# Evidence for Heterokaryosis in Phytophthora megasperma var. sojae

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## ABSTRACT

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Heterokaryosis in *Phytophthora megasperma* var. *sojae* has been proven by the synthesis and degradation of heterokaryons from various auxotrophic and drug-resistant mutants. Three auxotrophic mutants (*his*, *thr*, and *lys* = histidine-, threonine-, and lysine-requiring mutants, respectively) were obtained from wild-type strains of race 1, and two (*met* and *pur* = methionine- and purine-requiring mutants, respectively) were obtained from race 3. Fluorophenylalanine-resistant mutants were recovered from races 1, 2, and 3 and cycloheximide-resistant mutants from races 1 and 3. All drug-resistant mutants had pathogenicity, growth rates, and sporulation that were indistinguishable from the corresponding wild types. However, none of the auxotrophic mutants produced normal oospores; only two (*his* and *met*) were pathogenic on soybeans, and only *met* produced normal zoospores. A heterokaryon synthesized from a drug-resistant mutant and its corresponding wild type behaved normally. However, although several heterokaryons were synthesized from auxotrophs, only *met* + *pur* heterokaryons were pathogenic and had growth rates and sporulation properties similar to the wild types.

*Additional key words*: auxotrophic mutants, drug-resistant mutants, genetics, soybeans.

We have engaged in a program designed ultimately to allow deduction of the inheritance of genes governing the expression of race characters [virulence genes sensu Flor (4)] in the soybean pathogen, *Phytophthora megasperma* (Drechs.) var. *sojae* A. A. Hildb. Since the organism is homothallic, the most direct approach appeared to involve the introduction of appropriate genetic markers into wild-type strains of the various fungus races and to form interracial heterokaryons with these strains. Stable heterokaryons then could be selfed and recombinant progeny containing both genetic markers selected and tested for pathogenicity to obtain data on the segregation of virulence genes. Although indirect evidence previously indicated that *Phytophthora* spp. undergo heterokaryosis (2, 5, 8, 11, 15, 17, 19), critical heterokaryosis tests (12) using nuclear genetic markers had not been preformed heretofore. We therefore set out to obtain suitable mutants of *P. megasperma* var. *sojae* and to use these in the synthesis of heterokaryons.

## MATERIALS AND METHODS

Three inbred wild-type strains of *P. megasperma* var. *sojae* [one each of races 1, 2, and 3 (10)] were used in the present work. Race designations were determined by inoculation of differential soybean cultivars as previously described (10).

**Isolation of auxotrophic and drug-resistant mutants from wild-type strains of Phytophthora megasperma var. sojae.**— The mutagens used in this work were ultraviolet light, nitrous acid, ethyl-methanesulfonate, and N-methyl-N$^1$-nitro-N-nitrosoguanidine (NG). All mutants reported here resulted from the use of NG. Uninucleate zoospores of *P. megasperma* var. *sojae*, produced as previously described (10), were induced to encyst by vigorous agitation and treated with NG (30 $\mu$g/ml) for 20-30 min, which resulted in 5-20% survival. Several different techniques were tested to increase selectively the number of surviving auxotrophic mutants [filtration enrichment, direct plating, bromodeoxyuridine (1), and rescue techniques]. Direct plating and filtration enrichment were the two methods used most extensively. Filtration enrichment involved introducing NG-treated cysts into minimal liquid medium (10), incubating for 72-96 hr at 25 C, filtering to remove hyphal growth, concentrating nongerminated cysts in the filtrate by centrifugation, and overlaying them onto complete medium agar (CmA) (minimal medium supplemented with 2 mg/ml each of casamino acids and yeast extract, Difco) (10). The resultant colonies were transferred to minimal medium agar (MmA) and CmA and those that did not grow on MmA were tested four more times at weekly intervals by transferring to MmA. If there was no growth on MmA after these successive transfers, the auxotrophic mutant was tested for nutritional requirements by the method of Holliday (7). Auxotrophic mutants were maintained on supplemented MmA.

Drug-resistant mutants were selected by direct plating of NG-treated cysts on MmA supplemented with *p*-fluorophenylalanine (Fpa) (5 $\mu$g/ml) or cycloheximide (Cyx) (5 $\mu$M). Visible colonies were transferred and

maintained on MmA containing 10 μg/ml of Fpa or 10 μM of Cyx, the concentrations on which wild-type mycelium did not grow.

**Synthesis and degradation of heterokaryons.**—Two methods were used to construct heterokaryons. In the first, 4-mm plugs were transferred from cultures of two different auxotrophic or drug-resistant mutants onto MmA or MmA containing Fpa and Cyx. The plugs were placed about 10-mm apart with 10 pairs of plugs per petri plate. A variation of this method was to add minced mycelium of two different mutants to MmA or drug-supplemented MmA. The second method used was to add minced mycelium of two different auxotrophic mutants to liquid Mm; the flasks were agitated once daily for 10-14 days; and the resultant mycelium was recovered, washed by centrifugation, resuspended, and the fragments were overlaid onto MmA. The colonies which developed were transferred to MmA or drug-supplemented MmA and only those which continued to grow after five successive transfers were studied further. In this paper, these colonies will be referred to as presumptive heterokaryons.

Hyphal-tip isolates of auxotrophic or drug-resistant presumptive heterokaryons were established on cleared V8-juice agar (V8-JA) (18) and tested for growth on MmA or drug-containing MmA, respectively. Hyphal-tip isolates which grew on MmA or drug-containing MmA will be referred to as heterokaryons.

A modification of Ho and Hickman's method (6) was used for production of zoospores from the many different heterokaryons studied. Five agar plugs (4 mm in diameter) of cultures on V8-JA, MmA, or supplemented MmA were transferred to a petri plate containing 5 ml of noncleared V8-juice (diluted 1/5) (18) and incubated in the dark at 25 C for 24 to 28 hr. The fungus was washed in situ five times with sterile water and finally 9 ml of sterile water were added to each plate. Sporangia were produced and zoospores released within 6 hr in most cases. The zoospores were allowed to encyst and germinate on water agar (WA) [Bacto-agar and/or Noble agar (Difco)] and colonies which were established on V8-JA from individually transferred germinated cysts were tested for growth on supplemented and minimal medium.

## RESULTS

**Production of mutants.**—Five well-characterized auxotrophic mutants were recovered from 40 mutagenesis experiments, two from filtration enrichment, and three from direct plating. A large number (50-75) of NG-treated cysts initially did not grow on MmA and appeared to be auxotrophs, but subsequently grew on MmA after several transfers onto CmA. Therefore, putative auxotrophic mutants were not screened for specific nutrient requirements until they failed to grow after five separate transfers onto MmA.

*Phytophthora megasperma* var. *sojae* is not a prolific producer of zoospores; consequently difficulty was experienced in obtaining sufficient numbers of zoospores for mutagenesis experiments. Up to 50% of the nongerminated cysts were lost by handling in the filtration enrichment technique, and since *P. megasperma* var. *sojae* is a relatively slow-growing fungus, the time required for prototrophic cysts to establish filterable mycelium (3-4 days) decreased the number of auxotrophs that survived and were recovered. Utility of the filtration enrichment procedure also was diminished by the propensity of cysts to germinate and form substantial germ tubes in the absence of nutrients. This behavior, subsequently observed with both auxotrophic mutants and wild types, probably caused substantial loss of auxotrophs in the initial filtration.

Cycloheximide- and Fpa-resistant mutants were less difficult to obtain, and five drug-resistant mutants were recovered from direct plating in three mutagenesis experiments. All grew consistently after five separate transfers onto drug-supplemented MmA.

**Characterization of mutants.**—All five auxotrophic mutants [methionine (*met*)-, purine (*pur*)-, lysine (*lys*)-, threonine (*thr*)-, and histidine (*his*)- requiring] were "nonleaky" mutants that grew relatively well on MmA supplemented with 2 mM concentrations of the required nutrient (for the *pur* mutant, guanosine routinely was used, but adenosine was equally effective). They all grew relatively poorly on V8-JA. Three auxotrophs (*thr*, *lys*, and *his*) were mutants of race 1 and the other two (*met* and *pur*) were mutants of race 3. Only two of the five

TABLE 1. Colonies established and nutritional types of single-hyphal-tip isolates and their zoospore progeny from five *met* + *pur* presumptive heterokaryons[a] of *Phytophthora megasperma* var. *sojae*[b]

| Presumptive heterokaryon[c] | Hyphal-tip isolates | | Zoospore isolates | | |
|---|---|---|---|---|---|
| | Colonies established (%) | Growth on minimal medium (%) | Colonies established (%) | Proportion of | |
| | | | | *met* | *pur* |
| 1 | 28 | 74 | 32 | 57 | 0 |
| 2 | 63 | 84 | 38 | 94 | 12 |
| 3 | 18 | 93 | 24 | 24 | 5 |
| 4 | 38 | 92 | 70 | 487 | 262 |
| 5 | 25 | 100 | 64 | 237 | 183 |

[a]The presumptive heterokaryons were constructed from a *met* = methionine- and a *pur* = purine-requiring mutant of *P. megasperma* var. *sojae*.

[b]Colonies were established from hyphal tips or zoospores on V8-JA (V8, juice agar) and tested for growth on minimal medium and/or minimal medium supplemented with methionine or guanosine.

[c]Zoospores were tested from four, five, two, three, and three different hyphal-tip isolates obtained from presumptive heterokaryons one, two three, four, and five, respectively.

auxotrophs (*his* and *met*) were pathogenic on soybeans; as expected, the *his* auxotroph was race 1 and the *met* auxotroph was race 3. None of the auxotrophs produced normal-appearing oospores and only one (*met*) produced normal sporangia and zoospores under the same conditions as did the wild types (10). Increasing the concentration of the required nutrient up to 10 times in MmA or supplementing V8-JA with the required nutrient did not improve production of these structures. Sporangia formed by the auxotrophs (except *met*) germinated directly or liberated mostly nonmotile zoospores or masses of undifferentiated cytoplasm. The nonmotile zoospores produced short germ tubes, but rarely established colonies. However, the few zoospores and oospores which did establish colonies were all of the auxotrophic parental type from which they were derived. After 1 yr of continuous subculturing, all auxotrophic mutants remained stable and grew well only on Mm supplemented with the specific requirement.

All of the drug-resistant mutants exhibited pathogenicity on soybeans, growth rates, and sporulation properties which were nearly identical to those of the wild-type isolates. However, growth was better on media not supplemented with drugs. Zoospores from one Fpa-resistant ($Fpa^R$) mutant (race 1), were uniformly $Fpa^R$ through three generations, but many second-generation zoospores from the other drug-resistant mutants were drug-sensitive. The observed somatic segregation in all the drug-resistant mutants except one suggests the operation of extrachromosomal elements.

**Construction of heterokaryons.**—Attempts were made to synthesize heterokaryons from all different combinations of the five auxotrophic mutants. In 25 experiments, four of these combinations were recovered as presumptive heterokaryons (*his* + *pur*, *thr* + *met*, *thr* + *pur*, and *met* + *pur*) and single-hyphal-tip isolates from them grew consistently on minimal medium, indicating that they were indeed heterokaryons. However, only the hyphal-tip cultures of *met* + *pur* presumptive heterokaryons produced normal sporangia, zoospores, and oospores. Therefore, five different *met* + *pur* presumptive heterokaryons were tested further. Colonies grew from hyphal tips at the same frequency on MmA as on V8-JA, but the time required for establishment on MmA (5-20 days) was considerably longer than on V8-JA (2-3 days); therefore our standard procedure was to establish colonies on V8-JA and test for growth on MmA. With one exception, zoospores from hyphal-tip cultures were all either *met* or *pur* (Table 1). The one exception was a prototrophic colony derived from a single zoospore which grew consistently on MmA and produced zoospores that were all prototrophic.

There were noticeable differences between the five presumptive heterokaryons tested for colony establishment on V8-JA from hyphal tips, growth on MmA, and the proportion of *met* to *pur* zoospores from hyphal-tip cultures (Table 1). However, zoospores that germinated on a more purified water agar [Noble (NWA) instead of Bacto (BWA)] established colonies less often and the proportion of *pur* survivors was considerably lower (Table 2). Therefore, it is doubtful whether the data in Table 1 reflect the actual ratio of *met* and *pur* nuclei. Altering the culture conditions (Cm or Mm supplemented with methionine or guanosine) before zoospore production did not influence the observed ratio of *met* to *pur* zoospores.

Like the parent *met* auxotroph, the *met* single zoospore progeny from *met* + *pur* heterokaryons produced normal zoospores which germinated relatively well (46%, average of four experiments), established colonies well (84%) and, like zoospores of the parent auxotroph, all required methionine. The *pur* single zoospore recovered from the heterokaryon established colonies that behaved like the parent *pur* auxotroph and did not produce motile zoospores.

All *met* + *pur* heterokaryons were fully as pathogenic as the wild type and expressed the race 3 phenotype. As in the constituent auxotrophs, the *met* zoospore progeny were pathogenic with the race 3 phenotype, but the *pur* zoospore progeny were nonpathogenic. All of the *met* + *pur* heterokaryons were pathogenic, but those with an observed nuclear ratio of about 1:1 (*met*:*pur*) were more aggressive.

From the drug-resistant mutants, presumptive

TABLE 2. Comparison of germination, colony establishment, and nutritional requirements[a] or *p*-fluorophenylalanine sensitivity ($Fpa^S$) or resistance ($Fpa^R$) of zoospores overlain on Bacto water agar (BWA) or Noble water agar (NWA) and transferred to V8-JA at 20 or 72 hours after germination. Zoospores were from *met* + *pur* and $Fpa^R$ + $Fpa^S$ presumptive heterokaryons of *Phytophthora megasperma* var. *sojae*

| Heterokaryons[b] | Germination (%) of zoospores on | | Colonies established (%)[c] by zoospores germinated on | | | |
|---|---|---|---|---|---|---|
| | | | BWA | | NWA | |
| | BWA | NWA | 20 hr | 72 hr | 20 hr | 72 hr |
| *met* + *pur* | ... | ... | 34 | 34 | 34 | 16 |
| $Fpa^R$ + $Fpa^S$ | 81 | 54 | 62 | ... | 59 | ... |
| Ratio of *met*:*pur* | | | 4:1 | 6:1 | 12:1 | 65:1 |
| Ratio of $Fpa^S$ : $Fpa^R$ | | | 1:1 | ... | 2:1 | ... |

[a]Abbreviations: *met* = methionine-requiring, and *pur* = purine-requiring of *P. megasperma* var. *sojae* zoospores from *met* + *pur* heterokaryons.

[b]Data for *met* + *pur* heterokaryons is cumulative for zoospores from 11 hyphal-tip isolates of presumptive heterokaryons one, two, and three. Data for $Fpa^R$ + $Fpa^S$ heterokaryons is cumulative for zoospores from six hyphal-tip isolates of one presumptive heterokaryon.

[c]Percentage colony establishment on V8-JA (V8, juice agar) by germinated zoospores transferred to V8-JA at 20 or 72 hr after germination.

TABLE 3. Colonies established and *p*-fluorophenylalanine resistance ($Fpa^R$) or sensitivity ($Fpa^S$) of hyphal tips and zoospores from hyphal-tip isolates of a $Fpa^R + Fpa^S$ presumptive heterokaryon of *Phytophthora megasperma* var. *sojae*[a]

| Hyphal tip isolations | | | Zoospores | | | |
|---|---|---|---|---|---|---|
| Colonies established (%) | Growth on Fpa medium (%) | Hyphal-tip isolate number | Germination (%) | Colonies established (%) | Proportion of: | |
| | | | | | $Fpa^R$ | $Fpa^S$ |
| 70 | 82 | 1 | ... | 78 | 16 | 9 |
| | | 2 | ... | 87 | 2 | 12 |
| | | 3 | 65 | 73 | 32 | 15 |
| | | 4 | 66 | 78 | 27 | 23 |
| | | 5 | 60 | 48 | 0 | 31 |
| | | 6 | 74 | 42 | 3 | 24 |

[a]The presumptive heterokaryon was constructed between a Fpa-resistant mutant and the corresponding wild type. Colonies were established on V8-JA (V8, juice agar) from hyphal tips or zoospores and tested for growth on Fpa-supplemented (10 μg/ml) and minimal medium.

heterokaryons and/or heteroplasmons were synthesized that contained Cyx and Fpa markers; these also were constructed from drug-resistant mutants and their respective wild-type strains. The $Cyx^R + Fpa^R$ cultures synthesized from drug-resistant mutants which had shown somatic segregation were considered to be presumptive heteroplasmons and data obtained from them is not presented here. Therefore, only data from the presumptive heterokaryon synthesized from the nonsomatic segregating $Fpa^R$ mutant and its corresponding $Fpa^S$ race 1 wild type is reported in this paper (Table 3).

Most of the hyphal-tip isolates from this $Fpa^S + Fpa^R$ presumptive heterokaryon grew on Fpa medium. Single zoospore progeny from hyphal-tip isolates segregated into two groups, $Fpa^S$ and $Fpa^R$ (Table 3). Second-generation zoospore progeny uniformly reflected the phenotype of the parents. Zoospores from the six different hyphal-tip isolates that were tested germinated equally well, but colony establishment and the proportion of $Fpa^S$ to $Fpa^R$ varied considerably (Table 3). Handling of zoospores reduced the percentage germination, but had little or no influence on colony establishment and apparent nuclear ratio (Table 2). The $Fpa^S + Fpa^R$ heterokaryons and their zoospore progeny were all pathogenic and expressed the race 1 phenotype on soybeans.

## DISCUSSION

Our observation that heterokaryons can be constructed from drug-resistant and auxotrophic strains of *P. megasperma* var. *sojae* and that they can be degraded to the respective monokaryons by isolation of single zoospores, is proof that heterokaryosis occurs in this fungus (12). These results confirmed previous indirect evidence (2, 5, 8, 11, 15, 19) and were predictable from the observations of Stephenson et al. (17) that anastomosis bridges were formed between hyphae of *Phytophthora capsici*. Assuming that hyphal fusion was the means by which our heterokaryons were formed, the frequency of heterokaryosis in *P. megasperma sojae* does not appear to be high because the frequency of heterokaryon recovery from our experiments was relatively low even though forcing techniques were used. Therefore, the common occurrence of heterokaryosis in nature would seem questionable. However, if heterokaryons are formed naturally by means other than hyphal fusion, such as mutation, polyploidy, and/or somatic crossing over, heterokaryosis may be of considerable importance in the biology of the fungus.

Whereas drug-resistant mutants of *P. megasperma* var. *sojae* were readily isolated, it was much more difficult to obtain auxotrophic mutants. One reason for this may be that enrichment techniques were not sufficiently effective with *P. megasperma* var. *sojae*. Another reason may be that *P. megasperma* var. *sojae* is diploid and auxotrophs, being recessive, would be detected only when present in both homologues. *Phytophthora* spp. now appear to be diploid (3, 13, 14, 16) and indeed our genetic data with *P. megasperma* var. *sojae* (9) are consistent with this conclusion.

The auxotrophic mutants have been stable for more than 1 yr, but they exhibit some characters detrimental to survival such as poor formation and germination of zoospores and oospores and complete loss of pathogenicity in three of the five auxotrophic mutants, even when the required nutrient was added with the inoculum. However, as will be discussed more fully in the following paper (9), recombinant progeny from the *met* + *pur* heterokaryons were pathogenic, and had improved sporulation.

The observed clear-cut segregation of auxotrophic (Table 1) and drug-resistant (Table 3) heterokaryons into the component parental types by analysis of zoospore progeny indicates that the mutants used in our heterokaryosis studies are nuclear markers and encourages their use in genetic studies.

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