Variation in Cutinase, Esterase, and Chromosome Patterns in Nop Mutants of a Transformed Pathogenic Strain of *Phytophthora capsici*

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ABSTRACT

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We have designed a strategy to isolate nonpathogenic (Nop) mutants of the Oomycete fungus *Phytophthora capsici*. This strategy involves direct enrichment of Nop mutants by exposure of mutagenized zoospores to pepper (*Capsicum annuum*) seedlings and the isolation of germinating zoospores that show chemotaxis but are unable to penetrate the host tissue. After enrichment, 13 Nop mutants were isolated at a frequency of $2\text{–}5 \times 10^{-2}$ by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. The nonpathogenic phenotype of the mutants was tested by inoculating pepper seedlings as well as pepper fruits with zoospores and mycelia; in all cases no disease symptoms were observed. The Nop phenotype was stable for at least 25 asexual generations. In addition to the Nop phenotype, the Nop mutants showed reduced cutinase and esterase activities, and the electrophoretic karyotype of mutant strains was changed compared to the progenitor.

Additional keywords: chromosome rearrangement, MNNG mutagenesis.

The fungus *Phytophthora capsici* Leonian causes blight of peppers (*Capsicum annuum* L.). This disease has a destructive phase at the crown of the stem that results in wilting and development of a purplish-black canker advancing up the main stem, which eventually causes girdling of the stem and death of the plant (4).

The interaction of pepper plants with *P. capsici* has been a model system for investigation of host-pathogen interaction. Fungal signal proteins called elicitins, in particular capsicein (3) and enzymes such as those involved in phytoalexin biosynthesis (28), have been identified as components of this host-pathogen interaction. However, little is known about the molecular basis of pathogenicity of this fungus. To date, only the genes for cutinase production (9,33) and pisatin demethylation (29,39) from *Nectria haematococca* and the *b* locus from *Ustilago maydis* (18,30) have been isolated and characterized as important components of fungal pathogenesis.

The use of mutagens to induce specific mutations and the selection of survivors with an auxotrophy or resistance to an antibiotic or drug has demonstrated clearly its usefulness for genetic analysis in *Phytophthora* species (31). Strains of *P. capsici* resistant to the fungicide metalaxyl have been generated from zoospores in the laboratory by mass selection (6) after ultraviolet irradiation (7) or chemical mutagenesis (8), thus, making it possible to investigate the problem of fungicide resistance.

To address pathogenesis, it is necessary to generate stable nonpathogenic (Nop) mutants and characterize the mutated genes responsible for this phenotype. Moreover, the availability of transformation systems for *P. capsici* (1,2) makes the cloning of altered genes in Nop mutants feasible.

The application of pulsed field gel electrophoresis to separate intact chromosomes in agarose is useful for analyzing fungal genomes (22). By this analysis, major genome rearrangements have been detected in some fungi (15,21,27,35,36). Despite the importance of *Phytophthora* spp., there are only two reports of electrophoretic chromosome separation comparing the patterns of six species (12,37). The analysis of polymorphisms in chemically induced *Phytophthora* spp. mutants has not been reported. This paper presents a novel strategy for the isolation of Nop mutants of *P. capsici* by in planta selection after chemical mutagenesis. We also show that cutinase and esterase production in Nop mutants is reduced and that the mutant strains have alterations in chromosome length.

MATERIALS AND METHODS

Chemical mutagen. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was purshased from Aldrich Chemical Co., Milwaukee, WI.

Fungal isolates and growth conditions. *P. capsici* P1314-T1 pathogenic to peppers after transformation to hygromycin B resistance with plasmid pHL1 (1) was used to produce zoospores for the chemical mutagenesis. Zoospores were obtained by growing the strain on cleared V8-juice agar and hygromycin B at 200 μg/ml at 27 C for 3–4 days in petri plates. A 5-mm-diameter cork borer was used to cut disks from the agar plates placed (10 per plate) in 15 ml of 20% cleared V8 broth and grown for 2 days at 27 C. The mycelial mats were rinsed three times with sterile demineralized water, placed in 15 ml of sterile soil extract (10 g of soil per liter of water), and incubated for 2 days at 27 C to induce sporangia formation. To initiate zoospore release, the mats were placed in sterile demineralized water and chilled at 4 C for 30 min to 1 h. The zoospore suspension was collected and centrifuged at 9,000 *g* for 15 min.

Mutagenesis procedure. Chemical mutagenesis of *P. capsici* P1314-T1 hygromycin B-resistant zoospores was done (8) with a 10-min exposure to MNNG at 40 μg/ml and carried out in 30-ml corex tubes. Cysts exposed to the mutagen were collected by centrifugation at 9,000 *g* for 10 min after inactivation of MNNG with sodium thiosulfate (2.5%) and washed three times with sterile water. Cysts were resuspended in 200 ml of sterile water and placed in a 355-ml polystyrene cup containing three bare-rooted Serrano pepper seedlings. After incubation for 24 h at 27 C, the roots were washed with sterile water, and the suspension of

the attached zoospores was centrifuged at 3,000 *g* for 5 min and plated on selective media ([14]; PARPV) containing V8 agar, vancomycin (100 μg/ml), rifampicin (10 μg/ml), ampicillin (200 μg/ml), pentachloronitrobenzene (100 μg/ml) to control bacterial and fungal contamination, and hygromycin B (200 μg/ml). Plates were incubated at 27 C to allow the development of colonies on the agar surface. Plugs (5 mm diameter) were taken from each colony and transferred to V8 agar. A survival curve of zoospores treated with MNNG was done before the in planta experiments.

Screening for Nop mutants of *P. capsici* P1314-T1. Individual colonies resistant to hygromycin B were grown in V8 broth to obtain mycelium or 5×10^5 zoospores, as previously described (14), and then were used to inoculate Serrano pepper seedlings (grown in sterile soil) in polystyrene cups containing 200 ml of sterile water and incubated at 27 C in a growth chamber. Mycelium or zoospore suspensions were inoculated into 5-mm holes made on Serrano pepper fruits. The fruits were previously disinfected with a 3:1 bleach solution. Additionally, uninjured pepper fruits, disinfected as above, were inoculated with a zoospore suspension or mycelia in petri plates and kept at 27 C. All inoculated fruits were covered with plastic to maintain 100% relative humidity. Results of inoculation of fruits and seedlings were recorded after 10 days of incubation. Inoculation experiments were repeated at least three times with three replicates. In addition, an inoculation with the hygromycin-resistant progenitor *P. capsici* was carried out as a control.

Cutinase assay. For the cutinase assay, five petri plates containing 20 agar plugs of each strain were grown on V8 broth at 28 C. After 2 days, the cultures were washed with sterile-demineralized water to eliminate the V8 broth. The washed mycelium was used to inoculate 50-ml flasks containing 10 ml of fresh minimal medium (20) and 7 mg of tritiated apple cutin (H^3-cutin) (17) radiolabeled with $(H^3)NaBH^4$ (Amersham, Buckinghamshire, England) as a carbon source. The cultures were grown for 4 days, and 500 μl of each culture medium was used to measure the release of radioactive soluble material from H^3-cutin after centrifugation of the samples for 5 min in Eppendorf tubes. Protein concentrations were determined by the bicinchoninic acid method (32) with 50 μl of the culture medium. The same amount of total protein from each culture medium was used for the tritiated cutinase assay.

Esterase activity. The assay for esterase activity was performed with the culture medium, as described previously (16), after 4 days induction with apple cutin; esterase activity was measured spectrophotometrically by following the absorbance at 405 nm of the released *p*-nitrophenyl esters used as substrates. The activity was calculated (1 U is equal to 0.02 nmol/μg of *p*-nitrophenyl butyrate hydrolyzed per minute per milliliter of culture supernatant) based on equal protein concentrations and nanomoles of substrate hydrolyzed with a standard curve made with *p*-nitrophenol, a compound released after hydrolysis of *p*-nitrophenyl butyrate (25).

Preparation of chromosomal samples and gel electrophoresis conditions. Chromosomal DNA preparations of untransformed *P. capsici* P1314, P1314-T1 and 10 monozoospore cultures of the P1314-T1 strain, and mutant isolates for CHEF (contour clamped homogeneous electric field) gel electrophoresis were made from mycelial cultures grown in V8 broth at 27 C for 2 days (5). The mycelium, grown as above, was obtained by filtration, washed three times with sterile-demineralized water, and resuspended in 1 M mannitol/7 mM $MgSO_2$ (9:1) for the preparation of protoplasts (19), using Cellulysin (Behring Diagnostics, La Jolla, CA) at 20 μg/ml and Novozym 234 (Novo Laboratories, Wilton, CT) at 50 μg/ml. Protoplasts were centrifuged at 9,000 *g* for 10 min, and the pellet was resuspended in 1.4% low melting-point agarose (Bio-Rad, Richmond, CA), 250 mM EDTA (pH 7.5), and 1 M mannitol at 50 C. The sample plugs were incubated in NDS buffer (0.5 M EDTA, pH 8.0; 0.01 M Tris-HCl; 1% lauroylsarcosine; and proteinase K at 2 mg/ml [Boehringer Mannheim, Mannheim, Germany]) at 55 C overnight. After five washes in 0.5 M EDTA, pH 8.0, the plugs were stored at 4 C in 0.05 M EDTA. Chromosomal DNA samples of yeasts and *Neurospora crassa* were prepared as described by Gunderson and Chu (11). CHEF gels were prepared from 100 ml of 0.8% agarose (Bethesda Research Laboratories, Gaithersburg, MD) in 0.5× TAE buffer (1× TAE = 0.04 M Tris-acetate, 0.001 M EDTA; for 72 h at 50 min and 72 h at 30 min switching times, at 50 V). The gels were run in a CHEF mapper system (Bio-Rad) to attempt chromosome separation with the following conditions: 168 or 240 h running time, 20–70 min switching intervals with linear ramping, 120-degree constant angle, 1.5 V/cm, 14 C constant temperature. Chromosomal DNA samples also were run in a CHEF apparatus with vertical driving electrodes separated by 26.5 cm (donated by J. R. Ecker, University of Pennsylvania, Philadelphia) with the following conditions: 72 h at 50 min, 18 h at 45 min, and 72 h at 30 min switching times, 50 V at 14 C. At the end of the run, the gel was stained in 100 ml of 0.5× TAE containing ethidium bromide at 10 μg/ml for 20 min and destained in the same buffer for 30 min to 1 h.

Hybridization analysis. After electrophoresis, DNA was depurinated with 0.25 M HCl for 30 min then denatured for 30 min (0.5 M NaOH, 1.5 M NaCl) and neutralized for 30 min (0.5 M Tris-HCl, pH 7.0, 3 M NaCl). Chromosomes or digested genomic DNA were transferred to Hybond-N nylon membrane (Amersham) by blotting with 25 mM phosphate buffer, and the blot was baked at 80 C for 2 h. DNA hybridization was performed according to standard procedures (20), and probes were labeled by the random primer method (10). Probe pUC18 was used to identify the transformed plasmid pHL1 *P. capsici* P1314-T1 progenitor and the isolated Nop mutants. Plasmid containing a telomeric repeat from *Arabidopsis thaliana* (26) was donated by E. Richards (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

RESULTS

Isolation of Nop mutants of *P. capsici* P1314-T1. To enrich for *P. capsici* P1314-T1 mutants affected in pathogenesis, we developed a procedure that would enrich for a population capable of adhering to but not penetrating the plant cuticle. The mutagenized hygromycin B-resistant zoospores were placed in contact with bare-rooted seedlings of Serrano peppers (grown in sterile soil) in sterile water to allow penetration of the plant tissue and initiation of infection. After this incubation period, zoospores able to bind but not penetrate were collected by a light washing. The number of hygromycin B-resistant colonies isolated from the washed roots was between 100 and 150 out of an initial inoculum of $1–2 \times 10^8$ zoospores. Only three to six of the hygromycin B-resistant colonies were identified as stable Nop mutants after several tests on pepper plants and fruits. The survival frequency of zoospores treated with MNNG was 0.001%. In all three independent experiments with the chemical mutagen, the frequency of Nop mutants was $2–3 \times 10^{-8}$ (Table 1) from the total population and $3–5 \times 10^{-2}$ of the zoospores obtained after root washing. Similar experiments with nonmutagenized zoospores yielded no Nop isolates. Mutants isolated after MNNG mutagenesis and selection in pepper seedlings were retested for

TABLE 1. Mutation frequency of *Phytophthora capsici* P1314-T1 obtained in three independent experiments with the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

No. of zoospores treated	Isolates from washed roots[y]	Nop mutant isolates[z]	Mutation rate
2.0×10^8	150	6	3×10^{-7}
1.6×10^8	119	4	2.5×10^{-7}
1.3×10^8	100	3	2.3×10^{-7}

[y] Isolates recovered from washed roots on selective medium (14) containing V8 agar, vancomycin, rifampicin, ampicillin, pentachloronitrobenzene, and hygromycin B after mutagenesis and 24-h selection in planta.
[z] Nonpathogenic mutants on pepper seedlings and pepper fruits after 25 asexual generations.

pathogenicity on Serrano pepper seedlings and fruits and were nonpathogenic even after 25 asexual generations on V8 agar. The symptoms produced by the pathogenic transformed strain are shown in Figure 1B. We screened for mutant isolates causing no symptoms. Plants inoculated with Nop mutants showed the same phenotype as Nop 38 shown in Figure 1C.

A Southern blot hybridization analysis of the transformed hygromycin B-resistant isolate of *P. capsici* P1314-T1 and the Nop mutants was performed with pUC18 as the probe (pHL1 [38] is a derivative of pUC12 plasmid and contains the *hsp*70 promoter of *U. maydis* fused to the hygromycin B phosphotransferase gene from *Escherichia coli*) to demonstrate that the isolated mutants recovered from the pepper roots were identical to the progenitor strain, except for the mutant phenotype and genotype. When total DNA from pHL1 transformants was digested with *Hin*dIII, a single 2.6-kb band was observed, corresponding to the expected size of pUC12 of the plasmid (Fig. 2).

The production of zoospores by mutant strains was reduced 10-fold when compared to the parental strain. The sporangial morphology of the mutants seen under the microscope was very similar to that of the parental strain. The sporangia of *P. capsici* varied in shape, e.g., sphaerical, ellipsoid, ovoid, and obpyriform in morphology. We did not see any abnormal morphology in the mutants analyzed compared to the parental transformed or untransformed strains (data not shown).

Cutinase and esterase activities in Nop mutants. We compared the cutinase and esterase activity of the Nop mutants to that of the parental strain of *P. capsici* P1314-T1 and the *P. capsici* P1314 untransformed strain. The highest esterase activity was found in the parental strains (P1314-T1 and P1314); the activities of both strains were similar. The Nop mutants, on the other hand, had significantly lower esterase activity. The calculated values for enzyme activity at 3 min are shown in Table 2. The esterase activity among the Nop mutants was only 13–29% of that of the parental strain. Cutinase activity was assayed after 4 days of induction from cultures grown on H^3-cutin. Lower rates of cutinase activity, 0–18.5% of that of the parental strain, were obtained from the Nop mutants compared to the progenitor as seen in Table 2. The activities shown were measured from the release of soluble material from H^3-cutin as a specific cutinase assay. A *Fusarium* sp. isolated from soil was used as a positive control for cutinase and esterase production, and in both assays showed higher activities than did *P. capsici* P1314-T1 or P1314.

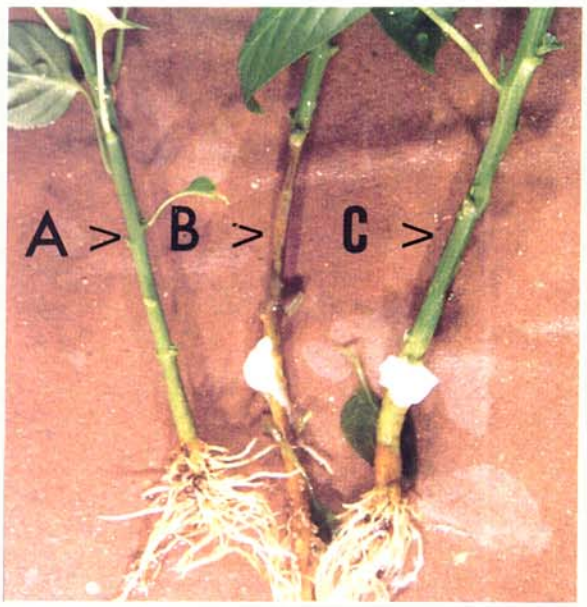

Fig. 1A–C. Pathogenicity tests of nonpathogenic (Nop) mutant isolates on bare-rooted pepper seedling. **A,** Uninoculated pepper plant. **B,** Seedling inoculated with transformed *P. capsici* P1314-T1 strain showing the typical symptom, black canker. **C,** Seedling inoculated with mutant Nop 38 showing no symptoms of necrosis or blight.

Electrophoretic karyotype analysis of *P. capsici* P1314-T1 and Nop mutants. The main problem encountered in the preparation of chromosomal DNA of *P. capsici* was the degradation of DNA during the production of protoplasts and the embedding in agarose. The same problems were observed in the preparation of *P. megasperma* (12) and other filamentous fungi (36). This problem was partially overcome by increasing the mannitol concentration to 90% during protoplast production and EDTA to 0.5 M during the embedding in agarose. DNA electrophoretic differences in mutants of several fungi have been correlated with colony morphology and virulence (27,35). Therefore, we investigated whether differences could be detected between Nop mutants and the parental *P. capsici* P1314-T1 strain at the chromosome level. Chromosome length differences were found between the parental P1314-T1 strain and mutant isolates even after 25 asexual generations.

The parental strain P1314-T1 (Fig. 3A, lanes 3 and 6) showed one high molecular weight band above 5.7 Mb; two diffuse bands, one above 4.7 Mb and one below 4.7 Mb; three or more faint bands approximately 3.5 Mb in size; two more diffuse bands above 2.2 Mb that may be composed of two chromosomes in each band; and two more below the 2.2-Mb chromosome of *Saccharomyces cerevisiae*. Three mutant isolates were separated under the same conditions used for the parental strain (Fig. 3A,

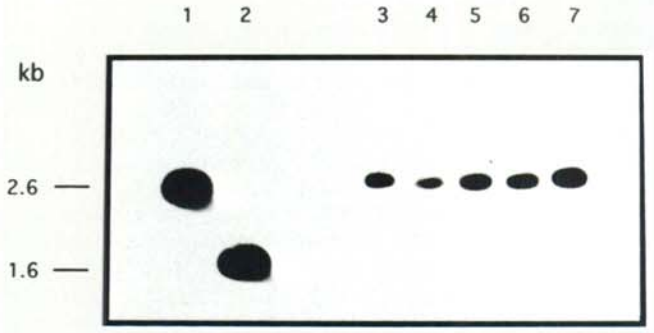

Fig. 2. Southern blot hybridization analysis of pHL1 *Phytophthora capsici* transformants. Total genomic DNA (23) from *P. capsici* transformants grown in V8 broth containing hygromycin B at 200 μg/ml was isolated and digested with *Hin*dIII and electrophoresed on 0.8% agarose gels at 59 V for 2 h. After electrophoresis, the gel was blotted and hybridized against ^{32}P-labeled pUC18. Lane 1, *Hin*dIII-digested pUC18; lane 2, λ 1-kb ladder molecular weight marker; lane 3, transformed pathogenic *P. capsici* P1314-T1; lanes 4–7, nonpathogenic mutants 13, 16, 30, and 38, respectively.

TABLE 2. Esterase and cutinase activities in culture medium from *Phytophthora capsici* P1314-T1 and the Nop mutants after 4 days of induction on cutin and H^3-cutin

Strain	Esterase[x]	Percent[y]	Cutinase[z] (cpm/mg protein)	Percent[y]
P1314-T1	510 (±90)a	100.0	8,876 (±157)a	100.00
Nop 13	150 (±35)b	29.4	3,586 (±90)b	18.50
Nop 16	66 (±10)c	12.9	2,375 (±50)c	0.00
Nop 30	90 (±16)d	17.6	2,384 (±120)c	0.07
Nop 38	96 (±20)d	18.8	2,375 (±90)c	0.00
Fusarium sp.	1,064 (±120)e	...	13,617 (±305)d	...

[x] Esterase activity was assayed with the culture filtrate, using *p*-nitrophenyl butyrate as a substrate. Results are the averages of three experiments with three replicates per test. Numbers in units = nanomoles of substrate hydrolyzed per minute per milliliter. Numbers in parens (±) represent standard deviation. Numbers followed by the same letter are not significantly different at $P = 0.05$, as determined by Tukey's test.

[y] Activities are given as the difference compared with *P. capsici* 1314-T1 strain activity (100%). A control with culture medium alone for esterase activity showed 0 units and for cutinase activity 2,381 cpm.

[z] Cutinase activity was measured by the release of radioactivity from H^3-cutin (counts per minute per milligram of protein). Results are the averages of three experiments with three replicates per test.

lanes 4, 5, and 7). Significant chromosome length variations were found between the mutants and the parental P1314-T1 strain. Two of the most conspicuous changes were the absence in the mutants of the highest molecular weight parental band and the presence of new smaller bands in the mutants.

To visualize the location of diffuse bands and the intensity of the bands in the parental strain and the Nop mutants, Southern blot hybridization was performed using the telomeric repeat from *A. thaliana* as a probe (Fig. 3B). We found that all the chromosomes hybridized to the telomeric repeat, suggesting that *P. capsici* contains telomeric sequences similar to other eukaryotes. We again observed that the highest molecular weight band in the mutants is not present, confirming major chromosomal losses and/or rearrangements in the Nop mutants (Fig. 3B). Chromosome variations also were seen when gels were run for 240 h in the CHEF mapper under the conditions described in Figure 3A and C. Chromosome variations also were seen between the parental P1314-T1 strain and the Nop mutants in the gel run in the CHEF apparatus

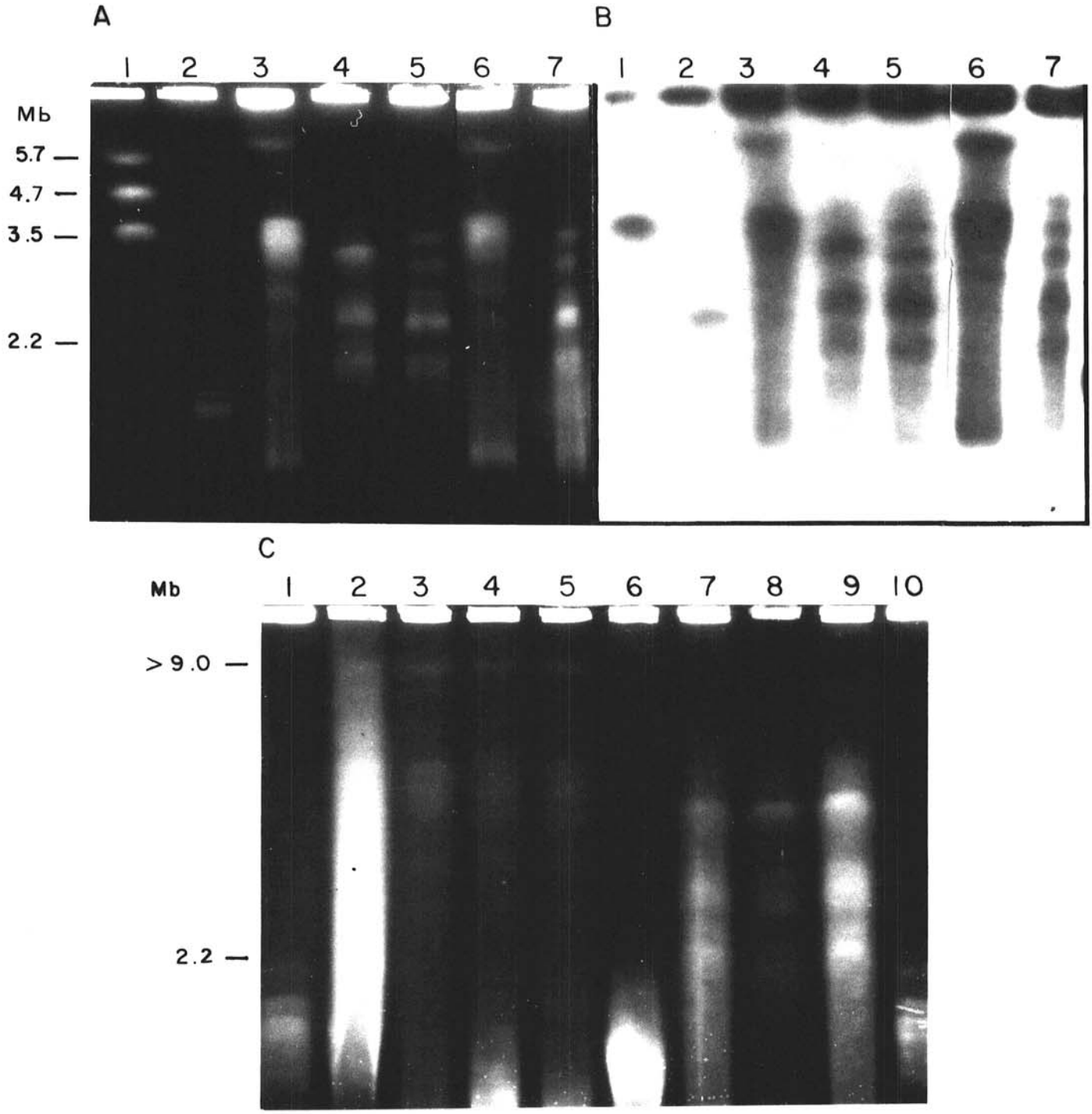

Fig. 3A–C. Comparison of *Phytophthora capsici* P1314-T1 and nonpathogenic (Nop) mutant chromosomes by **A and C,** pulsed field gel electrophoresis, using the contour clamped homogeneous electric field (CHEF) mapper apparatus, and **B,** hybridization of the resolved chromosomes. Lane 1, *Schizosaccharomyces pombe* and lane 2, *Saccharomyces cerevisiae* YPH274 were used as size markers. Lanes 3 and 6, two preparations of *P. capsici* P1314-T1 chromosome samples. Lanes 4, 5, and 7, Nop mutants 13, 30, and 38, respectively. **A,** a CHEF-gel run for 168 h with 20–70 min switching times, linear ramping, and stained with ethidium bromide. **B,** a Southern hybridization using *Arabidopsis thaliana* telomeric repeat as a probe. **C,** a CHEF-gel run for 240 h with the same switching times as **A,** in which lanes 1, 6, and 10 are *Saccharomyces cerevisiae* YPH274 size marker. Lane 2, *Neurospora crassa* as a size marker. Lanes 3, 4, and 5, three different chromosomal preparations of P1314-T1. Lanes 7, 8, and 9, Nop mutants 13, 30, and 38, respectively.

with vertical driving electrodes as shown in Figure 4A.

To evaluate the amount of variation that could occur among zoospore cultures of the transformed P1314-T1 strain, chromosomal DNA preparations of 10 monozoospore cultures, as shown in Figure 4B, were run in the CHEF apparatus with vertical driving electrodes under the same conditions as in Figure 4A. No variation among the zoospore cultures was observed.

DISCUSSION

Chemically induced Nop mutants in the diploid organism *P. capsici* were selected after screening colonies derived from zoospores. The screening method used for the isolation of the potential mutants was aided by an enrichment procedure that facilitated the recovery of the strains with the desired phenotype. In attempting to isolate Nop mutants of *P. capsici*, our rationale was to use the host plant as the selective agent for enrichment of mutants affected in the recognition of the host or in the production of penetration enzymes. The exposure of 1–2 $\times$ 10^8 mutagenized zoospores to pepper seedlings for 24 h was enough to select for mutants that were attracted to the roots but could not penetrate the pepper plants. Those zoospores attached to the roots were washed off and plated on selective PARPV medium with hygromycin B to ensure growth of only *P. capsici* colonies. The treat-

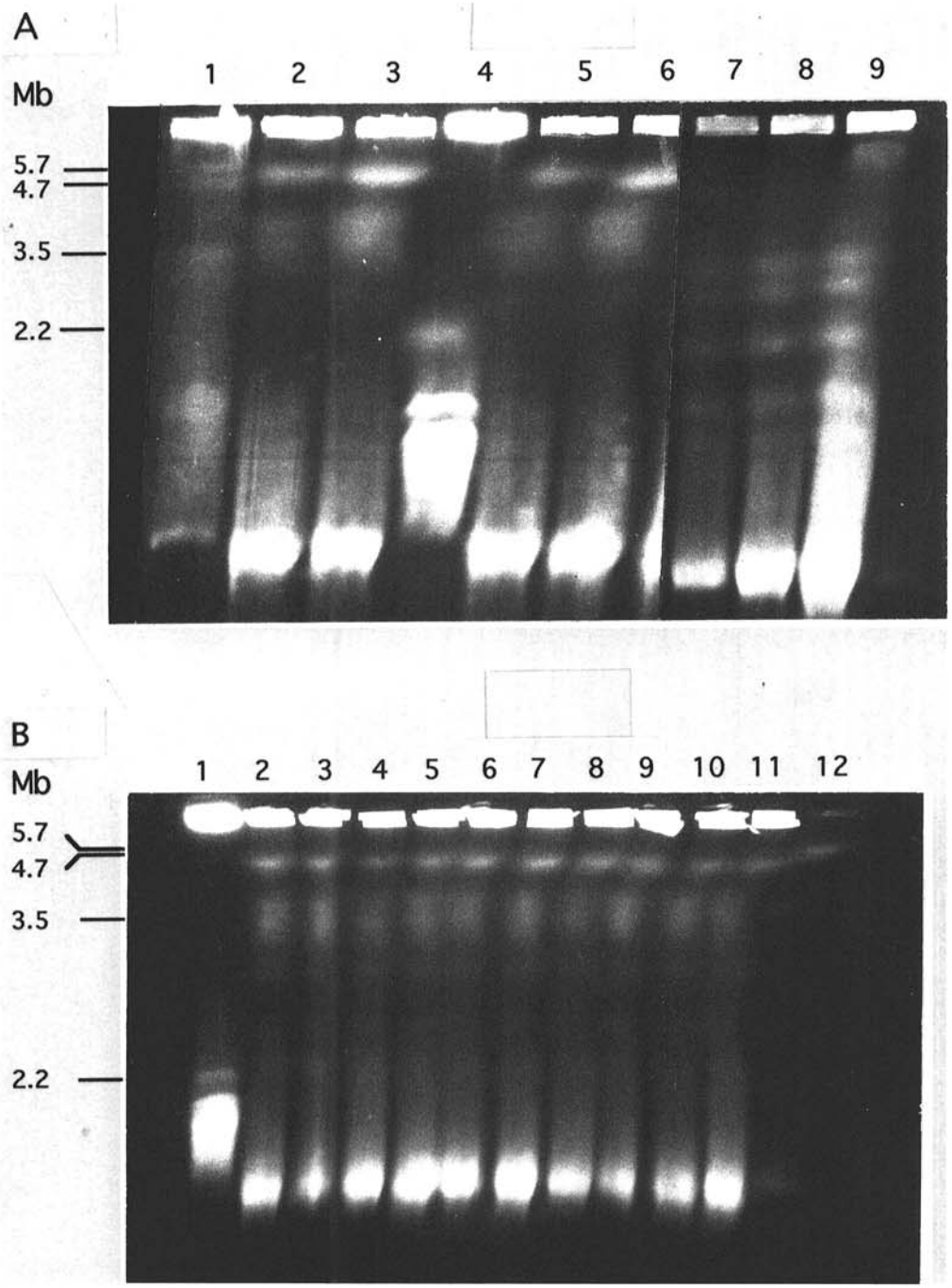

Fig. 4A and B. Comparison of untransformed *Phytophthora capsici* P1314, P1314-T1 monozoospore cultures, and nonpathogenic (Nop)-mutant chromosomes by the contour clamped homogeneous electric field (CHEF) mapper apparatus with vertical driving electrodes. **A,** a CHEF-gel run 72 h at 50 min, 18 h at 45 min, and 72 h at 30 min switching times. Lane 1, *Schizosaccharomyces pombe* and lane 4, *Saccharomyces cerevisiae* YPH274 as size markers. Lanes 2 and 3, two preparations of untransformed P1314; lanes 5 and 6, two chromosomal preparations of P1314-T1. Lanes 7–9, three chromosomal preparations of Nop mutant 27. **B,** a CHEF-gel run with the same conditions as **A**. Lane 1, *Saccharomyces cerevisiae* YPH274 and lane 12, *Schizosaccharomyces pombe* as size markers. Lanes 2–10, chromosomal samples of 10 monozoospore cultures of P1314-T1.

ment of *P. capsici* zoospores with the chemical mutagen MNNG and the selection in planta resulted in the generation of a few isolates exhibiting a Nop phenotype in vivo to pepper seedlings and fruits. The type of mutants that could perhaps be selected and isolated by this strategy are: 1) those defective in the recognition of the host, although still capable of attachment to the roots; 2) those that cannot produce enzymes involved in penetration; and 3) those that are nonpathogenic in inoculated pepper-fruit tissues. All mutants isolated were incapable of causing disease, even when inoculated inside of fruits, and they showed reduced cutinase and esterase activity. Because of the gross changes seen in these mutants, we suspect that more than one step in the pathogenesis process is affected.

There are no reports of Nop mutants of *Phytophthora* spp., only of mutants resistant to fungicides (6,8,13) for which the frequency of mutation reported is similar to the one reported here. Similar behavior was observed with metalaxyl-resistant mutants generated by the chemical MNNG (7,13).

Studies of the relationship between *P. capsici* oospore and zoospore inoculum levels and mortality of peppers (4) have shown that 75–95% of plants inoculated with 10–25 zoospores, respectively, died. Therefore, our results showing decreased zoospore production in the mutants, 10^6 compared to 10^7 for the parental strain, should not be a limiting factor for infection.

Cutinase is synthesized by many plant-pathogenic fungi to dissolve the plant cuticle during penetration. There is considerable evidence indicating the crucial role of cutinase in the penetration and establishment of disease by some pathogenic fungi (17), as well as controversial evidence indicating that it is unnecessary for pathogenicity of *F. solani* f. sp. *pisi* on peas (34). The results presented here indicate that cutinase gene expression in *P. capsici* Nop mutants is affected. The biological significance of cutinase in plant-pathogenic fungi has been demonstrated with mutants of *F. s. pisi* (17), in which reduced cutinase activity was correlated with lower efficiency of infection. On the other hand, Stahl and Schäfer (34) concluded in their gene-replacement work that the cutinase gene of *F. s. pisi* is not essential for the infection of pea. However, there are probably more enzymes involved in the penetration and colonization process, and suppression of only one enzyme may not be responsible for the loss of pathogenicity.

Based on the molecular weights of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* chromosomes, we estimated that *P. capsici* chromosomes range from 5.7 to 1.7 Mb. This size range is very similar to the range reported by Howlett (12) for *P. megasperma* and the six *Phytophthora* spp., including *P. capsici* shown by Tooley and Carras (37). The conditions we used to separate the chromosomes of *P. capsici* allowed us to see more chromosomal bands compared to the cluster of bands seen under the conditions previously reported (37), as well as more of the differences between the mutants and the parental strain.

The chromosome number of *P. capsici* may be more than 10, estimated on the basis of the faintly resolved bands. However, a more accurate estimate cannot be obtained until available genes from *Phytophthora* spp. can be used to establish molecular linkage groups and until further resolution of chromosomes by the improvement of the CHEF technique.

Telomeric repeats are highly conserved sequences among divergent eukaryotes, in most cases the repeat of one species cross-hybridizes with other unrelated species. The hybridization of *P. capsici* with a telomeric region of *A. thaliana* clearly showed the presence of chromosomal bands at different positions between the wild-type strain and the isolated mutants, as well as the existent homology in the telomeric sequences. There are previous reports of *Chlamydomonas* hybridization with telomeric sequences of *A. thaliana* (24,26).

The presence of chromosome length differences in the mutant isolates was unexpected. Nevertheless, chromosome polymorphisms have been observed in morphological mutants of *Candida albicans* (27,35) that were correlated with colony morphology, state of ploidy, and virulence. Chromosomal length differences also have been described in different isolates or races of *Cladosporium fulvum* (36) and *Colletotrichum gloeosporioides* (21). The chromosomal rearrangements may play a role in generating variation in the organism. In the case of *P. capsici* Nop mutants, chromosomal rearrangements could be correlated with low levels of cutinase and esterase activity and with the complete loss of pathogenicity.

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