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Occurrence of parasexual cycle in *Phytophthora parasitica* following protoplast fusion

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Abstract. Metalaxyl-resistant (M^r) and chloroneb-resistant (Cn^r) protoplasts of *Phytophthora parasitica* were fused, with heterokaryon formation rates ranging from 2.6×10^{-3} to 8.2×10^{-3}. The heterokaryosis was confirmed in six of seven putative heterokaryons by the production of both M^r zoospores and Cn^r zoospores, and/or $M^r + Cn^r$ zoospores. The 7th putative heterokaryon produced only Cn^r zoospores, probably because of the loss of the M^r chromosome during subculture. The heterokaryons produced normal-sized zoospores as well as large zoospores that were almost twice the size of normal-sized zoospores. Fifty percent of the normal-sized zoospores contained M^r and Cn^r genes. The results suggested an occurrence of diploidization after karyogamy of the heterokaryon and thus demonstrated the completion of events leading to a parasexual cycle following protoplast fusion.

Keywords: Diploidization; *Phytophthora parasitica*; Parasexual cycle; Protoplast fusion.

Introduction

Protoplast fusion has proved to be a highly efficient procedure for obtaining heterokaryons and enabling the parasexual cycle of filamentous fungi readily available for genetic studies and for industrial strain improvement (Anne and Peberdy, 1985; Peberdy, 1989). The procedure is especially valuable for *Phytophthora* because of the rare occurrence of hyphal fusion in this group of fungi (Judelson and Yang, 1998). However, the number of reports on this subject is very limited for *Phytophthora* (Layton and Kuhn, 1988; Lucas et al., 1990). Protoplast fusion has been used by Lucas et al. (1990) to generate somatic hybrids of *Phytophthora capsici* Leonian. Double drug-resistant strains were obtained when protoplasts of a metalaxyl resistant mutant of *Phytophthora sojai* Kaufmann & Gerdemann were fused with fluorotryptophan-resistant protoplasts (Layton and Kuhn, 1988). Fifteen of the hybrids produced zoospores of each parental phenotype, indicating a heterokaryon formation, while eleven hybrids produced a large number of zoospores that were resistant to both metalaxyl and fluorotryptophan, suggesting that karyogamy had possibly occurred. However, evidence that the selective markers represented nuclear genes has been lacking (Layton and Kuhn, 1988).

Chang and Ko (1990) obtained mutants of *Phytophthora parasitica* Dastur that were resistant to either metalaxyl or chloroneb and found that metalaxyl resistance and chloroneb resistance in these mutants were each conferred by a single dominant gene in heterozygous condition. Because of the availability of these nuclear gene markers, we used the mutants to perform protoplast fusion. Afterwards, we were able to detect events leading to a parasexual cycle, similar to those occurring in haploid fungi (Burnett, 1975; Pontecorvo, 1956). Details of the study are reported here.

Materials and Methods

Mutant Selection

Single-zoospore isolates P991 and 6134 of *P. parasitica* (Chang and Ko, 1990) were cultured on V-8 agar (10% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) containing either 25 ppm metalaxyl (subdue 2E, 25.1% active) or 200 ppm chloroneb (Terraneb WP, 65% active). Fast-growing sectors that appeared after 3 weeks were transferred to the same fungicide medium. Single-zoospore cultures were isolated from those that retained resistance after five successive subcultures, and zoospore cultures that displayed resistance after four subcultures on fungicide-free medium were selected. Fungicide resistance was determined by placing culture discs (4 mm) on V-8 agar amended with 25 ppm metalaxyl or 200 ppm chloroneb. Cultures that were sensitive to metalaxyl did not grow in 7 days at 24°C on medium containing metalaxyl, while those that were resistant grew continuously, up to 18-20 mm in diameter, during the same incubation period. Similarly, cultures that

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were sensitive to chloroneb did not grow in 3 days on chloroneb medium, and those that were resistant grew continuously, up to 9-10 mm in diameter, during the same incubation period.

Protoplast Isolation and Fusion

The method of Layton and Kuhn (1988) was modified for protoplast isolation and fusion. Fifty ml of zoospore suspension were mixed with 10 ml of V-8 broth in a 250-ml flask. The V-8 broth was prepared by filtering V-8 juice through two layers of cheesecloth. After incubating at 24°C for 2 days, the culture was filtered through a 90-µm sieve. Mycelia collected on the sieve were washed with 200 ml of sterile distilled water and re-suspended in 6 ml of stabilizer solution of 1 M mannitol and 7 mM $MgSO_4$ contained in a 25-ml centrifuge tube. After 1 h, 1 ml of Driselase (Sigma, St. Louis, MO) solution (100 mg /ml water) was added to the tube, and the suspension was shaken at 80 rpm for 4-5 h at 24°C. Protoplasts were separated from non-digested germlings by filtering through a 45-µm sieve and concentrated by centrifuging at 1,000 *g* for 10 min. The entire procedure was performed under aseptic conditions.

For protoplast fusion, equal numbers of protoplasts (2×10^6-5×10^6) of P991M^r-2 and 6134Cn^r-3 were mixed and pelleted at 1,000 *g* for 10 min. The protoplasts were re-suspended in 400 µl of fusion solution, consisting of 40% w/v polyethylene glycol (PEG) 8000, 50 mM $CaCl_2$, and 20 mM Tris. After incubating at 24°C for 15 min, the protoplast concentration was adjusted to 5-8 protoplasts/µl with 1 M mannitol. A marking pen was used to draw 50 circles (ca. 3 mm diam.) on the bottom of each regeneration agar (20% V-8 juice, 1 M mannitol and 1.5% Bacto agar) plate. A 2 µl drop of the protoplast suspension was placed on the surface of the agar, above each circle (Ho and Ko, 1997). The protoplasts were allowed to regenerate at 24°C for 12 h and were then overlaid with a selective medium consisting of V-8 agar amended with 25 ppm metalaxyl and 200 ppm chloroneb. Colonies which grew through the selective medium within 7 days were transferred to 10% V-8 agar plates and considered to be putative heterokaryons. Twice the number of protoplasts of P991M^r-2 and 6134Cn^r-3 were separately processed through the same fusion procedure to detect the appearance of any spontaneous mutants.

Resolution of Putative Heterokaryons

Since most zoospores of *Phytophthora* are uninucleate (Layton and Kuhn, 1988; Zheng and Ko, 1996), zoospore progenies were produced to verify the heterokaryons. For each putative heterokaryon, 20-60 single-zoospore cultures were established (Ko, 1981). The phenotype of each culture was determined by testing four subcultures (3 mm diam. culture discs) on media containing metalaxyl, chloroneb, metalaxyl plus chloroneb, or neither chemical. Symbols employed to represent the phenotypes for fungicide resistance were M^r, the subculture of which was resistant to metalaxyl, but not to chloroneb or metalaxyl plus chloroneb; Cn^r, the subculture of which was resistant to chloroneb but not to metalaxyl or metalaxyl plus chloroneb; M^r, Cn^r, but not M^r+Cn^r, the subculture of which was resistant to metalaxyl or chloroneb, but not metalaxyl plus chloroneb; M^r, Cn^r, M^r+Cn^r, the subculture of which was resistant to metalaxyl, chloroneb, and combination of metalaxyl and chloroneb; and M^s + Cn^s, the subculture of which showed no resistance to metalaxyl, chloroneb, or metalaxyl plus choroneb. Three replicates were used. Data were recorded after incubating at 24°C for 7 days for medium containing metalaxyl or metalaxyl plus chloroneb, and 3 days for that containing chloroneb.

Characteristics of zoospores of four putative heterokaryons that maintained their resistance to both metalaxyl and chloroneb after being stored in test tubes of distilled water (Boeswinkle, 1976) at 24°C for 18 months were examined in detail. Zoospore sizes were measured microscopically after encystment was induced by vortexing for 1 min.

Staining of Protoplast Nuclei

Nuclei in protoplasts were stained with acridine orange, following the method of Zheng and Ko (1996). A drop of protoplast suspension was mixed with one drop of acridine orange solution on a glass slide, covered with a cover slip, and examined immediately with a Zeiss fluorescence microscope.

Results

Germlings from 9×10^6 zoospores of *P. parasitica* released 1.8×10^6 - 7.3×10^6 protoplasts when digested with Driselase. Protoplasts sizes ranged from 11.7 to 28 µm in diameter. The number of nuclei in each protoplast ranged from 0 to 5, with an average of 1.4. About 80% of the protoplasts had 1 or 2 nuclei, and approximately 10% contained no nuclei (Figure 1).

Fusion between 720 to 750 protoplasts of P991M^r-2 and 6134Cn^r-3 resulted in 2 to 6 colonies appearing on the selective medium in 7 days with heterokaryon formation rates

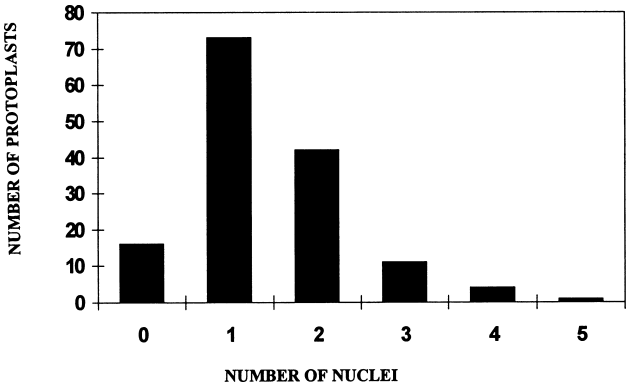

Figure 1. Distribution of nuclear numbers in protoplasts of *Phytophthora parasitica*.

in three experiments ranging from 2.6×10^{-3} - 8.2×10^{-3} (Table 1). No colonies developed on selective medium for the self-fusion controls of 6134Cn^r-3 and P991M^r-2 protoplasts.

The putative heterokaryons frequently produced sectors on V-8 agar or V-8 agar supplemented with metalaxyl and chloroneb (Figure 2). When subcultured on medium without fungicide for two months, 11 of the 19 heterokaryons retained their ability to grow normally on the medium containing both metalaxyl and chloroneb, while eight failed to grow. After storage in sterile distilled water for 18 months, five of the 11 double fungicide resistant heterokaryons retained their ability to grow on the medium containing both metalaxyl and chloroneb.

Among seven putative heterokaryons tested, selections HK-7, HK-8, HK-12, and HK-13 produced both M^r zoospores and Cn^r zoospores (Table 2). Some zoospores

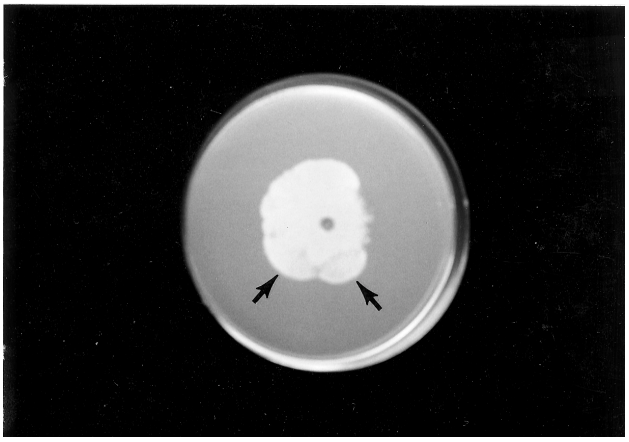

Figure 2. A heterokaryon with sectors (arrows) on V-8 agar resulting from fusion between P991M^r-2 and 6134Cn^r-3 protoplasts of *Phytophthora parasitica.*

Table 1. Heterokayon formation resulting from protoplast fusion between P991M^r-2 and 6134Cn^r-3 mutants of *Phytophthora parasitica.*

Exp.	No. of heterokaryons produced (No. of protoplasts used)		
	P991M^r-2 × 6134Cn^r-3	P991M^r-2 × P991M^r-2	6134Cn^r-3 × 6134Cn^r-3
I	5 (720)	0 (750)	0 (560)
II	2 (750)	0 (760)	0 (710)
III	6 (730)	0 (720)	0 (730)

Table 2. Phenotypic characteristics of zoospore progenies of putative heterokaryons resulting from fusion between P991M^r-2 and 6134Cn^r-3 of *Phytophthora parasitica*.

Progeny phenotype[a]	Zoospore cultures (No.)						
	HK-7	HK-8	HK-11	HK-12	HK-13	HK-15	HK-16
M^r	14	4	0	4	6	0	0
Cn^r	35	17	21	15	11	5	3
M^r, Cn^r, but not M^r+Cn^r	8	0	0	0	15	17	4
M^r, Cn^r, M^r+Cn^r	0	0	0	0	7	7	13
M^s, Cn^s	0	1	3	1	1	1	0

[a]Phenotype of each zoospore culture was determined by testing four subcultures (3 mm diam. culture discs) on media containing metalaxyl, chloroneb, metalaxyl plus chloroneb or none of these two chemicals. M^r, with subculture resistant to metalaxyl but not chloroneb or metalaxyl plus chloroneb; Cn^r, with subculture resistant to chloroneb but not metalaxyl or metalaxyl plus chloroneb; M^r, Cn^r, but not M^r+Cn^r, with subculture resistant to metalaxyl, chloroneb, but not metalaxyl plus chloroneb; Mr, Cn^r, M^r+Cn^r, with subculture resistant to metalaxyl, chloroneb, and metalaxyl plus chloroneb; M^s+Cn^s, with no subculture resistant to metalaxyl, chloroneb, or metalaxyl plus chloroneb.

Table 3. Sizes of zoospores produced by three heterokaryons and their parental isolates P991M^r-2 and 6134Cn^r-3 of *Phytophthora parasitica.*

Isolate[a]	Zoospores (No.)				
	Total scored	Normal spore (9.5-14 µm) Avg. size		Large spore (15-19 µm) Avg. size	
		No.	(µm diam. ± SD)	No.	(µm diam. ± SD)
HK-15	86	54	11.2 ± 1.90	32	18.2 ± 1.36
HK-7	18	18	10.9 ± 1.42	0	
HK-13	22	22	10.3 ± 1.33	0	
P991M^r-2	26	26	10.2 ± 0.92	0	
6134Cn^r-3	29	29	11.2 ± 1.84	0	

[a]Tested after storage in sterile distilled water in test tubes for 18 months at 24°C.

of HK-13, HK-15 and HK-16 were resistant to the mixture of metalaxyl and cloroneb. HK-11 produced only Cn^r zoospores and a few fungicide sensitive zoospores. HK-8, HK-12, HK-13 and HK-15 also produced one fungicide sensitive zoospore each. Subcultures derived from some of the zoospore cultures of HK-7, HK-13, HK-15 and HK-16 were resistant to either metalaxyl or chloroneb, but not to the combination of the fungicides (Table 2).

After storage in sterile distilled water for 18 months, HK-16 lost its ability to produce zoospores. HK-7 and HK-13 produced zoospores with sizes that were similar to their parents (Table 3). However, 37% of the zoospores produced by HK-15 were almost twice the parental size (Table 3; Figure 3). One of 30 cultures derived from normal-sized zoospores of HK-15 was resistant to metalaxyl plus chloroneb, and 11 of 27 derived from the large zoospores were resistant to both fungicides (Table 4). Subcultures derived from 14 normal-zoospore and 4 large-zoospore cultures were resistant to either metalaxyl or chlroneb, but not metalaxyl plus chloroneb. Zoospore cultures of $P991M^r$-2 and $6134Cn^r$-3 displayed the same fungicide resistance as their parents. The culture derived from a normal-sized zoospore of HK-15 produced only normal-sized zoospores, whereas zoospores produced by a large-sized zoospore culture were composed of 37% normal-sized and 63% large-sized zoospores (Table 5).

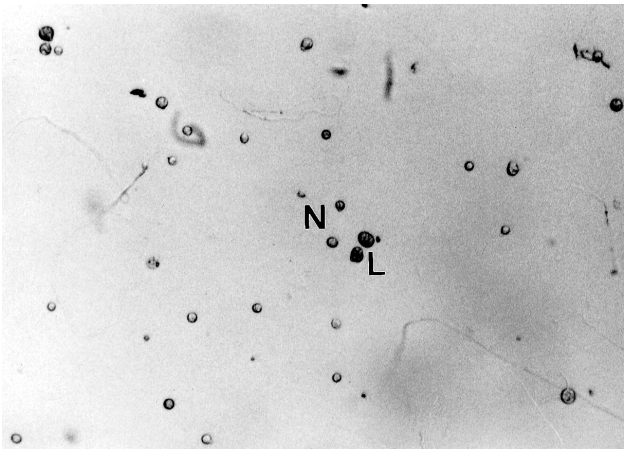

Figure 3. Normal-sized (N) and large-sized (L) encysted zoospores produced by heterokaryon HK-15 resulting from fusion between $P991M^r$-2 and $6134Cn^r$-3 protoplasts of *Phytophthora parasitica*. ×200.

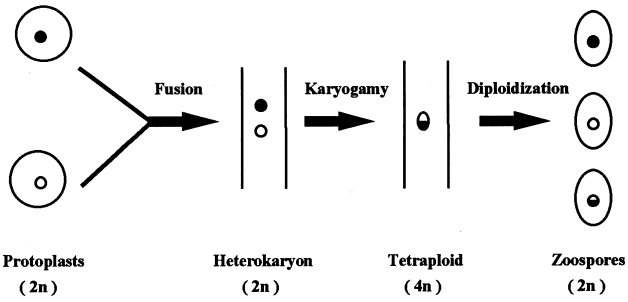

Figure 4. Diagrammatic illustration of events leading to the parasexual cycle of a diploid fungus *Phytophthora parasitica* following protoplast fusion.

Discussion

In six of seven putative heterokaryons examined, heterokaryosis was confirmed by the production of both M^r zoospores and Cn^r zoospores, and/or M^r+Cn^r zoospores. The protocol used in this study is, therefore, suitable for

Table 4. Relationship between zoospore size and fungicide resistance among zoospore progenies of heterokaryon HK-15 and parental isolates $P991M^r$-2 and $6134Cn^r$-3 of *Phytophthora parasitica*.

Progeny phenotype[a]	Zoospores (No.)					
	Normal spores (9.5-14 μm)			Large spores (15-19 μm)		
	HK-15	$P991M^r$-2	$6134Cn^r$-3	HK-15	$P991M^r$-2	$6134Cn^r$-3
M^r	0	26	0	0	0	0
Cn^r	15	0	29	12	0	0
M^r, Cn^r, but not M^r+Cn^r	14	0	0	4	0	0
M^r, Cn^r, M^r+Cn^r	1	0	0	11	0	0
M^s+Cn^s	0	0	0	0	0	0

[a]The same as Table 2.

Table 5. Size of zoospores produced by cultures derived from large and normal spores of heterokaryon HK-15 of *Phytophthora parasitica.*

Origin of culture	Zoospores (No.)				
		Normal spores (9.5-14 μm)		Large spores (15-19 μm)	
		Avg. size		Avg. size	
	Total scored	No.	(μm diam. ± SD)	No.	(μm diam. ± SD)
Large spore	68	25	11.3 ± 1.54	43	18.7 ± 0.74
Normal spore	27	27	10.7 ± 1.35	0	

obtaining heterokaryons. Among the heterokaryons examined, five produced small numbers of zoospores sensitive to both metalaxyl and chloroneb (Table 2). This is probably due to the loss of the chromosome that contained the resistant gene, or to occurrence of a back mutation in some nuclei. Production of only Cn^r zoospores and fungicide sensitive zoospores by HK-11 was probably attributable to a loss of M^r nuclei during subcultures resulting from uneven frequencies of the different types of nuclei in mycelia. Uneven distribution and continuous segregation of the different types of nuclei in mycelia during growth may also explain the observation that subcultures from some zoospore cultures derived from heterokaryons were resistant to either metalaxyl or chloroneb, but not to a mixture of both fungicides (Table 2).

In order to use the double drug resistance in uninucleate zoospores produced by fusion products as evidence for karyogamy formation, the drug resistant phenotypes must be controlled by nuclear genes (Layton and Kuhn, 1988). Metalaxyl resistance and chloroneb resistance in *P. parasitica* are encoded by nuclear genes because the phenotypes are transmitted to the sexual offspring according to Mendelian inheritance (Chang and Ko, 1990). Conclusive evidence has also been obtained in a nuclear transfer study (Gu and Ko, 1998). When metalxyl sensitive protoplasts of *P. parasitica* received the nuclei isolated from a metalaxyl resistant mutant, they also acquired drug resistance. Therefore, the production of $M^r + Cn^r$ zoospores by HK-13, HK-15 and HK-16 (Table 2) clearly demonstrated the occurrence of karyogamy in these heterokaryons. The proposal for occurrence of karyogamy is also supported by the production of zoospores by HK-15 that are about twice the size of normal zoospores (Table 3). Diploid spores were reported to be about twice the size of haploid spores in haploid fungi, and spore size was considered to be a reliable criterion for karyogamy (Bos, 1996).

Each large zoospore of *P. parasitica* tested contained a single nucleus, about twice the size found in normal sized zoospores (Gu and Ko, 1998). Since *Phytophthora* species are diploids (Sansome, 1965), the $M^r + Cn^r$ large-sized zoospores produced by HK-15 were probably tetraploids. The Cn^r large-sized zoospores probably represented aneuploids derived from tetraploids by a loss of chromosomes carrying the M^r gene. The observation that subcultures derived from four large zoospore cultures were either resistant to metalaxyl or chloroneb, but not to metalaxyl plus chloroneb, indicated that tetraploids are probably unstable and that loss of chromosomes may be common during mitosis. About 50% of the normal-sized zoospores produced by HK-15 contained both M^r and Cn^r genes (Table 4), suggesting that diploidization had occurred after karyogamy. Occurrence of diploidization is also supported by the observation that 37% of spores derived from a large-sized spore were of normal size (Table 5). The results demonstrate the fulfillment of events that lead to a parasexual cycle (Figure 4) similar to those observed in haploid fungi (Burnett, 1975; Pontecorvo, 1956). The only previous instance of parasexual cycle in Oomycetes was observed when isolated nuclei of *P. parasitica* were transferred into protoplasts of the species (Gu and Ko, 1998). The resulting nuclear hybrids also underwent karyogamy and diploidization.

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Phytophthora parasitica 之無性生殖週期發生於原生質體融合之後

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Phytophthora parasitica 之抗 metalaxyl (M^r) 原生質體與同菌種之抗 Chloroneb (Cn^r) 原生質體進行融合，其形成異核體的機率為 2.6×10^{-3}-8.2×10^{-3}。在 7 個可能異核體中有 6 個可產生 M^r 游走子和 Cn^r 游走子和／或 M^r+Cn^r 游走子。另一個可能異核體僅產生 Cn^r 游走子，可能是在培養過程中失去 M^r 染色體。原生質體融合之異核體菌株可產生正常大小之游走子，同時產生比正常游走子大約兩倍之較大游走子。50% 之正常游走子具有 M^r 和 Cn^r 基因。本研究結果表示變兩倍體現象發生於異核體核融合後，因此顯示原生質體融合可引起無性生殖週期之發生。

關鍵詞：變兩倍體現象；*Phytophthora parasitica*；無性生殖週期；原生質體融合。