–21 (25, and R. I. Buzzell and T. R. Anderson, *unpublished*). The lines OX676, OX678, OX681, and OX682 were developed by making one or two backcrosses of *Rps* genes from PI 171.442, Kingwa, PI 84.637, and PI 54.615-1, respectively, into Harosoy. The derivation of the other *Rps* lines was described previously (31).

When tested by the insertion of mycelium into hypocotyl wounds, race 1 is virulent on Harosoy and Wayne; race 3 is virulent on Harosoy, Wayne, and Harosoy 63 (*Rps*$_1$); and race 6 is virulent on all cultivars except OX681 (*Rps*$_1^b$), OX682 (*Rps*$_1^c$), and OX678 (*Rps*$_1^k$).

Seedlings were grown in the dark for 6 days, then placed horizontally in glass trays and inoculated by placing a 10-μl drop of a zoospore suspension (10^5 per milliliter) on the hypocotyl surface 1.5–2.0 cm below the cotyledons as described previously (32). Inoculated seedlings were incubated in the dark at 25 C for 48 hr.

The hypocotyl responses were considered to be resistant (R) when the resulting lesions were restricted to the area covered by the inoculum drop or, if more extensive, remained brown and necrotic, and the underlying tissue remained firm. A susceptible response (S) was characterized by rapidly extending colorless, water-soaked lesions resulting in collapse of the hypocotyl. Occasionally, responses that could not be clearly assigned to either category were obtained and these are referred to as intermediate (I). In the experiments for which lesion size is reported, measurements were made along the length of the hypocotyl as in a previous study (31). All observations and measurements were obtained from at least eight, and usually 10, seedlings.

Glyceollin was determined in extracts of tissues excised from inoculated sites as described previously (32). Data are based on three separate experiments and expressed as micrograms of glyceollin per gram of tissue (fresh weight).

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RESULTS

Three single-zoospore isolates obtained from a stock culture of race 6 differed distinctly in relative virulence (Table 1). Isolate "A" was similar to the parent culture, whereas isolate "B" differed markedly and was virulent only on Wayne, Harosoy, and OX681 ($Rps_1{}^b$) (a pattern characteristic of race 2). Isolate "C" was similar to the parent culture except that it was avirulent on Wayne and PRX27-108-2 (Rps_4). Further variability was displayed by a second generation of isolates derived from isolates "A," "B," and "C." Thus, one isolate from "A" was identical to "A," but four others had gained virulence against OX678 ($Rps_1{}^k$). Four isolates from "B" exhibited the same pattern as "B," but a fifth was completely avirulent. From "C," no isolates with an identical pattern of virulence to "C" were obtained; one, however, apparently reverted to the original parent stock culture and the others were similar to "C" except that in addition they were virulent against OX678 ($Rps_1{}^k$).

Cultivar responses to a completely avirulent isolate from race 3, and to three successive single-spore generations again were found to vary (Table 2). The first single-zoospore isolate (#1) displayed virulence on Wayne and Harosoy that was not expressed by the avirulent stock culture. A second-generation, single-spore isolate #2 (obtained from #1) was also virulent on Harosoy 63 (Rps_1) thus displaying the characteristics of race 3. Two third-generation isolates, #3A and #3B (obtained from #2), differed in that #3B had the characteristics of race 3, like its immediate parent, but #3A was almost completely avirulent except that it induced an intermediate response on Wayne.

Much less variability in virulence was detected in successive (random) mass transfers of these race 3-derived isolates (Table 2). Apart from intermediate responses, only two changes in virulence were observed (#1, mass transfer I on Harosoy 63 (Rps_1) and #3A, mass transfer II on Wayne). It is noteworthy that all the variability observed with these isolates following single-spore isolations or mass transfers was related to cultivars Wayne, Harosoy, and Harosoy 63 (Rps_1), the three cultivars on which race 3 is virulent. No changes in virulence were observed on the cultivars carrying genes $Rps_1{}^b$, $Rps_1{}^c$, $Rps_1{}^k$, Rps_2, Rps_3, Rps_4, Rps_5, or Rps_6.

Completely avirulent isolates from races 1 and 3 could not be differentiated from isolates that retained the virulence characteristics of these races by their morphology and growth rate on V-8 juice, potato-dextrose, or carrot agar or by their ability to produce high numbers of zoospores ($10^5 \cdot ml^{-1}$). Furthermore, the completely avirulent isolates infected hypocotyls and caused the development of lesions and the accumulation of glyceollin typical of incompatible interactions involving normal races and resistant cultivars (Table 3). The completely avirulent race "1," for example,

TABLE 1. Deviation from expected soybean hypocotyl reactions in response to inoculation with single-zoospore isolates of *Phytophthora megasperma* f. sp. *glycinea*, race 6

	First single-zoospore generation	Second single-zoospore generation	Hypocotyl response[y]										
			W	H	1	1^b	1^c	1^k	2	3	4	5	6
Parent[z]			S	S	S	R	R	R	S	S	S	S	S
	Isolate "A"		S	S	S	R	R	R	S	S	S	S	S
		Isolate "A"-1	S	S	S	R	R	R	S	S	S	S	S
		Isolate "A"-2,-3,-4,-5	S	S	S	R	R	S	S	S	S	S	S
	Isolate "B"		S	S	R	S	R	R	R	R	R	R	R
		Isolate "B"-1,-2,-3,-4	S	S	R	S	R	R	R	R	R	R	R
		Isolate "B"-5	R	R	R	R	R	R	R	R	R	R	R
	Isolate "C"		R	S	S	R	R	R	S	S	R	S	S
		Isolate "C"-1	S	S	S	R	R	R	S	S	S	S	S
		Isolate "C"-2,-3,-4	S	S	S	I	R	S	S	S	S	S	S

[y] Hypocotyl response was determined following inoculation with zoospore suspensions and rated as resistant (R, a brown restricted lesion), susceptible (S, a water-soaked spreading lesion) or intermediate (I, either mixed R and S responses or indeterminate) for W (Wayne), H (Harosoy), 1 (Rps_1), 1^b ($Rps_1{}^b$), 1^c ($Rps_1{}^c$), 1^k ($Rps_1{}^k$), 2 (Rps_2), 3 (Rps_3), 4 (Rps_4), 5 (Rps_5), and 6 (Rps_6). Hypocotyl responses differing from those to the original parent culture are underlined.

[z] Parent is a stock culture maintained by mycelial transfer. Isolates "A," "B," and "C" are three cultures derived from single-zoospores from the parent. Similar isolates "A"-1, "B"-1, "C"-1, etc., are derived from single zoospores from "A," "B," and "C."

TABLE 2. Changes in virulence generated by single-zoospore isolations and mass mycelial transfers from a completely avirulent stock culture of *Phytophthora megasperma* f. sp. *glycinea* race 3

Inoculum[x] source		Hypocotyl response[y]											
						Mass transfer[z]							
						I				II			
		W	H	1	1^b-6	W	H	1	1^b-6	W	H	1	1^b-6
Parent		R	R	R	R	R	R	R	R	I	R	R	R
Single-zoospore generations													
#1		S	S	R	R	S	S	S	R	S	S	S	R
#2		S	S	S	R	S	S	S	R	S	S	S	R
#3	Isolate A	I	R	R	R	R	I	R	R	S	I	R	R
	Isolate B	S	S	S	R	S	S	S	R	S	S	S	R

[x] A single zoospore was isolated from the parent culture to get single-zoospore isolate #1. From the resulting culture a single zoospore was isolated to get #2, from which isolates #3A and #3B were obtained.

[y] Hypocotyl response was determined following inoculation with zoospore suspensions and rated as resistant (R, restricted brown lesion), susceptible (S, water-soaked spreading lesion) or intermediate (I, either mixed R and S responses or indeterminate) for W (Wayne), H (Harosoy), 1 (Rps_1), and 1^b-6 ($Rps_1{}^b$, $Rps_1{}^c$, $Rps_1{}^k$, Rps_2, Rps_3, Rps_4, Rps_5, and Rps_6).

[z] Mass transfer indicates the response following inoculation with zoospores of each of the cultures after I and II successive random mycelial transfers from the parent culture and the derived single-zoospore cultures.

produced similar (although slightly larger) brown, limited lesions on Harosoy and Wayne (normally susceptible) and on Harosoy 63 (normally resistant). High levels of glyceollin were produced in all three cultivars, although again there were differences in amount. With the race "3" isolates the interactions were typically compatible for isolate B and generally incompatible for isolate A. However, the expression of resistance to the latter evidently was less effective than to race "1" in terms of both lesion length and glyceollin accumulation.

DISCUSSION

A major difficulty in pursuing the line of study described here is a logistic one. There is a physical limit to the number of standardized zoospore suspensions that can be prepared and to the number of plants that can be inoculated in a single experiment. Clearly, the limited sampling that this dictates is insignificant in relation to the population of zoospores produced by a single colony. The degree of variation observed varied widely among random single-zoospore samplings and while the data of Table 1 may represent one extreme, single-zoospore isolates of races 4 and 5 did not differ in virulence from their parents, although this cannot be considered to be evidence that these races are inherently more stable. In addition, single-zoospore virulence cannot be tested directly, but only after colony formation and generation of a new crop of zoospores, a process that may introduce a further degree of variability.

The data indicate that variation in virulence is expressed following single-zoospore propagation (and to a lesser degree following mass mycelial transfers) in *Pmg*. These observations are consistent with other reports of variability in virulence in *Pmg* (13,14,22) and in other species of *Phytophthora* (10,30).

The observed variation following single-zoospore isolation contrasts to the relative stability associated with mass transfers. Routinely, races of *Pmg* maintain cultivar-specific virulence patterns through many such transfers over a period of years, although rejuvenation by passage through the host appears to be necessary to prevent eventual loss of virulence. Even with the highly variable single-zoospore isolates of the completely avirulent race "3" described here (Table 2), subsequent mass transfers were much more stable. These responses mimic patterns of variability obtained by Caten and Jinks (7) who, in a study of factors determining the inheritance of growth rate in *P. infestans*, found greater variation expressed among the progeny of single-zoospore isolations than among the progeny of sporangial or mass mycelial transfers. Apparently, in their study as in ours, the greater the volume of cytoplasm (and number of nuclei) sampled, the greater the stability of the phenotype.

Previous genetical studies of *Pmg* (22,24) indicate that like other *Phytophthora* spp., *Pmg* is vegetatively diploid. Shaw (30) discusses the possibility that mitotic crossing-over, by exposing accumulated mutations or modifier or recessive genes, may act as a potential source of variability in a diploid fungus. An example of this in our study may have been the observed change in virulence from a race 6 phenotype to a race 2 phenotype following zoosporogenesis (isolate "B" in Table 1). Assuming a gene-for-gene system, this change would involve seven loci, and it is highly improbable that it could result from mutation.

In higher fungi, mitotic mechanisms generating variation including mutation, crossing-over, and gene conversion arise infrequently in large populations (11). Conventional mitotic mechanisms are thus unlikely to account for the high frequency of variants we have obtained following random single-zoospore isolations. An alternative possibility discussed by Shaw (30) is the random distribution of cytoplasmic genes during zoosporogenesis. While much of the variation could be explained in this way, the alternate loss and recovery of virulence in successive single-zoospore generations could not be explained.

Regardless of the mechanisms involved, the implications of the examples of variability described here are of significance both for control of the disease in the field and for an understanding of the host-pathogen interaction. Evidently, new races differing from their parent cultures in cultivar specificity can be obtained by single-zoospore isolation. This can involve either a loss or gain of virulence with respect to individual cultivars (Table 1). From race 6, isolates with virulence against all resistance genes except Rps_1[c] were obtained. Such random generation of new races is entirely consistent with the evidence of Hobe (14) that a wide range of new races may be obtained from soybean field soils. Additional races also were obtained by single-zoospore isolation from Hobe's field isolates (14). From this type of evidence, control based solely upon major resistance genes is likely to be transitory at best.

Loss of virulence after prolonged periods in laboratory culture is commonly observed and the completely avirulent stock cultures of races 1 and 3 (Tables 2 and 3) are presumably examples of this phenomenon. A completely avirulent single-zoospore isolate was obtained also from race 6 (Table 1, B-5) so it is possible that such variants are randomly produced and that conditions in culture favor their selection. These completely avirulent cultures appeared to differ little from cultures with cultivar-specific avirulence from which they were derived. They grew normally, produced abundant zoospores, and infected soybean hypocotyls with the development of restricted brown lesions and glyceollin levels characteristic of incompatible interactions. They must, therefore, be considered to have genes for avirulence with respect to all known *Rps* genes including that of Harosoy and also to a gene in cultivar Wayne, in which no gene for resistance has been identified. According to various hypotheses, this could be due to loss of aggressiveness, although this was not distinguishable from avirulence, acquisition of avirulence genes by *Pmg* in culture that govern the elicitation of host-defense mechanisms (17), or loss of genes that suppress such mechanisms (5). The last of these could be a specific loss with respect to the host *Rps* genes involved or a general loss of basic compatibility (8).

TABLE 3. Hypocotyl response, lesion size, and glyceollin accumulation in soybean cultivars inoculated with single-zoospore isolates derived from completely avirulent cultures, originally isolated from race 1 or 3 of *Phytophthora megasperma* f. sp. *glycinea*

Inoculum[w]	Cultivar	Hypocotyl[x] response	Lesion[y] length (mm)	Glyceollin[z] ($\mu g \cdot g^{-1}$ fr. wt.)
Race "1"	Wayne	R	8.1 ± 0.2	1,506 ± 188
	Harosoy	R	8.9 ± 0.6	1,767 ± 133
	Harosoy 63	R	5.6 ± 0.1	1,182 ± 106
Race "3" (A)	Wayne	I	33.7 ± 4.7	506 ± 203
	Harosoy	R	21.0 ± 7.3	990 ± 170
	Harosoy 63	R	22.7 ± 4.8	857 ± 130
(B)	Wayne	S	50.9 ± 1.9	142 ± 27
	Harosoy	S	51.4 ± 3.5	343 ± 106
	Harosoy 63	S	42.1 ± 7.1	403 ± 170

[w] Race "1" is a completely avirulent culture of race 1 that normally would be virulent on cultivars Wayne and Harosoy and completely avirulent on Harosoy 63. Race "3" (A) and (B) are two single-spore isolates of a completely avirulent race 3 culture (see Table 2). Isolate B behaves like a typical race 3, i.e., virulent on all three cultivars.

[x] Hypocotyl response was rated as resistant (R, restricted brown lesion), susceptible (S, spreading water-soaked lesion), or intermediate (I, mixed or indeterminate).

[y] Lesions were measured along the length of the hypocotyl, means and standard deviations are from three replicates of 10 plants each.

[z] Glyceollin was determined in tissue excised from the inoculated site, values are means and standard deviations of three replicate groups of 10 plants each.

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