Genetics

In Planta Formation of Heterokaryons of *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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The generation of heterokaryons in planta could represent a mechanism to account for the high rate of occurrence of new virulent races of *Phytophthora megasperma* f. sp. *glycinea*. Zoospores of drug-resistant mutants of *P. m. glycinea* races 1 and 3 were inoculated on a susceptible soybean cultivar (Williams), and fungal colonies were reisolated onto plates selecting for double drug resistance. One heterokaryon was reisolated from a mixed inoculation with *P. m. glycinea* race 1 metalaxyl resistant and race 1 fluorotryptophan-resistant zoospores. This colony grew on plates containing both metalaxyl and fluorotryptophan and gave rise to zoospores of either metalaxyl or fluorotryptophan resistance. The drug-resistant mutants were also used to investigate the effects of simultaneous or sequential inoculation of both races (one metalaxyl resistant and one fluorotryptophan resistant) and competition between races early in infection. Two virulent races were inoculated on a susceptible cultivar (Williams), and a virulent (fluorotryptophan-resistant) and an avirulent (metalaxyl-resistant) race were inoculated on cultivar Harosoy 63. *P. m. glycinea* was reisolated from the inoculated roots and characterized for drug resistance. When susceptible roots were sequentially inoculated, the race that was inoculated first was reisolated more frequently. When resistant roots were inoculated with an avirulent race 2 hr before inoculation with the virulent race, the number of roots from which *P. m. glycinea* could be reisolated was reduced. The importance of the response of the susceptible and resistant cultivars to sequential inoculation is discussed.

Phytophthora megasperma Drechsler f. sp. *glycinea* is a fungal pathogen of soybeans causing root rot (7,11). *P. m. glycinea* and soybean are proposed to have a gene for gene interaction (3,9). The appearance of new virulent races of *P. m. glycinea* can be quite rapid (11), but the source of these new *P. m. glycinea* races is unknown. Although *Phytophthora* races may already exist in great diversity in the soil, we wanted to investigate the possibility of formation of heterokaryons in planta as a mechanism to generate new races. Heterokaryon formation between avirulent and virulent races could lead to heterozygosity at a number of virulence loci. Oosporogenesis or somatic recombination and zoosporogenesis could then lead to novel combinations of homozygous recessive virulence loci in a single nucleus and a new virulent race.

We wanted to investigate in planta heterokaryon formation because we had developed a sensitive and stringent assay for heterokaryons. Discovery of heterokaryons formed in planta has been impossible until now because distinguishing characteristics of *P. m. glycinea* races other than virulence phenotype have not yet been found (4,8). Virulence phenotype is not a useful distinguishing characteristic because heterokaryons formed between a virulent and an avirulent race of *P. m. glycinea* were predominantly avirulent (9). Thus, heterokaryons could not be distinguished from the parental races solely by virulence phenotype. We produced mutants of races of *P. m. glycinea* that maintained their virulence phenotype and could be identified easily by fungicide or amino acid analog resistance (8,9). With these mutants, we investigated the formation of interracial and intraracial heterokaryons in planta.

We also used these mutants to study the interaction between an avirulent and a virulent race on plants susceptible to both races and on plants resistant to one race and susceptible to the

other. Because the drug resistance and virulence phenotypes of our mutants are stable through five generations of zoospores (8), the drug-resistant races provide a convenient and sensitive method to investigate the effects of mixed inoculum on susceptible and resistant plants. The drug resistance phenotype can be assayed directly on agar plates without redetermining virulence phenotype. Thus, we could determine which races had infected the root by reisolating the fungus from root pieces directly onto drug-containing agar plates.

We used this reisolation assay to determine how the zoospores of different races interacted in the early stages of infection. By determining the effect of either simultaneous or sequential inoculation on the number of plants from which we could reisolate *P. m. glycinea* as well as the drug resistance phenotype of the reisolated colonies, we could observe if virulent and avirulent races had any effect on each other during the infection of a cultivar susceptible to both races or of a cultivar susceptible to one race and resistant to the other. A portion of this work was referred to briefly in a previously published article (8).

MATERIALS AND METHODS

Fungal isolates. The following *P. m. glycinea* drug-resistant mutants were obtained after mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine as described previously (8): 1 Mex (5-58 Mex 6), race 1, metalaxyl resistant; 1 Ftp (5-58 Ftp 4), race 1, fluorotryptophan resistant; 3 Pfp (76-4-4 Pfp 2), race 3, fluorophenylalanine/fluorotryptophan resistant; and 3 Mex (76-4-4 Mex 2), race 3, metalaxyl resistant. Cultures were grown and zoospores produced as described previously (8).

Soybean cultivars. *Glycine max* L. Merr. 'Williams' and 'Harosoy' have the *rps* gene and are susceptible to both *P. m. glycinea* race 1 and 3 (1). Cultivar Harosoy 63 has the Rps1 gene which confers resistance to *P. m. glycinea* race 1 but not *P. m. glycinea* race 3. Cultivar Williams seed was obtained from Purdue Agriculture Alumni Foundation, Romney, IN. Cultivar Harosoy and Harosoy 63 seed were obtained from Dr. James Wilcox, USDA, ARS, Agronomy Dept., Purdue University, West Lafayette, IN.

Seeds were soaked for 5 min in tenfold diluted commercial chlorine bleach, washed in tap water, and allowed to imbibe in tap water for 1 hr at room temperature. Imbibed seeds were planted in vermiculite and incubated in a germinator at 27 C in an 18-hr daylight regime. Two-day-old seedlings were used for root inoculations.

Inoculation. Zoospore suspension concentrations were determined by counting on a hemacytometer after fixing the zoospores in Lactophenol Blue (lactic acid/phenol/glycerol/water, 1:1:1:1, by vol), 0.05% aniline blue). Zoospore suspensions were diluted to 1 to 4×10^4 per milliliter with sterile water and counted again. Two-day-old seedlings were removed from vermiculite and the roots washed in sterile water. Seedlings with roots about 2.5 cm long were placed in 1.5-ml Eppendorf tubes with 1.0–1.5 ml of *P. m. glycinea* zoospore suspension to give 1 to 2×10^4 zoospores total and the tubes were filled with sterile water. Seedlings were incubated at 100% relative humidity and 25 C in a germinator. The first 18 hr were dark followed by a 12-hr light/dark cycle. Seedlings were approximately 20 cm from four 40W fluorescent bulbs. For simultaneous inoculations, tubes contained 1 to 2×10^4 zoospores of each race. For sequential inoculations, zoospore suspensions were recounted, and seedlings were transferred either 2 or 8 hr after the beginning of the first inoculation to Eppendorf tubes with the other race as described above.

Reisolation of *P. m. glycinea*. Two days after inoculation, seedlings were scored for color, root length, and number of secondary roots. Seedlings were surface disinfested in tenfold-diluted commercial chlorine bleach for 15 sec and rinsed thoroughly in sterile distilled water. Roots were sliced into 1-mm sections with a flamed razor blade, and adjacent sections were transferred onto V-8 agar plates containing 20 μg/ml of chloramphenicol, 50 μg/ml of streptomycin, 50 μg/ml of benomyl, and 50 μg/ml of natamycin and onto V-8 agar plates with the above antibiotics and either

10 μg/ml of fluorotryptophan or 20 μg/ml of metalaxyl. Plates were incubated for 5 days and scored for number of root sections that gave rise to a colony on each plate from each plant. In general, *P. m. glycinea* of only one drug resistance phenotype was reisolated from each infected root. Roots were separated into classes as follows. The race 1 class were roots where fungal colonies were observed only on plates containing no Ftp or Mex and plates with Mex. The race 3 class were roots where fungal colonies were observed only on plates containing no Ftp or Mex and plates with Ftp. The race 3 and race 1 classes were roots where fungal colonies were observed on all plates. In preliminary experiments with seedlings inoculated with either 1 Mex or 3 Pfp alone, the virulence phenotype of the reisolated fungus was tested by hypocotyl inoculation as described previously (9). No change in virulence phenotype and no new virulence phenotypes were observed.

To test for heterokaryon formation, equal numbers of zoospores (1 to 2×10^4) of two differing drug-resistant mutants were inoculated onto 2-day-old seedlings of cultivars Harosoy or Williams, both susceptible to race 1 and race 3, as described above. One-millimeter sections were plated on V-8 agar plates with the above antibiotics and V-8 agar plates with the above antibiotics and both 10 μg/ml of fluorotryptophan and 20 μg/ml of metalaxyl. After 5 to 7 days, any sections with fungus on the plates with both metalaxyl and fluorotryptophan were scored as potential heterokaryons and transferred to V-8 agar plates with antibiotics and metalaxyl and fluorotryptophan to determine if they continued growing in the presence of both drugs.

Statistical analysis. Chi-square analysis of the data in Tables 2, 3, and 4 was with the Number Cruncher Statistical System Version 4.2.1 (Dr. Jerry Hintze, Kaysville, UT). The Mantel-Haenszel analysis was done by Dr. Myra Samuels, Purdue University, West Lafayette, IN.

RESULTS

Formation of heterokaryons in planta. Three different attempts were made to observe the formation of heterokaryons in planta (Table 1). In previous experiments where heterokaryons were produced by protoplast fusion (8), fusing metalaxyl- and fluorotryptophan-resistant isolates of the same race produced the greatest number of heterokaryons. The number of putative heterokaryons formed in planta was greater when fluorotryptophan- and metalaxyl-resistant zoospores of the same race were used to inoculate roots (Table 1). These putative heterokaryons appeared as small, fuzzy colonies on the root pieces on plates containing both fluorotryptophan and metalaxyl. One of these putative heterokaryons continued to grow when mycelia without

TABLE 1. Heterokaryon formation in planta in susceptible cultivars Harosoy and Williams after inoculation with virulent races of *Phytophthora megasperma* f. sp. *glycinea*

Races inoculated[a]	Plants inoculated	Plants reisolated[b]	Heterokaryons reisolated[c]	Heterokaryons transferred[d]
A. 1 Mex + 3 Pfp	56	44 (79)	2 (4)	0
B. 1 Mex + 1 Ftp	60	36 (60)	10 (17)	1
C. 3 Mex + 3 Pfp	73	60 (82)	11 (15)	0

[a] Races of *P. m. glycinea* inoculated. A. 1 Mex (race 1, metalaxyl resistant) and 3 Pfp (race 3, fluorotryptophan resistant) zoospores were inoculated simultaneously. B. 1 Mex (race 1, metalaxyl resistant) and 1 Ftp (race 1, fluorotryptophan resistant) zoospores were inoculated simultaneously. C. 3 Mex (race 3, metalaxyl resistant) and 3 Pfp (race 3, fluorotryptophan resistant) zoospores were inoculated simultaneously.
[b] Number of plants from which colonies of *P. m. glycinea* were reisolated. Number in parentheses is percentage of plants inoculated from which colonies were reisolated.
[c] Number of plants from which putative heterokaryon colonies were reisolated. Putative heterokaryons grew on V-8 agar with both fluorotryptophan (10 μg/ml) and metalaxyl (20 μg/ml). Number in parentheses is percentage of plants inoculated from which putative heterokaryons were reisolated.
[d] Number of putative heterokaryon colonies that continued to grow when transferred from the reisolation plate to a V-8 agar plate with both fluorotryptophan and metalaxyl.

root sections were transferred from the initial reisolation plate to a second V-8 plate with both drugs. This colony grew slowly on plates without drugs and was lost after a number of transfers.

Zoospores were generated from this slow-growing putative heterokaryon. Zoospore germination on plates with no drugs was 39% of the number of zoospores plated (189 colonies from 480 plated as determined by counting with a hemacytometer). Seventy of 480 zoospores germinated on plates with 10 μg/ml of fluorotryptophan, 89 of 480 germinated on plates with 20 μg/ml of metalaxyl, and 2 of 480 germinated on plates with both fluorotryptophan and metalaxyl.

Inoculation and reisolation controls. The virulence phenotype of 1 Mex and 3 Pfp was previously determined by hypocotyl inoculation (9). To characterize the response of susceptible and resistant roots to zoospore inoculation, 2-day-old seedlings of Harosoy and Harosoy 63 were inoculated with zoospores of race 1 or race 3 for 2 hr, then planted in vermiculite. Preliminary results indicated that a suspension of 1 to 4×10^4 zoospores per milliliter was sufficient to achieve infection but did not overcome resistance, similar to the range previously observed for root inoculations (5,6). After 5 days, the seedlings were scored for disease symptoms. About 50% of the Harosoy seedlings inoculated with race 1 or race 3 and 50% of the Harosoy 63 seedlings inoculated with race 3 were dead. The remainder had brown, soft taproots and few secondary roots which were also brown and soft. Harosoy 63 seedlings inoculated with race 1 had a dark brown region at the taproot tip, but were otherwise unaffected. Secondary roots of Harosoy 63 inoculated with race 1 were abundant and showed no signs of browning or softening.

The susceptible cultivar Harosoy was inoculated with zoospores of either 1 Mex or 3 Pfp to test the effect of reisolation on drug resistance phenotype. All reisolated colonies from 1 Mex-inoculated plants grew on plates containing metalaxyl, and none grew on plates with fluorotryptophan. All reisolated colonies from 3 Pfp inoculated plants grew on plates containing fluorotryptophan and none grew on plates with metalaxyl.

Inoculation of a susceptible cultivar (Williams) with two virulent races. Because there was no change in the pathogenicity of the reisolated fungus, we were able to inoculate soybean roots with mixtures of race 1 and race 3 zoospores and score the reisolated colonies for race by determining drug resistance phenotype. The results of the reisolation of race 1 and race 3 from cultivar Williams are given in Table 2. Williams is susceptible to both race 1 and race 3. When we consider the total number of both race 1 and race 3 colonies reisolated, it is independent of the inoculation treatment (treatments A–E) ($\chi^2 = 4.5807$, df = 4, P = 0.3331). However, when only the reisolation of race 1 colonies from soybean roots inoculated with both race 1 and race 3 is considered (treatments A, B, and C), the inoculation treatment does significantly affect the reisolation of race 1 from Williams ($\chi^2 = 13.0422$, df = 2, P = 0.0015). After pairwise comparison of the inoculation treatments, we found that race 1 is reisolated significantly less frequently when race 3 is inoculated first than when

both races are inoculated simultaneously (treatments A and C) ($\chi^2 = 3.1053$, P = 0.039). Race 1 is reisolated significantly more frequently when it is inoculated first than when both races are inoculated simultaneously (treatments A and B) ($\chi^2 = 2.8712$, P = 0.0451).

When the reisolation of only race 3 from Williams roots is considered, the inoculation treatment also has a significant effect ($\chi^2 = 7.2501$, df = 2, P = 0.0266). The reisolation of race 3 was significantly less frequent when race 1 was inoculated first than when both races were inoculated simultaneously (treatments A and B) ($\chi^2 = 3.1972$, P = 0.0369).

Inoculation of cultivar Harosoy 63 with an avirulent (race 1) and a virulent race (race 3). In Table 3, the reisolation of race 1 and race 3 from cultivar Harosoy 63 is reported. Harosoy 63 is resistant to race 1 and susceptible to race 3. When the reisolation of both race 1 and race 3 colonies from Harosoy 63 is considered, the number reisolated is not independent of the inoculation treatment ($\chi^2 = 19.2877$, df = 4, P = 0.0007). In pairwise comparisons of treatments, significantly more colonies were reisolated when race 3 was inoculated 8 hr before race 1 than when race 1 and race 3 were inoculated simultaneously (treatments E and A) ($\chi^2 = 4.3279$, P = 0.0375). Significantly fewer colonies were reisolated when race 1 was inoculated 2 hr before race 3 than when both races were inoculated simultaneously (treatments B and A) ($\chi^2 = 8.4724$, P = 0.0036).

When the reisolation of only race 1 from Harosoy 63 roots inoculated with both race 1 and race 3 is considered, the reisolation of race 1 is not independent of the method of inoculation ($\chi^2 = 10.107$, df = 4, P = 0.0387). Comparing pairs of treatments, we observed that there was significantly more race 1 reisolated when race 1 was inoculated 8 hr before race 3 than when both races were inoculated simultaneously (treatments C and A) ($\chi^2 = 5.4536$, P = 0.0195).

When the reisolation of only race 3 from cultivar Harosoy 63 inoculated with both race 1 and race 3 is considered, the reisolation of race 3 is also not independent of the inoculation treatment ($\chi^2 = 18.7146$, df = 4, P = 0.009). Race 3 reisolation was significantly decreased when race 1 was inoculated two hours prior to race 3 than when race 1 and race 3 were inoculated simultaneously (treatments B and A) ($\chi^2 = 7.3384$, P = 0.0067).

We also tested the interaction of race 1 and race 3 using the Mantel-Haenszel statistic. This procedure attempts to determine if one race has an attraction or repulsion effect on the other race when both are present. We analyzed the data from both Williams and Harosoy 63 and found only one significant interaction, a positive dependence between race 1 and race 3 when race 3 was inoculated 2 hr prior to race 1 on Williams. All other treatments showed no significant interaction.

TABLE 2. Inoculation of a susceptible cultivar (Williams) with two virulent races of *P. m. glycinea*

Inoculation treatment[a]	Plants inoculated[b]	Plants reisolated[c]	Race 1[d]	Race 3[e]	Races 1 and 3[f]
A. Race 1 + Race 3	59	33	16	14	3
B. Race 1 − Race 3 (2 hr)	75	42	30	7	5
C. Race 3 − Race 1 (2 hr)	74	30	4	16	10
D. Race 1	65	32	32	0	0
E. Race 3	55	28	0	28	0

[a] Race 1 and race 3 inoculated simultaneously. B. Race 1 inoculated 2 hr before race 3. C. Race 3 inoculated 2 hr before race 1. D. Race 1 only. E. Race 3 only.
[b] Total number of plants inoculated.
[c] Total number of plants from which *P. m. glycinea* could be reisolated.
[d] Number of plants from which only race 1 was reisolated.
[e] Number of plants from which only race 3 was reisolated.
[f] Number of plants from which both race 1 and race 3 were reisolated.

TABLE 3. Inoculation of cultivar Harosoy 63 with an avirulent (race 1) and a virulent (race 3) race

Inoculation treatment[a]	Plants inoculated[b]	Plants reisolated[c]	Race 1[d]	Race 3[e]	Races 1 and 3[f]
A. Race 1 + Race 3	68	35	7	25	3
B. Race 1 − Race 3 (2 hr)	30	6	2	4	0
C. Race 1 − Race 3 (8 hr)	45	24	10	9	5
D. Race 3 − Race 1 (2 hr)	37	17	5	10	2
E. Race 3 − Race 1 (8 hr)	45	32	5	21	6
F. Race 1	47	13	13	0	0
G. Race 3	42	24	0	24	0

[a] A. Race 1 and race 3 inoculated simultaneously. B. Race 1 inoculated 2 hr before race 3. C. Race 1 inoculated 8 hr before race 3. D. Race 3 inoculated 2 hr before race 1. E. Race 3 inoculated 8 hr before race 1. F. Race 1 only. G. Race 3 only.
[b] Total number of plants inoculated.
[c] Total number of plants from which *P. m. glycinea* could be reisolated.
[d] Number of plants from which only race 1 was reisolated.
[e] Number of plants from which only race 3 was reisolated.
[f] Number of plants from which both race 1 and race 3 were reisolated.

In Tables 2 and 3, reisolation of either race 1 or race 3 is more common than reisolation of both races from a single plant. This is primarily due to the reisolation of only a single colony from a root in the majority of cases (Table 4). Of interest, the number of colonies reisolated per root was independent of the inoculation treatment for Harosoy 63 ($\chi^2 = 9.8442$, df = 6, P = 0.1314). The number of colonies reisolated per root was not independent of the inoculation treatment in Williams ($\chi^2 = 9.8338$, df = 4, P = 0.0433). More than one colony was reisolated in 47% of the Williams roots and from only 31% of the Harosoy 63 roots.

DISCUSSION

To date, no distinguishing morphological (4) or biochemical (8) differences have been reported for races of *P. m. glycinea*. Thus, our drug-resistant mutants afforded the first opportunity to select for heterokaryons formed in planta. Because the drug resistance and virulence phenotypes did not change after passage through the plant, we also were able to use selection on agar plates with fluorotryptophan or metalaxyl as a rapid means of determining the virulence phenotype of the reisolated fungus. This circumvented problems in determining virulence phenotype in a mixed population of races and was a quicker, easier, and more sensitive method to characterize the reisolated fungus than testing the material by hypocotyl inoculation on soybean differential cultivars. For the first time we could attempt to determine the effect of one race on another during the early period of the infection process.

We were able to identify one true heterokaryon that had been formed in planta. This colony grew on plates containing both fluorotryptophan and metalaxyl after transfer from the original reisolation plate. In previous experiments, no colonies were observed on plates containing both drugs when homogenized mycelium or zoospores of two different drug-resistant mutants were mixed and plated. Thus, growth on both drugs is a stringent selection for heterokaryon formation. Also, zoospore progeny of this heterokaryon were of both drug resistance types, indicating that there was a mixture of nuclei in the mycelium. Because this isolate was formed from two race 1 isolates, no further characterization of the zoospore progeny was possible.

Four to 17% of the inoculated plants gave rise to small colonies that grew on the root sections in the presence of both drugs and may have been transient heterokaryons. In previous experiments using protoplast fusion to form heterokaryons, we noted that the ratio of parental nuclei and the virulence phenotype in some heterokaryons changed over a period of months (8,9). Thus, heterokaryons may be unstable soon after formation. Our reisolation of the heterokaryons from the plants may have been too early and thus may have led to the high number of transient heterokaryons. Reisolation of *P. m. glycinea* from older plants

was more difficult due to bacterial contamination. Therefore, we chose to reisolate at 48 hr after inoculation to increase the efficiency of reisolation, even though this may not be the best time to reisolate stable heterokaryons.

Ward (13) reported that he could isolate the avirulent race from the inoculation site up to 1 wk after inoculation but was unable to detect the avirulent race in the lesion away from the site, even when the virulent race had been inoculated first. Since we inoculated the entire root and then sectioned it for reisolation, we cannot determine whether the presence of the virulent race affected proliferation of the avirulent race. Perhaps we observed such a high reisolation rate (28%) of the avirulent race from resistant plants because we used a strong selection that did not require characterization of virulence phenotype. In Ward's experiments (13), a low concentration of the avirulent race mixed with the virulent race would have gone undetected because the mixture would have caused disease and been characterized as the virulent race alone.

Heterokaryon formation depends on proximity of both mycelial types and may be favored during growth in the plant. Passage through the plant of either of our two mutated races of *P. m. glycinea* alone did not alter their drug resistance or virulence phenotype. With regard to the formation of new virulent races, heterokaryon formation between a virulent and an avirulent race in a resistant plant would be significant. We observed that both races can be reisolated from resistant plants. Beagle-Ristaino and Rissler (2) observed that both the avirulent and virulent races will penetrate and form oospores inside resistant plants. If a heterokaryon forms between an avirulent and a virulent race, the possibility exists for a sexual cross between the races if different parental nuclei are in the oogonium and antheridium during oosporogenesis. This would generate heterozygosity at the avirulence locus and the potential for meiotic recombination. Thus, heterokaryon formation in planta could provide a means to generate new virulent races.

Inoculation of Williams and Harosoy 63 with both race 1 and race 3. We wanted to find out if zoospores of different races could affect each other during the inoculation and perhaps affect the outcome of the plant-pathogen interaction. We were surprised at the difficulty in reisolating *P. m. glycinea* from the roots and that we could successfully reisolate from less than half of the inoculated plants. In most cases, we only reisolated a single colony from the root. We used approximately 10,000 zoospores in each inoculation and recovered both races from inoculated roots regardless of the inoculation method or the cultivar used. Thus, we conclude that our results cannot be explained by a limited number of infection sites or a direct competition between race 1 and race 3 zoospores for such sites. There is indeed little evidence for any direct interaction of races, as seen by the results of the Mantel-Haenszel analysis of the inoculation data. The simplest explanation of our data is that the zoospores of both races act independently of each other and that the alteration of the response of the plant is the most important factor in determining the reisolation of a race.

Consider the results of the inoculation of Williams. Overall, there is no reduction in the reisolation of *P. m. glycinea* due to the method of inoculation. This suggests that both races are equally virulent and vigorous and that the interaction of the plant with either race is similar. When race 3 is inoculated before race 1, more race 3 is reisolated. When race 1 is inoculated first, more race 1 is reisolated. Thus, the inoculation of a susceptible plant with a virulent race appears to induce some resistance in the plant to a subsequent inoculation with another virulent race. This would agree in general with the observations that the phytoalexin glyceollin is produced in plants after inoculation with a virulent race (6).

Results from the inoculation of Harosoy 63 can also be adequately explained by our hypothesis that the reaction of the plant is the most important factor. As expected, when the root is first inoculated with the avirulent race, the number of colonies of either race reisolated is reduced. The timing of the inoculation is critical. If the avirulent and virulent races are inoculated simul-

TABLE 4. Number of colonies reisolated from cultivars Williams and Harosoy 63

| | Williams | | Harosoy 63 | |
Inoculation[a]	1[b]	>1[c]	1[b]	>1[c]
A. Race 1 + Race 3	21	12	30	5
B. Race 1 − Race 3 (2 hr)	15	27	5	1
C. Race 1 − Race 3 (8 hr)	nd	nd	14	10
D. Race 3 − Race 1 (2 hr)	15	15	12	5
E. Race 3 − Race 1 (8 hr)	nd	nd	17	14
F. Race 1	22	10	10	3
G. Race 3	17	11	16	8
Totals	90	75	104	46

[a] A. Race 1 and race 3 inoculated simultaneously. B. Race 1 inoculated 2 hr before race 3. C. Race 1 inoculated 8 hr before race 3. D. Race 3 inoculated 2 hr before race 1. E. Race 3 inoculated 8 hr before race 1. F. Race 1 only. G. Race 3 only.
[b] Number of roots from which one colony was reisolated.
[c] Number of roots from which more than one colony was reisolated.

taneously, there is no significant decrease in resolation of either race; there also is no significant effect if the avirulent race is inoculated 8 hr before the virulent race. Thus, the plant response must be activated within a certain period to be effective. Interestingly, when the virulent race is inoculated 8 hr after the avirulent race, it improves the reisolation of the avirulent race. Because we reisolated from the entire root, we cannot conclude that the subsequent inoculation of the virulent race had a suppressive effect on the plant's resistance response to the avirulent race. It is more likely that the successful infection by the virulent race killed enough cells to nourish the avirulent race. If a suppression of the resistance response had occurred, prior inoculation of the virulent race should have also significantly increased the frequency of reisolation of the avirulent race. We observed no significant increase in reisolation of race 1 in Harosoy 63 roots that had been previously inoculated with race 3.

Our general conclusion is that the interaction of roots with zoospores tends to increase the plant's resistance to a subsequent inoculation that occurs within 2 hr of the initial inoculation, independent of the virulence of the subsequently inoculated pathogen or the resistance of the cultivar inoculated. Further, because we were able to reisolate both races from both Williams and Harosoy 63, the opportunity for different races to be in close proximity in a plant is greater than previously imagined. This could increase the frequency of in planta formation of heterokaryons between races in the field, and, hence, the relative importance of the contribution of this mechanism to the formation of new races.

LITERATURE CITED

1. Athow, K. L., and Laviolette, F. A. 1984. Breeding soybeans for race-specific *Phytophthora* resistance. Pages 12-21 in: World Soybean Research Conference III: Proceedings. R. Shible, ed. Westview Press, Boulder, CO.

2. Beagle-Ristaino, J. E., and Rissler, J. F. 1983. Histopathology of susceptible and resistant soybean roots inoculated with zoospores of *Phytophthora megasperma* f. sp. *glycinea*. Phytopathology 73:590-595.

3. Ellingboe, A. H. 1983. Genetical aspects of interaction between plant hosts and their soilborne pathogens. Phytopathology 73:941-944.

4. Erwin, D. C. 1983. Variability within and among species of *Phytophthora*. Pages 149-165 in: *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, eds. Am. Phytopathol. Soc., St. Paul, MN. 392 pp.

5. Eye, L. L., Sneh, B., and Lockwood, J. L. 1978. Inoculation of soybean seedlings with zoospores of *Phytophthora megasperma* var. *sojae* for pathogenicity and race determination. Phytopathology 68:1769-1773.

6. Hahn, M., Bohnert, A., and Grisebach, H. 1985. Quantitative localization of the phytoalexin glyceollin I in relation to fungal hyphae in soybean roots infected with *Phytophthora megasperma* f. sp. *glycinea*. Plant Physiol. 77:591-601.

7. Keen, N. T., and Yoshikawa, M. 1983. Physiology of disease and the nature of resistance to *Phytophthora*. Pages 279-287 in: *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, eds. Am. Phytopathol. Soc., St. Paul, MN. 392 pp.

8. Layton, A. C., and Kuhn D. N. 1988. Heterokaryon formation by protoplast fusion of drug-resistant mutants in *Phytophthora megasperma* f. sp. *glycinea*. Exp. Mycol. 12:180-194.

9. Layton, A. C., and Kuhn, D. N. 1988. The virulence of interracial heterokaryons of *Phytophthora megasperma* f. sp. *glycinea*. Phytopathology 78:961-966.

10. Long, M., and Keen, N. T. 1977. Evidence for heterokaryosis in *Phytophthora megasperma* var. *sojae*. Phytopathology 67:670-674.

11. Schmitthenner, A. F. 1985. Problems and progress in control of *Phytophthora* root rot of soybean. Plant Dis. 69:362-368.

12. Shaw, D. S. 1983. The cytogenetics and genetics of *Phytophthora*. Pages 81-94 in: *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, eds. Am. Phytopathol. Soc., St. Paul, MN. 392 pp.

13. Ward, E. W. B. 1983. Effects of mixed or consecutive inoculations on the interaction of soybeans with races of *Phytophthora megasperma* f. sp. *glycinea*. Physiol. Plant Pathol. 23:281-294.