POSSIBLE CYTOPLASMIC INHERITANCE AND ITS EFFECT ON THE PHENOTYPIC VARIABILITY IN PHYTOPHTHORA MEGASPERMA VAR. SOJAE.

by

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INTRODUCTION

Phytophora megasperma (Drech.) var. *sojae* A. A. Hildeb. (*Pms*), a fungal pathogen of soybean, *Glycine max* (L.) Merr., causes a root and stem rot of soybeans. *Pms* was first isolated by Suhovecky and Schmitthenner in 1951 from diseased soybeans growing in cultivated former lake beds in Ohio. The pathogen has since been found in every major soybean producing area in the United States and Canada, although not all races, distinguished by their pathogenicity on standard differential cultivars, have been found in one given area (23, 29, 33, 34, 35, 38).

In the last approximately 25 years, there has been considerable interest and research concerning the mechanisms causing asexual variation in various fungal groups: Ascomycetes (6, 18), Fungi Imperfecti (6, 15, 16, 19, 20, 23), Basidiomycetes (2), and Phycomycetes (4, 7, 26).

There is an increasing amount of research in the genus *Phytophthora* with respect to asexual variation. A study of cultural variation in this genus was made by Leonian (25). Adaptive changes in virulence of strains of *P. infestans* were reported by a number of workers, including Reddick and Mills (30), and Bruyn (3). Other species have been studied and several mechanisms have been proposed to explain asexual

variation: mutation and physiological adaptation in various species of Phytophthora (11); simple heterokaryosis in Pms (26); parasexuality in P. cactorum (4) and in P. infestans (9, 32); polyploidy in P. infestans (9); and cytoplasmic inheritance in P. cactorum (36), in P. infestans, (9, 40) and in Pms (26).

When Pms races 2 and 3 were cultured together, pathogenicity of colonies derived from the intersection of the two races was sometimes altered. The purpose of this study was to determine the most probable cause of this alteration in pathogenicity.

MATERIALS AND METHODS

Cultures of race 2 (UCR #406) and 3 (UCR #892) of Phytophthora megasperma var. sojae were received from N. T. Keen, University of California at Riverside. The race 2 culture was derived from a single zoospore, but the history of the race 3 culture is not known; therefore it cannot be inferred that the culture is homokaryotic as is the case for the race 2 culture.

Sanga and Calland, soybean cultivars used to distinguish Pms races 2 and 3, were used in pathogenicity tests. Sanga is susceptible to race 2 and resistant to race 3 (23) while Calland is resistant to race 2 and susceptible to race 3 (35). All plants were inoculated 10 days of age. The inoculation procedure involved making a longitudinal incision in the hypocotyl of the soybean, placing a small piece (approximately 3 mm. on a side) of fungus grown on Difco lima bean agar (LBA) in the incision, and applying petrolatum over the wound to prevent drying of the fungus (21). Numbers of dead plants were recorded at 4 and 10 days post-inoculation. A plant was considered dead if the hypocotyl had collapsed and the leaves had wilted or had begun to wilt.

The procedure of culturing the two Pms races together is a modification of the procedure used by Long and Keen in a

heterokaryosis study involving Pms (26). The two races were mass-transferred to individual plates of synthetic media developed by Ribiero et al. (31) in which all ingredients were included except B-sitosterol in order to prevent oospore production and hence, any possibilities of sexual reproduction. This media will be referred to as SYN-S. Ten days later, a sterilized #2 cork bore was used to make uniform 5 mm. plugs around the edge of the two colonies; these plugs were removed and paired with the opposite race on fresh SYN-S plates, with a distance of 1 cm. between members of a pair. Seven pairings were made. The cultures were grown at room temperature, approximately 27° C for 10 days. A block approximately 2x3x5 mm. was then taken from the agar midway between each of the seven pairs and plated on LBA. Three days later about 10 hyphal tips were taken from around each of the seven resultant colonies and transferred into fresh LBA plates to develop into colonies. Three extra hyphal tips were taken from one particular colony, referred to as the S-colony, that showed definite sectoring (Figure 1).

All of the hyphal-tip colonies were monitored for sectoring and relative oospore density differences. In addition, inoculations were made to Sanga and Calland to determine possible changes in pathogenicity from those of the parent cultures (races 2 and 3). Colonies derived from three hyphal tips of each of the six normal colonies, five hyphal tips of the S-colony (designated S-1 through S-5), and mass

Figure 1. Flow diagram of cultures used for inoculations.

1 plate (SYN-S) with 7 pairs of race 2 and race 3

*

7 plates (LBA) with 7 blocks taken from the points of intersection of races 2 and 3

*

hyphal tips from each of the 7 resultant colonies

*

inoculations---hyphal tip colonies of the other 6 colonies

*

hyphal tips of S-colony (7th colony)

S-1, S-2, and S-3

1) Original S-1 and S-2 cultures
   a) inoculation of the hyphal tips from 7 colonies into Sanga and Calland (7 days old)[a]
   b) inoculations of the other S-colony hyphal tips including S-1 into Sanga and Calland (21 days old)
   c) 1st inoculation with S-1 and S-2 (S-3 included) into differentials (23 days old)
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   b) S-1-1 into differentials (8 days old)
6) Reisolations
   a) 1st inoculation to Sanga and Calland (3 days old)
   b) 2nd inoculation on Sanga and Calland (3 days old)

S-4 and S-5

1) Original S-4 and S-5 cultures inoculated into Sanga and Calland (7 days old)
2) Inoculation of single zoospore isolate cultures
   a) 3 S-4 and 1 S-5 zoospore cultures inoculated into Sanga and Calland (2 days old)
   b) 2 of the same S-4 and the same S-5 zoospore cultures inoculated into differentials (4 days old)

[a]Age of culture used as inoculum

transfers of the original race 2 and race 3 isolates were used to inoculate sets of 10 Sanga and 10 Calland soybean plants (all cultures 7 days old). The race 2 and race 3 isolates alone, along with a mixture of race 2 and race 3 were used for controls. Apparent deviations from the pathogenicity pattern of the parent races were detected only in the three S-derived colonies (S-1, S-2, and S-3). Therefore, five more S-colony hyphal tip cultures (21 days old) were tested for their reaction on Sanga and Calland. Four hyphal tips of each of two colony isolates, S-1, and S-2, were made following a mass transfer from each of the respective original hyphal tip colonies. Each of the eight resultant colonies (4 days old) was inoculated into at least 10 plants of each of the cultivars. A previous loss in pathogenicity in some of the S-colony isolates and then an apparent reversion to pathogenicity in these hyphal tip colonies indicated the need for greater numbers, so more inoculations were made. Accordingly, 8 and 9 days after the previous inoculations one of the colonies derived from an S-1 hyphal tip and one derived from an S-2 hyphal tip (12 and 13 days old) were used to inoculate at least 22-24 plants each of Sanga and Calland, and the following day 40-44 more plants of each cultivar were inoculated with each of the same two hyphal tip colonies.

The possibility existed that changes in pathogenicity might be better detected by extending the host range tested. Accordingly, isolates S-1, S-2, and S-3 (23 days old) along with race 2 and race 3 as controls were inoculated into seven

of the standard differental cultivars: Columbus, Sanga, Calland, Mack, Altona, P.I. 171 442, and Tracy (23, 35) with not less than 10 plants for each differential cultivar being inoculated per culture. Five days later the original S-1 and S-2 cultures (28 days old) were inoculated into 10 plants of each of the differential cultivars for each isolate.

In the first S-1 and S-2 inoculations pathogenicity appeared to be considerably reduced, however, in subsequent inoculations with the same cultures pathogenicity was reduced. The following inoculations were performed to get a better idea of the pathogenicity of these cultures. The same S-1 and S-2 cultures (now 28 days old) were used as inoculum on 98-103 plants of both Sanga and Calland. In addition one-month-old race 2 and race 3 cultures were used as controls with 10-11 plants of Sanga and Calland being inoculated for each isolate.

A mass transfer was made from each of the original S-1 and S-2 colonies and these (15 days old) were used to inoculate 10 Sanga and Calland plants in order to see if pathogenicity was regained and to what extent. This was repeated four days later using the same inoculum on 53-75 Sanga and Calland plants. One more mass transfer was made from the original culture and the resulting colony was used as inoculum after two days of growth on a set of 10 Sanga and Calland soybeans to see if pathogenicity might be regained in the young mycelium.

S-1 and S-2 were reisolated from Sanga plants 10 days after inoculation to see if there might be a change in pathogenicity. One S-1 and three S-2 reisolates were obtained.

The S-1 and S-2 reisolates (3 days old) along with mass transfers from the original cultures of races 2 and 3 were inoculated into standard sets of 10 Sanga and Calland plants. This inoculation was repeated using a 3-day-old transfer from the same colony for inoculum as before. The same number of plants was inoculated.

A study of the pathogenicities of single zoospore isolates was made to see if simple heterokaryosis was involved. Zoospores were produced by the technique developed by Eye and Sneh (12) for races 2 and 3 and isolates S-1, S-2, S-4, and S-5. S-5 is an S-colony hyphal tip culture having race 3 pathogenicity. The isolates were grown out on LBA for 4-5 days in the dark at 20° C at which time the colonies were washed every 20 minutes for five washings with 15 ml. of sterilized tap water. The plates were returned to the 20° chamber after each washing. Following the last washing, they were returned to the 20° C chamber for 6 hours at which time the zoospores were removed by a sterile Pasteur pipet and spread out onto water agar to await germination. Six hours later, germinated zoospores were removed by the use of a fine-tipped scalpel removing a small cube of agar with the zoospore on top of it and each placed on an individual plate of LBA. Over a period of 4 days, colonies (3-6 days old) arising from 10 race 2 and race 3 zoospores and from 20 S-1 and S-2 zoospores were inoculated into sets of 10 Sanga and 10 Calland soybeans. To check for possible changes in pathogenicity with

colony age, single zoospore isolate S-1-1, the first single zoospore isolate (8 days old) inoculated of the S-1 isolate, was inoculated 5 days later into 5 plants of each of the differential cultivars.

It seemed useful at this time to extend the range of isolates tested, though on a smaller scale. Accordingly, the original S-4 and S-5 cultures (21 days old) were inoculated into a set of 10 Sanga and Calland soybean plants each. Three S-4 and one S-5 zoospore isolates (2 days old) were inoculated into sets of 10 Sanga and Calland soybeans. Two of the three S-4 single zoospore isolates and the same S-5 zoospore isolate (4 days old) were also inoculated into the seven differential cultivars with five of each cultivar being inoculated with each isolate.

In order to see if SYN-S could have induced the colony variation observed, races 2 and 3 were grown on SYN-S for 10 days, then 40 mass transfers each were planted onto LBA plates to look for sectoring effects similar to those seen in the S-colony.

In a search for other possible indicators of possible hyphal anastomosis, differences were sought in two measurable colony characteristics, oospore density and colony diameter. S-1, S-4, S-5, and races 2 and 3 were plated onto clarified V-8 juice agar (CV-8A) (39) to get uniform age cultures, then 5 mm. diameter plugs were transferred to other CV-8A plates. Three replications were made for each isolate, and all plates

were randomized and placed in a dark growth chamber at 20° C for 8 days. At the end of 8 days the colony diameters were measured three times for each colony and the average of the three measurements was recorded as the colony diameter. Oospore density measurements were made for each replication by the use of a Bio Tran II Automated Colony Counter (New Brunswick Scientific Co., Inc.). Twenty 40X fields were counted for each colony around a 20 mm. diameter circle centered at the center of the colony. Colony diameter and oospore densities were analyzed statistically to determine differences among isolates.

To detect changes in the ability of the single zoospore isolates to produce oospores and to sector during zoospore formation, transfers of 15 different single zoospore isolates of race 2, 15 of race 3, 21 of S-1, 25 of S-2, 23 of S-4 and 29 of S-5 were made onto LBA plates. These were observed for sectoring over a period of six days and at the end of 30 days relative oospore numbers were recorded.

RESULTS

Of the seven colonies that resulted from transfers of the seven race 2 and race 3 intersections occurring on SYN-S only one colony showed any obvious cultural peculiarity. The colony, referred to as the S-colony, had several sectors; one large sector formed a "wedge" protruding from the main body of the colony. This latter sector is the area from which the hyphal tips, S-1 and S-2, were taken.

Colonies that resulted from the hyphal tips of each of the six normal colonies grown from the agar block taken at the intersection of races 2 and 3 did not show any sectoring and the relative oospore densities were similar to those of races 2 and 3. The 13 hyphal-tip-derived colonies from the S-colony sectored by irregular projections from the main body of the colony except one, regardless of where on the S-colony the hyphal tips were taken, designated S-5. Cultures all of the same age of the S-colony derivatives had approximately 5 oospores per 20X field in comparison to about 21 oospores per field for the other six normal colonies derived from hyphal tips and (for the race 2 and 3 colonies all of the same age), again except for the S-5 which had approximately 21 oospores per field. The statistical analysis of oospore density and colony diameter for races 2 and 3, S-1, S-4, and S-5 showed

that at the .05 level there were no significant differences among isolates for rate of growth, as determined by colony diameter; but that there were significant differences among oospore densities. An lsd .05 comparison indicated that the only significant differences were between races 2 and 3, S-4 and S-1, S-4 and S-3, and between S-4 and S-5 (Table 1). All previous observations of oospore densities indicated that the oospore density of S-1 was similar to S-4, but they differed in the study in which actual counts were made.

In the initial pathogenicity tests of the hyphal-tip-derived isolates of the seven different intersections of races 2 and 3 (7 days old), the colonies derived from hyphal tips of the six normal colonies gave typical race 2 reactions. The colonies derived from the hyphal tips of the S-colony gave mixed reactions; three, designated S-1, S-2, and S-3, killed 20, 40, and 40 percent of Sanga soybeans, and none of the Calland soybeans; one (S-4) was pathogenic only to Sanga (race 2); and another (S-5) was pathogenic only to Calland (race 3). The controls behaved normally; race 2 killed only Sanga plants, race 3 killed only Calland plants, while the mixture of races 2 and 3 killed both (Table 2).

When space became available, five more colonies (21 days old) derived from S-colony hyphal tips were tested for their pathogenity on Sanga and Calland plants; reactions were not clear cut, but resembled race 2 pathogenicity. The original S-1 colony was used as a control; it killed 8 out of 10 Sanga

TABLE 1. Comparison of the mean oospore densities of races 2 and 3 and three S-colony isolates.

Isolate	Mean	
Race 3	28.6	a*
S-1	21.2	a b
S-5	20.7	a b
Race 2	14.2	b c
S-4	4.7	c

*means followed by a common letter are not significantly different at the .05 level; lsd .05 = 9.929.

TABLE 2. Results of initial pathogenicity tests of hyphal-tip isolates from colonies derived from the 7 points of intersection of race 2 and race 3 when grown together on SYN-S.

Colony source[ab]	Perentages[c]	
	Cultivar	
	Sanga	Calland
1	100	0
1	100	0
2	100	0
2	90	0
2	100	0
3	100	0
3	100	0
3	100	0
4	100	0
4	100	0
4	100	0
5	100	0
5	100	0
5	100	0
6	100	0
S-1	20	0
S-2	40	0
S-3	40	0
S-4	90	0
S-5	30	100
Control Race 2	100	0
Control Race 3	0	100
Control Race 2 and Race 3 combined	100	100

[a]Colonies 1-7 derived from the seven points of intersection; 7th colony also labeled as S-colony.

[b]All colonies 7 days old.

[c]Percent of 10 plants killed (7 days old).

plants and no Calland plants indicating that the pathogenicity to Sanga was regained since the same inoculum source 12 days earlier was nonpathogenic (Table 3). Yet, 7 days later, the original S-1 and S-2 cultures (28 days old) were used to inoculate 98-103 plants of each cultivar; the pathogenicity toward Sanga was again lost while the controls behaved normally (Table 4).

When S-1 and S-2 (23 days old) were inoculated into a full set of seven differentials, both isolates behaved like race 2 by killing Columbus and Sanga, but nothing else (Table 5). After 5 more days S-1 and S-2 (28 days old) from the original two cultures were again inoculated into the differentials; pathogenicity was lost even on Columbus which is susceptible to all known races (Table 6).

Four day old cultures derived from hyphal tips of S-1 and S-2 were inoculated into Sanga and Calland; all were pathogenic on Sanga (Table 7). In subsequent inoculations, when the same hyphal tip cultures (12 and 13 days old) their pathogenicity was essentially lost again (Table 8).

Fifteen-19-day-old colonies derived from the first mass transfers of the original S-1 and S-2 cultures were used to inoculate Sanga and Calland plants; no Calland plants were killed. This was also the case with inoculation involving the original S-1 and S-2 colonies. However, the S-1 mass transfer killed 10 and 8 percent of the Sanga plants instead of 2 percent and the S-2 mass transfer colony killed 10 and 4

TABLE 3. Inoculation results of additional S-colony isolates.

Cultivar	Percentages[a]				Additional S-Colony Isolates				
	Isolate[b]								
	Race 2	Race 3	S-1	S-5					
Sanga	100	20	80	0	70	100	90	100	100
Calland	0	100	0	100	0	0	0	0	0

[a]Percentage of ten plants killed.

[b]21 days old.

TABLE 4. Results of the inoculation of Sanga and Calland with the original S-1 and S-2 isolates.

	Percentages			
	Isolate[a]			
Cultivar	Race 2	Race 3	S-1	S-2
Sanga	100[b]	14[b]	3[c]	13[c]
Calland	0[b]	100[b]	0[c]	0[c]

[a]28 days old.

[b]Percentage of plants killed out of 21 plants inoculated.

[c]Percentage of plants killed out of 98-103 plants inoculated.

TABLE 5. Inoculation results of S-1, S-2, and S-3 on differentials for the first inoculation.

	Percentages[a]						
	Cultivar						
Isolate[b]	Columbus	Sanga	Calland	Mack	Altona	P.I.171 442	Tracy
Race 2	100	100	0	0	0	0	0
Race 3	100	0	100	0	58	0	0
S-1	100	80	0	0	0	0	0
S-2	100	73	0	0	0	0	0
S-3	100	93	0	0	0	0	0

[a]Percentage of plants killed out of at least 10 plants inoculated.

[b]23 days old.

TABLE 6. Inoculation results of S-1 and S-2 on differentials for the second inoculation.

Isolate[a]	Percentages[b]						
	Cultivar						
	Columbus	Sanga	Calland	Mack	Altona	P.I.171 492	Tracy
S-1	9	0	0	0	0	0	0
S-2	0	0	0	0	0	0	0

[a]28 days old.

[b]Percentage of plants killed out of 10 plants inoculated.

TABLE 7. First hyphal tip inoculation results.

	Percentages[a]											
	Isolates[b]											
				Hyphal Tip					Hyphal Tip			
Cultivar	Race 2	Race 3	S-1	S-1a	S-1b	S-1c	S-1d	S-2 parental	S-2a	S-2b	S-2c	S-2d
Sanga	100	7	21	100	100	100	100	17	100	100	100	90
Calland	0	100	0	0	0	0	0	0	0	0	0	0

[a]Percentage of inoculated plants killed out of 10 plants inoculated.

[b]4 days old

TABLE 8. Second hyphal tip inoculation results.

Isolate[a]	Percentages[b]	
	Cultivar	
	Sanga	Calland
S-1	5	0
S-2	2	0

[a]12 and 13 days old.

[b]Percentage of plants killed out of 62-68 plants inoculated.

percent of the Sanga plants instead of 13 percent on the two respective dates. The pathogenicities of these cultures were still lower than the race 2 control which killed 100 percent of the Sanga plants inoculated (Table 9). The two day old colony of an additional mass transfer from S-1 did not kill any of the Sanga or Calland plants inoculated.

From the results of inoculations with the single zoospore isolates of race 2, race 3, S-1, and S-2, race 2 retained its normal pathogenicity with respect to Sanga and Calland except that some of the zoospore isolates killed up to 4 out of the 10 Calland plants rather than not killing any. The race 3 zoospore isolates killed up to 10 out of 10 Sanga plants rather than not killing any while still remaining pathogenic to Calland. The number of Sanga plants killed by the 20 S-1 single zoospore isolates ranged from 2-9 out of 10 plants and that for the 20 S-2 single zoospore isolates ranged from 3-9 out of 10 plants while none of the Calland plants were killed (Table 10).

When zoospore isolate S-1-1 (8 days old) was used 5 days later to inoculate a set of differential cultivars, the race 2 pathogenicity pattern had been regained (Table 11).

Inoculation of Sanga and Calland using single zoospore isolates (2 days old) of S-4 and S-5 (Table 12) indicated that the three S-4 zoospore isolates behaved like race 2 as usual. The S-5 single zoospore isolate killed 5 Sanga plants instead of the 2 out of 10 observed in the inoculation of Sanga plants

TABLE 9. Results of the two inoculation separated by 4 days with a culture derived from the first mass transfer of the original S-1 and S-2 colonies.

	Percentages[a]			
	Isolates			
	S-1		S-2	
	1st[b]	2nd[c]	1st[b]	2nd[c]
Sanga	10	8	10	4
Calland	0	0	0	0

[a]Percentage of plants killed out of 53-75 plants inoculated.

[b]15 days old.

[c]19 days old.

TABLE 10. Results of single zoospore isolate inoculations for race 2 and 3 and S-1 and S-2.

Zoospore Number Designation	Percentages[a]							
	Isolates[b]							
	Race 2		Race 3		S-1		S-2	
	Sanga	Calland	Sanga	Calland	Sanga	Calland	Sanga	Calland
1	100	0	100	100	40	0	50	0
2	100	10	80	100	60	0	40	0
3	100	0	90	100	70	0	60	0
4	100	0	40	100	40	0	50	0
5	100	30	50	100	60	0	50	0
6	100	10	70	100	60	0	30	0
7	100	20	50	100	80	0	90	0
8	100	40	40	100	60	0	80	0
9	100	10	30	100	90	0	70	0
10	100	40	60	100	30	0	60	0
11					30	0	40	0
12					90	0	30	0
13					70	0	60	0
14					60	0	50	0
15					90	0	30	0
16					20	0	30	0
17					70	0	60	0
18					60	0	80	0
19					40	0	60	0
20					20	0	50	0

[a]Percentage of plants killed out of 10 plants inoculated.

[b]3-6 days old.

TABLE 11. Results of the inoculation of the differentials with an S-1 single zoospore isolate.

	Percentages[b]						
	Cultivar						
Isolate[a]	Columbus	Sanga	Calland	Mack	Altona	P.I. 171492	Tracy
S-1 Isolate	100	100	0	0	0	0	0

[a]8 days old.

[b]Percentage of plants killed out of 10 plants inoculated.

TABLE 12. Results of inoculation of Sanga and Calland with single zoospore isolates of S-4 and S-5.

Cultivar	Percentages[a]					
	Isolate[b]					
	S-4 Original	S-4-1 Zoospore	S-4-2 Zoospore	S-4-3 Zoospore	S-5 Original	S-5-1 Zoospore
Sanga	0	100	100	100	20	50
Calland	0	0	0	0	100	100

[a]Percentage of plants killed out of 10 plants inoculated.

[b]2 days old.

with the original S-5 colony. Otherwise S-5 gave a normal race 3 reaction. When the S-4 zoospore isolates (4 days old) were inoculated into the seven differentials, they gave a normal race 2 reaction except that the first single zoospore isolate of S-4 killed 3 out of the 5 Altona plants. S-5, when inoculated into the seven differentials, gave an unusual pathogenicity reading with all the differential cultivars being susceptible, even Tracy using the same culture of the S-5 zoospore and on the same date as the inoculation of the seven differential cultivars (Table 13).

One culture of S-1 and three cultures of S-2 reisolated from Sanga plants ten days after inoculation were used to inoculate 10 plants each of Sanga and Calland; races 2 and 3 were used as controls. The controls behaved normally, while the reisolated cultures (3 days old) killed all of the Sanga plants, but any Calland plants (Table 14). When this was repeated with another 3 day old reisolated culture the same results were obtained.

When races 2 and 3 were grown on SYN-S media and then plated onto LBA to see if the SYN-S media was causing the sectoring and decrease in oospore density, no sectoring or reduction in oospore density was observed in the 40 race 2 and 40 race 3 isolates after seven days.

It was also observed that the single-zoospore-derived colony morphology of race 2 and race 3 were all nearly the same. However, 24 percent of the S-1 zoospore isolates

TABLE 13. Results of the inoculation of differential varieties with S-4 and S-5 zoospore isolates.

Isolate[a]	Percentages[b]						
	Cultivar						
	Columbus	Sanga	Calland	Mack	Altona	P.I. 171492	Tracy
S-4-1 Zoospore	100	100	20	20	60	0	0
S-4-2 Zoospore	100	100	0	0	20	0	0
S-5 Zoospore	100	100	100	80	100	100	80

[a] 4 days old.

[b] Percentage of plants killed out of 5 plants inoculated.

TABLE 14. Results of the inoculation with reisolates of S-1 and S-2.

Cultivar[a]	Percentages[b]					
	Isolate					
	Race 2 Original	Race 3 Original	S-1a Reisolate	S-2a Reisolate	S-2b Reisolate	S-2c Reisolate
Sanga	100	14	100	100	100	100
Calland	0	100	0	0	0	0

[a] 3 days old.

[b] Percentage of plants killed out of at least 10 plants inoculated.

sectored, 32 percent of the 25 S-2 isolates sectored, 41 percent of the 23 S-4 isolates sectored, while none of the 29 S-5 single zoospore isolates sectored. The oospore densities of all of the race 2, race 3, and S-5 zoospore isolates were high (approximately 20 oospores per 40X field) and those of the S-1 and S-2 zoospore isolates were all low (approximately 5 oospores per field), while 21 of the 23 S-4 zoospore isolates were low and the other two had high densities (Table 15).

TABLE 15. Results of zoospore colony observation to look for sectoring and changes in oospore density.

	Percentages[a]					
	Isolate					
	Race 2	Race 3	S-1	S-2	S-4	S-5
Sectoring	0	0	24	32	59	0
Oospore Density	high[b]	high	low[c]	low	91%[d] low	high

[a]Percentage of cultures sectoring out of 15-79 zoospore colonies for each percent culture.

[b]high = approximately 21 oospores/40X field.

[c]low = approximately 5 oospores/40X field.

[d]91% had a low density but the rest (2 cultures) had a low density.

DISCUSSION

As mentioned in the Introduction, several mechanisms have been proposed to explain asexual variation in the genus Phytophthora: mutation, polyploidy, physiological adaptation, simple heterokaryosis, parasexuality, and cytoplasmic inheritance.

Most of the work reported has been in the area of simple zoospore variation and variation due to hyphal anastomosis, and most have been done with P. infestans. However, both types of studies can be used to help interpret the results of this present study.

Mutation might explain some asexual variation. Stamps (36) suggests nuclear mutation as a cause for colony variation in single zoospore isolates of P. cactorum, but due to the gradual nature and ready reversibility of some of the transformations, he concluded that cytoplasmic change explained the variation better. No confirmation of either was possible at the time (36). Buddenhaggen (4), who worked with artificially induced mutants of P. cactorum, concluded that the wide variability of single zoospore isolates could not be completely explained by artificially induced mutants in view of Stamp's previously cited work, and he suggested other possibilities. Mutation is not a probable explanation for the observations in the present study because the reversion of S-1 and S-2

isolates from nonpathogenic to pathogenic and then back to nonpathogenic indicates that it was not involved.

Polyploidy and aneuploidy are other possible explanations for the variation observed. Denward (9), in a study involving P. infestans, proposed that if single pathogenicity genes are allelic and manifest phenotypic specificity independently, the acquisition of complex pathogenicity would be restricted to increased polyploidy or through aneuploidy with manifold repetition of the chromosome or linkage group in which the pathogenicity locus was situated. This, however, implies that the organization of Phytophthora nuclei deviates from the traditional pattern of nuclei in higher organisms. There is some evidence that Pms is a polyploid (41), but pathogenicity studies with various races of Pms have shown that there is an allelic series that determines complex pathogenicities (1, 14, 34).

Caten and Jinks (7) briefly considered the possibility that single zoospore variation was due to physiological adaptation, but since at no time during the experiment were the zoospore isolates exposed to different environments which might have led to differentiation through adaptation, this possibility was discounted. S-1 and S-2 variation due to this does not seem probable because all cultures were maintained at an ambient room temperature of approximately 27° C. SYN-S could have caused a stress situation that in turn caused the

sectoring, the reduced oospore density and the alteration in pathogenicity; but when races 2 and 3 were grown separately on the SYN-S, no sectoring or reduced oospore density occurred out of 40 plates used for each race. Alteration in pathogenicity was not tested for because of the previous negative results.

The three possibilities mentioned do not appear to be of considerable importance. Three other mechanisms are similar in that each requires an exchange of genetic material: nuclei in the case of simple heterokaryosis and parasexuality, or a cytoplasmic factor in the case of cytoplasmic inheritance. The exchange of genetic material that could have occurred in this present experiment was probably through hyphal anastomosis. Evidence for this is given by Stephenson et al. (37) for P. capsici. Since this is probably the only method of genetic exchange pertinent to this study considering the procedure followed, it will be the only one discussed further.

The formation of single heterokaryons and formation of complex heterokaryons through the parasexual cycle is very similar in that in the simplest case both require at least two different kinds of nuclei.

When this situation exists simple heterokaryosis is complete, but parasexuality involves further nuclear fusion, somatic recombination, mitotic crossing-over, and eventual haploidization of the fused, diploid nuclei. A simple heterokaryotic mycelium, therefore, has only the two parental type

nuclei, but a simple heterokaryotic mycelium that has undergone the parasexual cycle will have more than the two parental type nuclei. The best way to determine if simple heterokaryosis or parasexuality is involved is to look at the phenotype of the single zoospore progeny. If parasexuality is involved there will be several different phenotypes in addition to the parental phenotypes.

There are no clear reports of simple heterokaryosis occurring in the genus Phytophthora except in the work of Long and Keen with Pms (26). It is not considered a likely mechanism in the present study because the single zoospore isolates did not show both parental types. The S-1 and S-2 zoospore isolates were all pathogenically more closely related to the race 2 parental culture than that of race 3, in that they killed some Sanga plants but no Calland plants (Table 10). This indicates that the race 3 nucleus was not present in the S-1 and S-2 mycelia. It is possible that simple heterokaryosis did occur and as Jinks has suggested, the parental genotypes of the zoospore derived cultures could be masked by the cytoplasmic effects (20). It is doubtful, however, that the race 3 pathogenicity would have been inhibited so completely and not be seen in any of the single zoospore isolates of S-1 and S-2.

Work with P. infestans indicates that a parasexual cycle could be involved (9, 24, 32). The presence of several different nonparental nuclei resulting from mitotic crossing-over

and haploidization would explain the non-reversion of S-1 and S-2 single zoospore cultures back to the parental pathogenicity types and also the wide range of plants being killed; but Long and Keen have shown with Pms that the two different nuclei in a simple heterokaryon remain distinct at least up to the time they caused zoospore production (26). In addition, there were no zoospore-derived colonies that pathogenically resembled race 3 indicating that a race 3 nucleus was probably not involved. There is also a question as to whether the vegetative phase of the Phytophthora species is haploid or diploid. This question is discussed in more detail by Elliot and Mac Intyre (10). The importance of knowing the answer to this question is that the parasexual cycle requires a haploid vegetative stage. Long and Keen (27) report from their work with heterokaryons that Pms appears to be vegetatively diploid. To be more certain that parasexuality is not involved in the S-1 and S-2 isolates, it would be necessary to test the pathogenicity of successive generations of asexually produced zoospores. If the variability would continue through several generations this would indicate cytoplasmic inheritance. If, however, there were little variability in the pathogenicity of the zoospores then the parasexual cycle would be the most likely explanation.

The last mechanism to be considered is cytoplasmic inheritance. This involves the addition to or alteration of

cytoplasmic genetic factors in a given mycelium. A body of evidence exists in support of cytoplasmic inheritance. MacIntyre and Elliot (28) concluded that growth rate in P. cactorum is controlled by a polygenic system and by cytoplasmic determinants. Upshall (40) interpreted the variation among single zoospore progeny of P. infestans to be caused by cytoplasmic inheritance, but there was no critical proof. Caten and Jinks (7) claimed that cytoplasmic control gave the best agreement with the observed results and involves the fewest assumptions to explain spontaneous variability of cultural characteristics. Leach and Rich (24) proposed as an alternative to heterokaryosis and parasexuality that the mixing of cytoplasm may have caused the increased pathogenicity similar to that occurring in the rust fungi indicating the possibility of genetic interaction involving cellular components of two genetically different mycelia. Sectoring of the S-colony while the others remained normal indicated that there was some genetic alteration of that particular Pms isolate, presumably due to the procedure followed to bring about hyphal anastomosis. The subsequent sectoring of all hyphal tip colonies of the S-colony and their reduced oospore density, except for S-5 in both cases, further indicated that there was a genetic alteration that caused them to be different from the controls and the other hyphal tips from the other six colonies. The reversion of the S-1 and S-2 isolates from nonpathogenic to pathogenic and back to

nonpathogenic, as seen in the inoculations of Sanga and Calland as well as in the inoculation of differentials (Tables 2, 3, 4, 5, and 6), points to an unstable genetic situation apart from the nucleus. The same pathogenicity reversion occurred in the hyphal-tip-derived colonies of the S-1 and S-2 isolates (Tables 7 and 8). The reversion of the S-1 and S-2 reisolates (Table 14) back to the pathogenic condition was apparently irreversible; subsequent inoculations of S-1 and S-2 into Sanga and Calland with consistent retention of nonpathogenicity indicate that nonpathogenicity became more stable with age of the culture. This could be due to the establishment of a genetic factor throughout the colony cytoplasm.

Jinks (20) describes three aspects of extranuclear change that could have occurred in this experiment. An extrachromosomally located cell component could have resulted from hyphal anastomosis and cytoplasmic exchange. Next, the cell component could have been physically lost when the S-1 and S-2 isolates were in the Sanga plants from which they were re-isolated. Finally, there could have been a change in the relative frequency or activity of the cell component when single zoospores were produced. This last aspect was seen with respect to pathogenicity when 20-90 percent of the Sanga plants were killed by the S-1 and S-2 zoospore colonies (Table 10) rather than the 3-13 percent killed by the parent

cultures (Table 4). This last aspect was also seen with respect to sectoring in culture when 24-41 percent of the S-1, S-2, and S-4 zoospore colonies sectored (Table 15), but all of the parent cultures sectored. These observations indicate the possibility that the cytoplasmic factors caused the inhibition of pathogenicity and the sectoring in some of the zoospores when the zoospores were formed. (More than one cytoplasmic factor is considered to be involved because not all of the S-colony isolates with low oospore densities were nonpathogenic to Sanga. At least two factors would be required to allow for the observed alteration of pathogenicity without the increase in oospore density.) This is possible because each zoospore could receive a random portion of cytoplasm during their formation that may or may not contain a cytoplasmic factor, although each would contain a nucleus.

There has been some speculation as to what the cytoplasmic component could be. Croft (8) proposed an autonomous self-replicating particle or a part of a self-replicating metabolic system. Jinks (20) suggested mitochondria, plastids, kinetosomes, or kinetoplasts.

Jinks (20) also proposed among other interrelationships of the chromosomal and extrachromosomal system that there could be suppression of the nuclear chromosome by the non-nuclear chromosomal material. In addition, just as Leach and Rich (24) suggested that there could be a combination of cytoplasmic components of the two races of P. infestans studied which

results in the utilization of a new food source (such as an amino acid) which could in turn increase pathogenicity, there could also be a similar combination of race 2 and race 3 components in Pms that could result in a loss in pathogenicity. Either one of these explanations could account for the loss of pathogenicity observed.

The reversible loss of pathogenicity as seen in this study might be used to study the relationship of virulence to aggressivity as they contribute to pathogenicity and also the possible role of cytoplasmic and nuclear inheritance in determing pathogenicity. Virulence, Knutson and Eide (22), refers to the spectrum of host varieties on which a compatible host:pathogen relationship can be established. Thus, virulence is determined by the number of different varieties on which a particular pathogen is pathogenic. Aggressivity (22) refers to the pathogen's ability to increase to epidemic proportions in a compatible host population. A compatible host relationship is one in which the pathogen can grow in the host.

Ninety-four single zoospore isolates of Pms by Hilty and Schmitthenner (17) had pathogenic variability ranging from nonpathogenic on all soybean varieties tested to highly aggressive on the susceptible varieties. The complete non-pathogenicity of some of the zoospore isolates is similar to that observed after inoculations of the S-1 and S-2 isolates into differential cultivars. The same factor could be involved.

In terms of the definition of aggressiveness and virulence, it appears that what Hilty and Schmitthenner refer to as virulence is what fits within the definition of aggressivity proposed by Knutson and Eide. The single zoospore isolates that they worked with varied considerably in aggressivity, but very little with respect to virulence. They observed that at least one plant of each of the cultivars was killed by all except one single zoospore isolate (virulence), but that the number of plants killed of a certain cultivar ranged from one to all of the plants inoculated (aggressivity). Caten and Jinks (7) suggest that cytoplasmic factors could be responsible for differences in aggressivity; but according to Gallegly (13), nuclear genes are responsible for virulence. A race in P. infestans and Pms is determined by the reaction of the differential host cultivars to the pathogen. This fits the definition of virulence. Therefore, it appears that like P. infestans, Pms virulence could be determined by chromosomal factors, but that aggressivity may be controlled by cytoplasmic factors. The evidence for this is that the virulence of races 1, 2, and 4 appear to be determined by nuclear genes because of the apparent Mendelian segregation of the progeny (14, 23, 34). The evidence in the present study implies that, although pathogenicity was apparently lost in the S-1 and S-2 isolates, virulence was not lost genetically because it was regained phenotypically. This could be interpreted to mean that aggressivity was lost in the S-1 and S-2 isolates giving a

nonpathogenic reaction. However, virulence was still there in the nuclear chromosomes, but it was masked almost completely by a cytoplasmic factor. When the cytoplasmic factor was lost or its effect was reduced in some way, the virulence was manifest. In the case of the complete loss of the cytoplasmic factor's influence, a high severity of infection would be observed; but if there was an intermediate cytoplasmic influence, then aggressivity would be intermediate and some plants would not be killed.

The evidence provided by these and other experiments with Pms and other species of Phytophthora cited suggest that cytoplasmic factors could be the cause for the complete loss of pathogenicity in the S-1 and S-2 isolates. However, the evidence is not conclusive.

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Appendix A. Oospore germination.

An attempt was made to germinate individual oospores of Phytophthora megasperma var. sojae so that they would produce sporangia and zoospores. The colonies derived from these single germinated oospores were then to be studied for variation within oospore progeny.

The procedure for oospore germination was adapted from Erwin and McCormick (1) and Long et al. (2). Pms hyphal-tips were plated onto clarified V-8 juice agar (CV-8A) (6) or a synthetic media (3) and placed in a growth chamber in the dark for three to four weeks at 24° C; the plates were either frozen; the agar in one-half of a plate was homogenized in 40 ml. of deionized water by a Brinkmann Polytron homogenizer for two 15-second intervals or in a Waring blender for five 1-minute intervals. Oospores were then separated from most of the chopped mycelium by straining the suspension through a sterile #53 nylon screen. Oospores were washed five times in sterile deionized water by centrifugation. Following the final washing, 0.25 ml. of oospore suspension (approximately 100-200 oospores) was placed in a deep-well slide and the remainder of the well was filled with deionized water or of another germination-inducing substance such as double-distilled water, snail enzyme (4), or dung infusion broth (5), and sealed with petrolatum and a coverslip. The deep-well slides were placed in a dark chamber at 24° C or 35° C and observed daily for germination via a sporangium. When an oospore did produce a

sporangium, an attempt was made to remove the oospore and sporangium by drawing them up into a sterile 5 microliter pipet to await zoospore release, at which time the contents of the pipet was discharged onto a plate of water agar for germination. The germinated zoospores were then transferred to separate lima bean agar (LBA) plates.

The percentage of germination was always less than one percent. Deionized water gave better germination than did the double distilled water, snail enzyme or dung infusion broth. When germination did occur, it was usually within five days. After many attempts to improve germination, germination stopped completely. During this time, Falcon Cooper dishes were used to provide a larger volume of water than deep-welled slides, since it was believed that the more diluted the oospore suspension, the more germination might occur; however, no germination occurred.

Although several oospores germinated, only one race 5 oospore with sporangium was isolated so that all the zoospores could be plated out. Sixteen colonies were produced.

From the one set obtained several experiments were performed to see if there was variability within the zoospores of one oospore with respect to cultural aspects and pathogenicity.

In order to check for cultural variability, colony growth rate and oospore density were determined for the parental culture and each of the 16 zoospore progeny. Plugs made with a #2 cork borer (5 mm.) were taken from the edge of each colony growing on the synthetic media developed by Ribeiro et al. and

were plated onto CV-8A. Three plugs per culture were transferred, one per plate, the plates were randomized with respect to replication and placed into a growth chamber at 20° C for seven days at which time the colony diameters were measured and oospore density determined as described in the cytoplasmic inheritance study (p. 7). The analysis of variance of colony diameter and oospore density indicated that at the .05 significance level, there were no differences among colony diameters nor oospore densities of the isolates.

In order to test for variability in pathogenicity at least 10 plants of various soybean cultivars and experimental lines were inoculated with the 16 zoospore isolates and one mass transfer from the race 5 parent culture of the germinated oospore as the control. The cultivars used were: Amsoy, Amsoy 71, Bonus, Calland, Clark, Clark 63, Cutler 71, Dare, Essex, Ex Clark, Forrest, Harcor, Marshall, Pomona, Wayne, Wells, Williams; in addition, experimental lines K74-104-75-54 (Tracy x Williams), K74-108-75-114 (Williams x 060-9647), and K74-108-75-169 (Williams x D60-9647) were used. Seed of all varieties was obtained from C. D. Nickell, Department of Agronomy, Kansas State University. All of the cultivars and experimental lines except K74-108-75-114 and K74-108-75-169 were susceptible to the 17 isolates. K74-108-75-114 is expected to give a mixed reaction while K74-108-75-169 is expected to give a resistant reaction to race 5 according to

previous inoculation studies by C. D. Nickell. Aggressivity readings were assigned to each host:pathogen combination 10 days after inoculation. The aggressivity readings were made on a scale of 0.5-5.0 with 0.5 being least aggressive and 5.0 being most aggressive; dead plants were assigned a rating of 5.0. Analysis of variance demonstrated there were no significant differences in pathogenicity at the 0.05 level among zoospore isolates inoculated into K74-108-75-114, but there were significant differences in pathogenicity ranking among isolates inoculated with K74-108-75-169. An l.s.d. 0.05, indicated five groups with differing pathogenicities. However, these differences between groups was very slight.

In conclusion there seems to be little, if any, variation among the zoospore isolates or the parent culture tested with respect to culture or pathogenicity.

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Appendix B. Seed transmission of *Phytophthora megasperma* var. *sojae*.

A study was made on the possibility of seed transmission of *Phytophthora megasperma* var. *sojae* (*Pms*), based on the report of recovery of *Pms* from immature soybean seeds by Klein (1).

Two different studies were made. One involved observing young plants for *Phytophthora* rot symptoms that might have developed from seed harvested from plants naturally infected with *Pms*. The seed was provided by C. D. Nickell, Department of Agronomy, Kansas State University. The other study involved observing seed from artificially inoculated plants. The first study was performed three different times and the second study was done once.

For each of the three periods of observation of seed from naturally infected plants, the seeds were planted in autoclaved sand with eight seeds in each pot. Approximately 400 seeds were planted per soybean line used: an experimental line (A73-314 Amsoy x (Provar x (Magna x Dissoy)), from Ames, Iowa, and the cultivars Columbus and Belatti (L263). The plants were monitored daily for one month in order to find diseased soybeans. Pots of the first group were placed directly on sand on a greenhouse bench that had previously been used for *Pms* inoculation studies. The pots of the second and third groups were placed on inverted cottage cheese containers in order to keep the pots away from the sand, possibly infested with *Pms*.

Columbus soybeans were planted in a field at the Ashland Experiment Field Station, Manhattan. One month after planting, and every 10 days for 50 days thereafter, 25 plants were inoculated with a culture of race 5 isolated from one of the infected plants of the first seed transmission study. Seed was harvested at maturity, planted in sterilized sand in the greenhouse, and observed for one month for dead or dying plants.

In the first study of the naturally infected plant seed, Pms was isolated from six plants which developed a brown lesion girdling the hypocotyl. Of these isolates, four were used to inoculate differential varieties; all four were identified as race 5. The second and third time the experiment was repeated, no Pms could be isolated from the young soybean plants, but some unknown bacteria along with Diaporthe sp., Ostracoderma sp., Thamnidium sp., Alternaria sp. and Chaetomium sp. were isolated.

Seeds from the artificially infected Columbus plants developed into normal, healthy plants.

It is possible that the infected seedlings of the first trial were not caused by seed transmission since in the second and third trials, when the pots were separated from the Pms infested sand, no Pms was found. The Pms infested sand could have been the source of fungus found in the infected seedlings. Another factor to consider before discounting the possibility of seed transmission is that the three trials were performed at different times of the year. Of the trials with seed from

naturally infected plants, the first was done during early spring when the weather was cool; the second was done at the first of the summer when the weather was much hotter than in the first trial; and the third was done in the middle of autumn when the weather was beginning to cool off. It could be that Pms infection in the seed is best during the cooler temperatures of early spring than during the hot and warmer temperatures of the summer and fall. This could also explain why no infection of the seed from the artificially infected plants occurred.

More work using chambers at various controlled temperatures could help answer this question.

It appears from the study that if seed transmission of Pms occurs, it requires specific environmental conditions.

LITERATURE CITED

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POSSIBLE CYTOPLASMIC INHERITANCE AND ITS EFFECT ON THE PHENOTYPIC VARIABILITY IN PHYTOPHTHORA MEGASPERMA VAR. SOJAE.

by

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Races 2 and 3 of Phytophthora megasperma var. sojae were grown together on a synthetic media. Some colonies derived from the points of intersection lacked pathogenicity on Sanga and Calland soybeans while others behaved as normal races 2 and 3. The nonpathogenic cultures, regained pathogenicity to Sanga soybeans after 16 days, then lost it after 5 more days. Cultures derived from hyphal tips of the nonpathogenic cultures were pathogenic to Sanga soybeans when the cultures were 4 days old, but 8 days later pathogenicity was lost using the same cultures. This same nonpathogenic Pms, when reisolated from inoculated soybeans, was pathogenic to Sanga. No definite parental-type pathogenicity was observed from cultures derived from single zoospore isolates of the nonpathogenic cultures, although there was a closer resemblance to the race 2 pathogenicity for all isolates than to that of race 3. Cytoplasmic inheritance is indicated as the probable mechanism causing the loss of pathogenicity.