Creation of Species Hybrids of *Phytophthora* with Modified Host Ranges by Zoospore Fusion

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ABSTRACT

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Species hybrids of the fungal plant pathogens *Phytophthora capsici* and *P. nicotianae* were created that exhibited expanded host ranges compared with parent organisms. One hybrid lost its ability to infect host plants specific to either species. The hybrids were created by a novel method of fusing uninucleate zoospores of these pathogens in the presence of polyethylene glycol (PEG) and LiCl. Lithium ion inhibited cell wall formation and encystment of zoospores during fusion in the presence of PEG. After fusion, lithium ion was replaced with calcium and potassium ions to induce encystment and spore germination. The hybrid characters of the fusion offspring were confirmed by detection of DNA sequences specific to each parent organism. Repetitive DNA of *P. capsici* was detected readily in all species hybrids by hybridization with a species-specific DNA probe. DNA of *P. nicotianae* was detected in some of the hybrids after amplification of DNA from hybrids with species-specific primers derived from *P. nicotianae*. Zoospore fusion provided a means of generating a series of organisms with modified pathogenicity-related traits to be used in molecular and ecological analyses.

Pathogens in the fungal class Oomycetes destroy plants in commercial production systems worldwide. Among the more than 40 species of *Phytophthora* within this fungal class, *P. capsici* Leonian and *P. nicotianae* Breda de Haan (syn. *P. parasitica*) are two highly virulent pathogens that are sympatric in their distributions and have overlapping host ranges. These species are typical of many others in the genus that possess few, if any, major genes that determine virulence or host specificity. Because *P. capsici* and *P. nicotianae* coexist geographically (18), there may be opportunities for genetic exchange between populations. Genetic exchange by species hybridization in *Phytophthora* has been suspected in several instances (20). For example, Sansome et al. (17) suggested that *P. meadii* may have arisen as a species hybrid in nature. Goodwin and Fry (8) created viable hybrids through sexual crosses of the sympatric species *P. mirabilis* and *P. infestans*. Boccas (2) induced sexual crosses among numerous heterothallic *Phytophthora* species but detected only one possible species hybrid among 220 tested progeny. Finally, Brasier (3) also suggested the potential for rare somatic fusions to produce hybrids.

Hybridization of the heterothallic organisms *P. capsici* and *P. nicotianae* via sexual processes is likely to be rare because compatible mating types required for sexual reproduction often do not occur at a single geographic location (14,15). Additionally, it is difficult to consistently obtain viable, sexually produced offspring in vitro without prolonged incubation of cultures (1,10). Consequently, it has been difficult to perform genetic analyses of host-parasite interactions involving these important pathogens.

Although species hybridization of *P. capsici* and *P. nicotianae* is likely to occur rarely, the event may be an important source of variation in pathogen populations. Unfortunately, detection of rare new variants in nature, from whatever source, is difficult. An alternative approach to examining the importance of species hybrids as a source of variation would be to create such organisms artificially. This study describes the use of a novel zoospore fusion method to create, for the first time in Oomycetes, somatic species hybrids that exhibit expanded host ranges. The hybrid nature of these organisms was confirmed by the detection of species-specific DNA of each parental organism in putative hybrids.

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MATERIALS AND METHODS

Fungal isolates. Isolates *P. capsici* Mex^{r}5 and *P. nicotianae* Fpa^{r}10, derived from *P. capsici* 15399 (from pepper) and *P. nicotianae* W1 (from tomato), respectively, were used in all experiments. Both isolates were obtained from the culture collection of J. M. Duniway of the Department of Plant Pathology, University of California, Davis. Each mutant isolate expressed a unique drug-resistance phenotype. *P. nicotianae* Fpa^{r}10 was resistant to 180 ppm fluorophenylalanine (Sigma, St. Louis, MO), and *P. capsici* Mex^{r}5 was resistant to 30 ppm metalaxyl (Ciba, Greensboro, NC). These isolates were derived by chemical mutagenesis methods (12). Each isolate was similar to its respective wild-type parental organism in morphological traits and patterns of growth and virulence and has remained stable through at least 2 years of periodic transfer and maintenance on growth medium not amended with any drug (6). These isolates represented incompatible mating types (both A2), and thus their use precluded any possibility of generating hybrids via sexual processes in zoospore fusion experiments.

Zoospore fusion. Cultures of each organism were initiated on V8 juice agar (19) and incubated in the dark for at least 3 days at 25°C. Zoosporangia and zoospores were produced after cultures were incubated for at least 3 more days in a 16-h light, 8-h dark

cycle (6). In each of three experiments, 1-ml aliquots containing 10^6 zoospores of *P. capsici* Mex^{r}5 and *P. nicotianae* Fpa^{r}10 were combined in an Eppendorf tube and centrifuged at 1,000 × *g* for 40 s. To inhibit zoospore encystment, the zoospore pellet was resuspended immediately in 100 µl of a fusion solution containing 30% polyethylene glycol (PEG) 3350 (Sigma) in 50 mM LiCl. After 2 min of incubation at 20°C, 200 µl of a solution of 100 mM sorbitol containing 5 mM $CaCl_2$ and 500 µM KCl were added to the suspension to induce cell encystment and cell wall formation. After centrifugation at 1,000 × *g* for 30 s, the pellet was resuspended in 200 µl of this solution and incubated at 20°C for 2 h, and then the cells were incorporated in pea broth agar medium (19) containing 10 g of sucrose, 1 g of L-asparagine, 250 mg of $MgSO_4 \cdot 7H_2O$, 500 mg of KH_2PO_4, 1 mg of thiamine, and 12.5 g of agar per liter. The medium was supplemented with 0.5% L-sorbose to restrict colony diameter and with pimaricin, ampicillin, and rifampicin at concentrations of 10, 600, and 10 ppm, respectively. After 24 h of incubation at 25°C, the medium was overlaid with the same growth medium amended with metalaxyl plus fluorophenylalanine at 15 and 75 ppm, respectively. These concentrations are one-half the concentrations that are inhibitory to growth of each nonresistant species. After 2 to 3 days of further incubation, these plates were overlaid with a final layer of the medium supplemented with 30 and 150 ppm metalaxyl and fluorophenylalanine, respectively. After 5 to 8 days, the colonies exhibiting the most rapid growth were transferred to V8 juice agar that contained both drugs at fully inhibitory concentrations. Isolates of putative species hybrids derived from single zoospores were selected on the basis of stable growth after repeated transfer. Colonies that showed abnormal growth or reproduction or other indicators of instability were not selected. In control treatments, zoospores of *P. capsici* and *P. nicotianae* were combined and manipulated through all steps of the fusion protocol with the exception of treatment with the fusion solution.

Visualization of nuclei after zoospore treatment. Nuclei were examined after a 2-h incubation of zoospores in encystment solution. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) according to the procedure of Martin (13) with slight modification. Cysts (50 µl) were placed on a microscope cover slip, incubated in a moist chamber for 30 to 120 min to allow cysts to adhere to the surface, and then fixed for 5 min in 3% formaldehyde, washed in water for 2 min, stained in DAPI for 6 min, and washed again in water for 3 min. Specimens were epi-illuminated by a UV source to excite the DNA-DAPI complex and viewed in a model BH2-RFL microscope (Olympus, Lake Success, NY). Similar observations were made for spores of parent isolates not subjected to the fusion treatment.

Evaluation of species hybridization. The hybrid nature of selected isolates was confirmed by the detection of species-specific parental DNA sequences. Total DNA was extracted from putative species hybrids and parental organisms (5). To detect DNA of *P. nicotianae*, DNA sequences were amplified by using 24-mer primers derived from a 1,000-bp, species-specific DNA sequence contained in plasmid pPP33A (5). The reaction conditions and concentrations of fungal DNA and reaction components were as described by Érsek et al. (5). The 24-mer primers were shown previously to distinguish *P. nicotianae* from *P. capsici* and 10 other species of *Phytophthora* and *Pythium* (5).

Oligonucleotide primers specific to *P. capsici* were not available. However, a 2,000-bp, repetitive DNA sequence specific to this species has been defined (J. T. English and J. E. Schoelz, *unpublished*). To detect this sequence in putative hybrids, DNA was digested with *Hae*III, electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and hybridized with a P-32-labeled probe as described in Sambrook et al. (16). Plasmid pCAP12 that contained the species-specific DNA insert was used as a probe to detect the presence of DNA specific to *P. capsici* in putative hybrids.

Evaluation of pathogenicity. The virulence of hybrids and parental organisms was evaluated against tomato (*Lycopersicon esculentum* Mill.), a host common to *P. capsici* and *P. nicotianae*; lemon fruit (*Citrus limon* (L.) Burm. f.), susceptible only to *P. nicotianae*; and the storage taproot of radish (*Raphanus sativus* L.), susceptible only to *P. capsici*. Inocula were taken from 5-day-old cultures grown on drug-amended V8 juice agar. A 4-mm-diameter inoculum plug was applied on the surface of a tomato (three-leaf stage) stem that had been wounded by abrasion of the epidermis. To inoculate lemon fruit, an inoculum plug was inserted into a 2-mm-deep wound of the same diameter cut from the pericarp. To inoculate radish (cultivar Red Pak), the root was cut from the stem, and an inoculum plug was placed in the center of the freshly cut, washed, and air-dried surface. Inoculation points of each host species were wrapped in Parafilm. Replicate inoculated plants were maintained in moist chambers at 22°C, and lesion development was evaluated daily. All inoculation experiments were performed at least twice.

RESULTS

Zoospore fusion. In each experiment, zoospores of *P. capsici* Mex^{r}5 and *P. nicotianae* Fpa^{r}10 appeared typical of either species when first combined in the fusion protocol (Fig. 1A). However, zoospores aggregated immediately into clumps of various sizes after the addition of PEG and LiCl (Fig. 1B). When this fusion solution was replaced with an encystment solution containing $CaCl_2$ and KCl, aggregated zoospores formed cell walls rapidly and rounded into cysts (Fig. 1C). Within 1 to 2 h, approximately 30% of the cysts germinated (Fig. 1D). Many spores were remarkably larger than spores not treated with the fusion solution, and the majority of large, regenerating cysts contained either a single, enlarged nucleus (Fig. 1E and F) or multiple nuclei (Fig. 1F). Colonies that expressed double drug resistance were recovered at a frequency of 10^{-5}. We did not find viable double-drug-resistant colonies on plates inoculated with zoospores that had not been treated with the fusion solution. Additionally, zoospores of parental organisms were not enlarged, and they did not contain multiple or enlarged nuclei.

Four representative, single-spore isolates, designated H0, H1, H3, and H4, obtained from the three fusion experiments were retained for further analyses. These isolates expressed double-resistance markers through repeated transfer and maintenance on growth medium not amended with drugs over a period of at least 1 year. Morphological traits of each isolate were indistinguishable from those of *P. capsici*. For example, isolates H1, H3, and H4 developed appressed or submerged mycelia typical of the parental isolate of *P. capsici;* aerial mycelium typical of *P. nicotianae* did not occur in any culture. Similarly, although isolates H0, H1, H3, and H4 varied somewhat in growth rate and zoosporangial dimension, they showed more resemblance to *P. capsici* than to *P. nicotianae* (data not shown).

Evaluation of species hybrids. The creation of species hybrids by fusion of zoospores was confirmed by the detection of species-specific parental DNA sequences. DNA specific to *P. nicotianae* was detected by polymerase chain reaction (PCR) (Fig. 2). By this method, a 1,000-bp amplification product was detected in isolates H0 and H1. This product corresponded to the amplification product from the parental isolate of *P. nicotianae* and plasmid pPP33A (5) containing the 1,000-bp sequence from which the species-specific, 24-mer primer pairs were derived. The 1,000-bp DNA band was not visible in isolates H3 or H4 by PCR. However, the 1,000-bp band was detected in H4 after hybridization of amplification products with radiolabeled pPP33A (Fig. 3).

DNA specific to *P. capsici* also was detected in putative hybrid isolates when extracted DNA of each of these isolates was digested with *Hae*III and probed with a repetitive DNA sequence

specific to *P. capsici*. Multiple bands of similar intensity were visualized in *P. capsici* and isolates H0, H1, H3, and H4. No hybridization occurred in digested DNA from *P. nicotianae* (Fig. 4).

Evaluation of pathogenicity. In all experiments, all isolates of species hybrids were able to infect tomato, a host common to *P. capsici* and *P. nicotianae* (Table 1). Lesions developed within 2 days of inoculation by any isolate. By the third day after inoculation, lesion severity was noticeably greater in plants infected with either parental organism than with hybrids. Additionally, lesions caused by hybrids H0 and H4 were more severe than

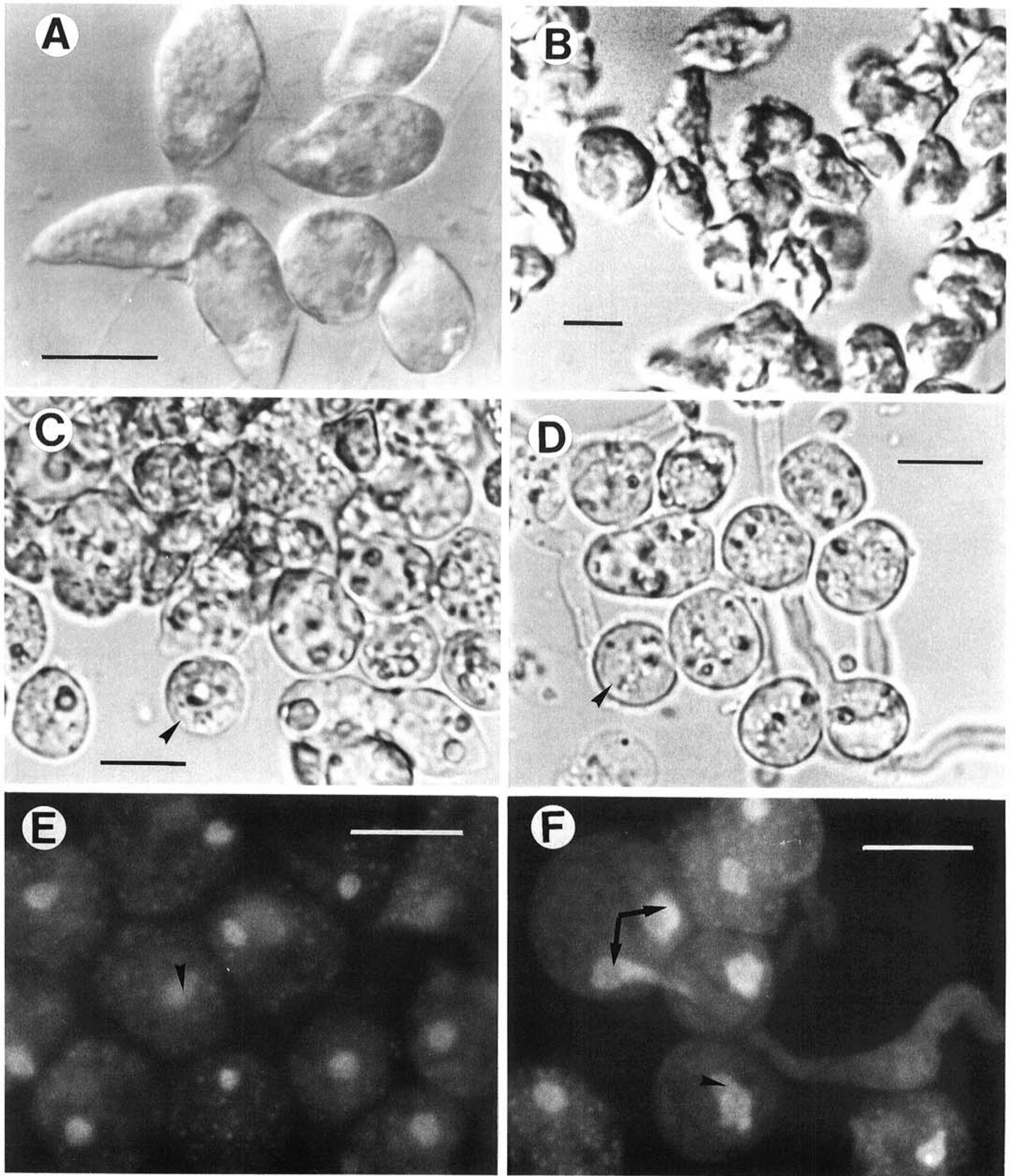

Fig. 1. Induction of zoospore fusion. **A,** Zoospores from a combined suspension of the metalaxyl-resistant mutant *Phytophthora capsici* Mex^{r}5 and the fluorophenylalanine-resistant mutant *P. nicotianae* Fpa^{r}10 prior to fusion. **B,** Aggregation of zoospores in the fusion solution. **C,** Onset of regeneration; arrowhead indicates a cell equivalent in size to those of nontreated parents. **D,** Zoospores after 2 h of regeneration; arrowhead indicates normal-sized cell. **E and F,** Nuclei of regenerating cells stained with DAPI (4′,6-diamidino-2-phenylindole) at the onset and after 2 h of regeneration, respectively; arrowheads indicate large nuclei, and arrows point to multiple nuclei. Bars = 10 μm.

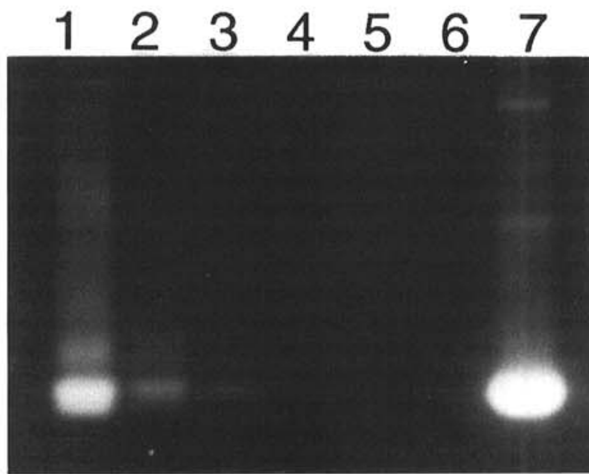

Fig. 2. Detection of *Phytophthora nicotianae* DNA sequences in putative hybrids H0 and H1 by amplification of DNA sequences specific to *P. nicotianae*. Lane 1, *P. nicotianae*; lanes 2 through 5, putative hybrids H0, H1, H3, and H4, respectively; lane 6, *P. capsici*; and lane 7, plasmid pPP33A containing a 1,000-bp repetitive sequence specific to *P. nicotianae* from which the 24-mer primer pairs were derived.

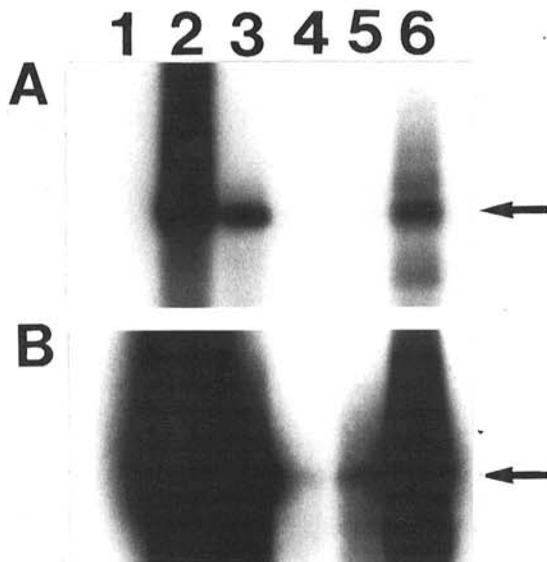

Fig. 3. Confirmation of the occurrence of *Phytophthora nicotianae* DNA sequences in putative hybrids. **A,** Hybridization of plasmid probe pPP33A to products amplified from total DNA of H0 and H1 with a 24-mer primer of *P. nicotianae*. **B,** Hybridization of pPP33A to products amplified from total DNA of putative hybrid H4 after further exposure of the autoradiograph. Plasmid pPP33A contained a 1,000-bp repetitive sequence specific to *P. nicotianae*. Lane 1, *P. capsici*; lanes 2 through 5, putative hybrids H0, H1, H3, and H4, respectively; and lane 6, *P. nicotianae*.

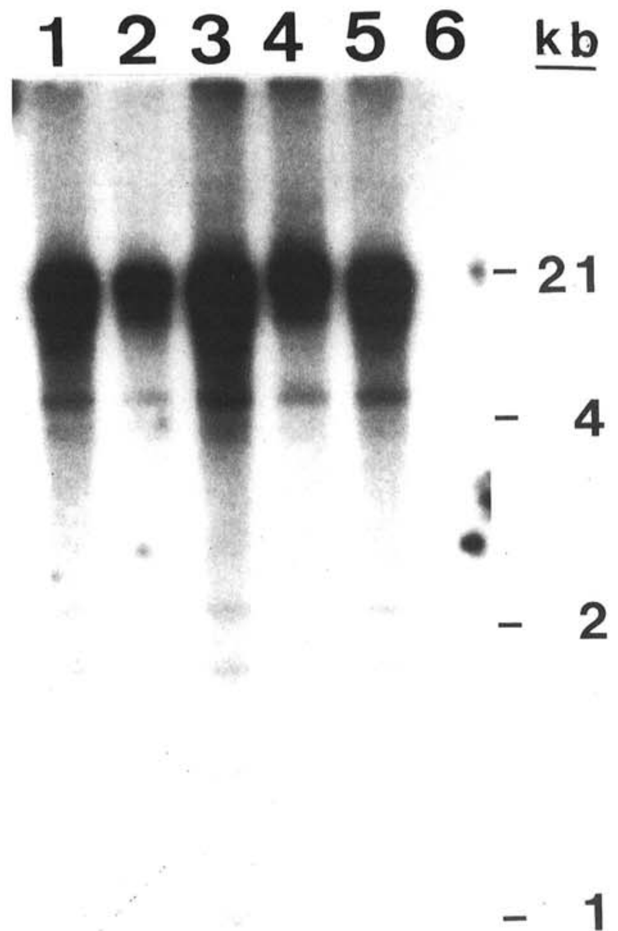

Fig. 4. Detection of *Phytophthora capsici* DNA sequences in putative species hybrids by hybridization of plasmid probe pCAP12 to a Southern blot of *Hae*III-digested total genomic DNAs. The plasmid contained a 2,000-bp repetitive sequence specific to *P. capsici*. Lane 1, *P. capsici*; lanes 2 through 5, putative hybrids H0, H1, H3, and H4, respectively; and lane 6, *P. nicotianae*.

those caused by H1. In contrast to tomato, radish was infected only by *P. capsici* and hybrids H0 and H4. Lesions were evident within 2 days of inoculation; and again, lesions caused by the parental organism were noticeably more severe. Similar reactions were observed on lemon, except that lesions formed only when fruit were inoculated with *P. nicotianae* and hybrids H0 and H4. No lesions formed when fruit were inoculated with hybrid H1, even after 6 days of incubation.

DISCUSSION

Species hybrids with expanded host ranges were created by somatic fusion of uninucleate zoospores of *P. capsici* and *P. nicotianae*. Because these species lack sexual reproductive systems that can be manipulated readily in vitro, it has been difficult to evaluate the genetic basis of processes related to pathogenesis. This unique fusion methodology provides a means to overcome this difficulty and readily generate variability in pathogenic traits.

Zoospores are discharged from sporangia in a wall-less state and thus are analogous to protoplasts. Unlike protoplasts, however, zoospores maintain their cellular integrity against large osmotic gradients (7). They form cell walls and encyst immediately in the presence of cations such as calcium ion or when exposed to physical forces such as agitation (9). These properties have made it difficult to utilize zoospores in fusion experiments. We circumvented undesired encystment by manipulating zoospores in the presence of lithium ion, a treatment that displaces calcium and potassium ions from the cells and inhibits cell wall formation (4). Thus, zoospores were maintained as protoplasts during fusion in the presence of PEG. Later displacement of lithium by calcium and potassium ions then induced encystment, and spore germination and colony development proceeded normally.

Protoplasts previously have been derived from mycelium of *P. megasperma* (11). A colony of a *Phytophthora* sp. in its vegetative state is coenocytic. Therefore, protoplasts derived from mycelium may be devoid of nuclei, or they may contain one or more nuclei. The use of zoospores to create hybrids increases the likelihood of fusion between uninucleate cells.

TABLE 1. Severity of lesions on host plants susceptible to either *Phytophthora capsici* or *P. nicotianae* after inoculation with parental organisms or putative species hybrids

Fungal isolate[a]	Radish	Lemon	Tomato
P. capsici Mex^{r}5	+++++[b]	–	+++++
P. nicotianae Fpa^{r}10	–	+++++	+++++
H0	++	++	+++
H1	–	–	++
H4	+++	++	+++

[a] *P. capsici* Mex^{r}5 and *P. nicotianae* Fpa^{r}10 are parental organisms. Isolates H0, H1, and H4 are somatic species hybrids of *P. capsici* and *P. nicotianae*.
[b] – = no lesion formation, and +++++ = very dark lesion of maximum size.

Similarities between the morphological and pathogenicity traits of hybrids H0, H1, and H4 and those of *P. capsici* and *P. nicotianae* suggested that species hybrids had been formed. However, the hybrid character of these isolates was demonstrated conclusively by the occurrence of DNA sequences unique to each parental species. Goodwin and Fry (8) stressed the importance of this line of evidence in confirming species hybridization. Because the genomic structure of fused spores could not be predicted, it was important to use repetitive DNA sequences, rather than low-copy sequences, of each parental species.

The events that occur after zoospores fuse in the presence of PEG are not known. In particular, it is not known whether the nuclei of the two organisms fuse or remain distinct. Observations of enlarged zoospores in fusion solution showed cells with either two nuclei or a single, enlarged nucleus (data not shown). Further observations are required to determine whether the occurrence of mixed populations of both types of zoospores represent stages in cellular reorganization or whether the interspecific multinucleate condition is stable. Changes in pathogenicity observed in species hybrids may be a consequence of the exchange of intact chromosomes, recombination, or differences in ploidy levels.

Regardless of their nuclear conditions, species hybrids, when reisolated from tomato or other test plants, remained stable in their hybrid characteristics and have remained stable through multiple inoculations and reisolations. These hybrids also were able to reproduce asexually through production of zoospores from colonized plant tissues. It will be of interest to determine whether the abilities to grow and reproduce are the minimum attributes required for establishment of new variants among populations of parental pathogen species.

With this zoospore fusion protocol, it is now possible to generate a series of hybrids with modifications in host range and other important traits related to pathogenesis. This protocol, in combination with unambiguous DNA markers, enables genetic analysis of pathogenesis in *Phytophthora* species that are recalcitrant to traditional approaches based on sexual crossing.

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