Induction of Auxotrophic Mutants in Phytophthora by Ultraviolet Light

Jesus Castro F., George A. Zentmyer, and William L. Belser, Jr.

Former Research Assistant and Professor, respectively, Department of Plant Pathology; and Associate Professor of Microbiology, Department of Life Sciences, University of California, Riverside 92502. Present address of senior author: Centro de Investigaciones Agrícolas de el Bajío, Apartado Postal No. 112, Celaya, Gto., Mexico. Supported by NSF Grants No. GB-658 and GB-7765. Accepted for publication 23 September 1970.

ABSTRACT

Zooospores of Phytophthora capsici and P. drechsleri were irradiated with ultraviolet (UV) while motile and after encystment, at a dosage that killed 99% of them. Mutants were screened by the “rescue method” and by the “starvation method,” which are discussed in relation to Phytophthora. Sensitivity of zooospores to UV light was different for motile and encysted zooospores, as shown by different survival curves and yield of mutants. No auxotrophic mutants were found among 685 survivors when zooospores were irradiated while motile. Four of 359 survivors of zooospores irradiated after encystment were auxotrophic mutants. Two (P. capsici) were methionine-dependent; two (P. drechsleri) required tryptophan. These results support the concept of haploidy in the vegetative stage of Phytophthora. Phytopathology 61:283-289.

Additional key words: genetic studies, methionine-deficient mutants, tryptophan-deficient mutants, ploidy.

RESUMEN

Zooosporas de Phytophthora capsici y P. drechsleri fueron irradiadas con luz ultravioleta (UV) en estado móvil (nado) y después de enquistadas, a una dosis que causó 99% de mortalidad. La selección de mutantes se hizo por el método de “rescate visual” y por el de “filtración sucesiva,” los cuales se discuten en relación con Phytophthora. Las curvas de sobrevivencia y el número de mutantes obtenidos indicaron una diferencia de sensibilidad a la luz UV entre las zooosporas móviles y las enquistadas. No se encontraron mutantes auxotróficas entre 685 sobrevivientes cuando las zooosporas fueron irradiadas en estado móvil. Cuando las zooosporas fueron irradiadas después del enquistamiento 4 de 359 fueron mutantes auxotróficas. Dos (P. capsici) requerían metionina y dos (P. drechsleri) requerían triptófano. Estos resultados soportan el concepto de que el estado vegetativo de Phytophthora es haploide. Phytopathology 61:283-289.

Genetic studies of Phytophthora have been hampered by the lack of good genetic markers, and the difficulty in germinating and establishing cultures from single zooospores. Markers used in such studies are: rate of growth; colony morphology; size and shape of sporangia; color of mycelium; pathogenicity; physiological races; and mating type (4, 6, 7, 15, 17). Unfortunately, none of these is single-gene dependent, and some are known to be under cytoplasmic control in other fungi (9).

Several workers have attempted to obtain mutants of this fungus by various means (2, 5, 6, 18, 19). Clarke & Robertson (5) and Sansome (16) have cited the failure to induce and isolate auxotrophic mutants as additional evidence to support their postulation of diploidy in the vegetative stage of the fungus.

This study was undertaken with the objective of isolating mutants of Phytophthora which would permit genetic studies of this genus.

MATERIALS AND METHODS.—Isolates.—Cultures of P. capsici Leonian, designated P-504 and P-505, were isolated from pepper in Chapingo, Mexico. The mating type of these cultures was determined by pairing them with known A1 and A2 types of P. infestans (Mont.) d. By. Culture P-504 was A1 and culture P-505 was A2 mating type. Strains of P. drechsleri Tucker, designated P-208 and P-209, were Galindo’s (6) strains 6500 and 6503, respectively.

Media.—The minimal medium (MM) was that of Bartnicki-Garcia (1) containing per liter: KH₂PO₄, 2 g; CaCl₂, 3.4 mg; FeSO₄·7H₂O, 1.0 mg; citric acid, 1.4 mg; ZnSO₄·7H₂O, 1.8 mg; MnSO₄·H₂O, 0.3 mg; CuSO₄·5H₂O, 0.4 mg; (NH₄)₂SO₄·4H₂O, 0.3 mg; MgSO₄·7H₂O, 0.5 g; thiamine·HCl, 1.0 mg; NaNO₃, 0.3 g; and Difco Bacto purified agar, 15 g.

The complete medium (CoM) consisted of MM supplemented with casein hydrolysate (2 g/liter) and the following vitamins (1 mg/liter): B₁, B₂, biotin, inositol, and folic acid. A partially enriched medium (0.1 CoM) was prepared by adding the above substances at one-tenth of the above concn to MM.

Clear V-8 juice (CV-8) was prepared by adding 5.0 g of CaCO₃ to 354 ml of Campbell’s V-8 juice and centrifuging the mixture at 100 g for 15 min. The supernatant was diluted 1:4 with deionized water. A solid medium (CV-8A) was prepared by adding 15 g/liter of Bacto agar.

Zooospore production.—All isolates were grown in 60 × 15-mm petri dishes containing CV-8 liquid medium at room temp (22-26°C) for 7-10 days. Mycelial mats bearing sporangia were washed 3 times in sterile deionized water to remove nutrients, and chilled at 4°C for 15-20 min. Zoospores were released at room temp 20-30 min after the chilling period, and the resulting suspension of zoospores was passed through a plastic screen or through several layers of cheesecloth to remove sporangia.

Ultraviolet irradiation.—Zooospores were irradiated while they were swimming and at intervals after encystment. Encystment was induced by shaking zooospores...
45-60 sec in a Vortex mixer. They were irradiated at the following time intervals after shaking: (i) 5 min, at which time about 99% of the zoospores were encysted; (ii) 15 min when 100% were encysted and 2-5% were beginning to germinate; and (iii) 25 min when about 10% of the zoospores were germinated. A zoospore suspension (3 ml) was irradiated under an ultraviolet lamp, G.E. 68T5 (wave length 2,537 A), at a distance of 20 cm for 3 min in 60 x 15-mm petri dishes with covers off.

**Screening of mutants.—**Two methods were used for isolation of “mutants” (or survivors) after irradiation. The “rescue” method consisted of plating irradiated zoospores on solid MM, 0.1 CoM, and CV-8A. Slow growers were identified by microscopic examination and transferred to CoM.

The “filtration enrichment” or “starvation” method (11) consisted of adding 4 ml of MM to each 60 x 15-mm petri dish of irradiated zoospores and incubating them in darkness to avoid photo reactivation for 2-3 days. The suspension was then passed through cheesecloth, plastic mesh, or muslin which removed some of the prototrophs which had produced mycelium. The filtrate was plated on solid CV-8A, CoM or 0.1 CoM.

The survivors were transferred from CoM to CV-8A slants for stock cultures and to MM for testing of nutritional requirements. Isolates producing sparse mycelium on MM were transferred serially twice on MM to eliminate the possibility that the growth resulted from nutrients carried with the inoculum plug. Inoculum plugs, 2 mm in diam, were used to prevent selection of “false prototrophs”. Isolates failing to grow on MM were plated on MM plus one of the following pools to determine nutritional requirements: pool 1—leucine, isoleucine, valine, methionine, lysine, threonine; pool 2—tryptophan, phenylalanine, tyrosine; pool 3—arginine and histidine; pool 4—glutamic acid, glycine, serine, alanine, cystine, cysteine, proline, hydroxyproline; pool 5—purines and pyrimidines; pool 6—B,H, biotin, inositol, folate acid.

In some tests, sodium thiosulfate (Na,S,O, was added to MM at concn from 10 to 50 mg/liter, in line with the report of Pontecorvo et al. (13) that auxotrophs obtained by the “starvation” technique included up to 60% of nonutilizers of sulfate which responded to methionine, cysteine, and inorganic thiosulfate.

**RESULTS.—**Response of zoospores to ultraviolet dosages.—When encysted zoospores were irradiated and plated on MM or 0.1 CoM, killing of zoospores was directly proportional to ultraviolet dosage up to 120 sec (99.4% kill). Further exposure of 90 sec gave only a 0.4% additional kill, indicating that a portion of the zoospore population was resistant to ultraviolet (Fig. 1). When motile zoospores were irradiated and plated on CV-8A, the killing was logarithmically related to the dosage. Irradiation for 180 sec killed 99.8% of the motile zoospores. Accordingly, most further irradiations were made at this dosage. In these tests, the survivors were counted after 48 hr incubation.

There was a higher percentage of survivors when zoospores were irradiated 5 min after encystment than when irradiated 25 min after encystment.

![Fig. 1. Effect of ultraviolet (U.V.) dosage on survival of zoospores of Phytophthora capsici. A = Zoospores irradiated while motile and plated on clear V-8 juice agar. B = Zoospores irradiated 5 min after encystment and plated on 0.1 CoM complete medium. C = Zoospores irradiated 5 min after encystment and plated on minimal media.](image)

Irradiation of zoospores at 5-15 min after encystment resulted in the production of four auxotrophic mutants among 359 survivors tested. Irradiation of motile zoospores did not yield any auxotrophic mutants among the 685 survivors tested.

**Methionine-dependent mutants.—**Mutants P505-6 and P505-32 of P. capsici failed to grow on MM but grew well on MM supplemented with methionine or cystine (Fig. 2). P505-32 also grew sparsely in the presence of threonine and serine, two precursors of methionine, but P505-6 did not grow in the presence of these two amino acids. P505-6 failed to grow on MM supplemented with sodium thiosulfate. Since P505-6 appeared more stable than P505-32, it was selected for further studies.

1) **Stability.—**The stability of methionine mutant P505-6 was studied. In 2 out of 40 plates on MM, P505-6 began to grow after 34 days. One of the “adapted” strains was found to grow well on both MM and MM + methionine. To determine the percentage of reverted nuclei, the “adapted” strain was passed serially through MM and sampled by plating zoospores on MM and MM + methionine after each passage. The survival of zoospores on MM after each of the transfers was 25, 60, 85, and 90%, respectively. The survival on MM + methionine was 95-98%.

Fifty-four single-zoospore cultures were isolated from P505-6 and tested for growth requirements. None grew on MM unless methionine was added. On CV-8A eight of these cultures grew slowly, and some sectored. When the sectors were transferred to fresh CV-8A they grew normally.

2) **Effect of inoculum size on growth.—**Three single
zoospore cultures from PS05-6 were grown on CV-8A. Discs of 2-, 4-, and 8-mm diam were transferred from the margin of the growing colony to MM and MM + methionine. Three replicate plates were incubated in darkness at room temp (24°C). There was no correlation of growth on MM with inoculum size.

3) Pathogenicity.—The pathogenicity of mutant PS05-6 was tested to determine whether or not ultraviolet irradiation had altered it, and to test the stability of the mutant after passage through leaf tissue. Detached leaves of Pimento pepper were inoculated with zoospores of PS05 and PS05-6 and incubated at room temp in petri dishes containing water-soaked filter paper. Four days after inoculation, symptoms were evident and both strains appeared equally pathogenic. PS05-6 was reisolated by cutting small pieces of leaf tissue about 20 mm from the site of inoculation and plating them on CV-8A. In this medium, the fungus had the same rate of growth and morphology as the original mutant. When tested for nutritional requirements, it was still methionine-dependent.

4) Survival of mutants.—Information on the length of survival of a mutant in MM would be valuable in the screening of mutants by the "starvation" method. To determine this, zoospores of PS05-6 were plated on MM and on MM + methionine as a control. Drops of a methionine solution were added to MM plates at different times after plating.

Germination of zoospores in MM appeared normal until the germ tube reached a certain length (approx 12 hr after encystment) when the rate of growth decreased. Germ tube length was variable. Fig. 3-A shows the appearance of zoospores on MM after 4 days. When methionine was added, many of the zoospores grew normally (Fig. 3-B). After the 6th day, the zoospores were vacuolated and many were dead. The survival rate decreased sharply and the germ tubes of a few zoospores continued to grow when methionine was added. A few zoospores grew even after 8 days on MM when methionine was added; nutrients released from the dead zoospores may have aided in survival.

Tryptophan-dependent mutants.—Mutants P209-105 and P209-151 of P. drechsleri failed to grow on MM but grew on MM + pool 2 (pool 2 = phenylalanine, tryptophan, tyrosine). When the individual components of this pool were tested, both isolates grew only in the presence of tryptophan (Fig. 4). P209-151 grew well at a concn of 0.1 mg tryptophan/plate. At 0.5 mg/plate, growth was slower and there were numerous
tested in CV-8A with, and without, tryptophan. Indoleacetic acid did not affect reproduction. β-sitosterol inhibited oosporic formation.

Since tryptophan mutants did not produce viable sorangia and zoospores could not be obtained, stability was tested by taking discs 4 mm in diam from the edge of colonies on CV-8A and plating on MM. Both mutants appeared stable, because no growth was found in any case.

Discussion.—The failure to induce and isolate auxotrophic mutants of Phytophthora by several authors (2, 5, 6) has been cited by Clarke & Robertson (5) as additional evidence of diploidy in the vegetative state. The main problem, however, was apparently technical, since we isolated biochemical mutants from encysted zoospores after irradiation with ultraviolet.

Buddenhagen's (2) main objective was to obtain mutants of any kind, and especially morphological mutants. His highest rate of mutation (50%) was obtained with ultraviolet irradiation for 45 sec, after which he recorded a survival of 25%. At these dosages of ultraviolet and percentage of survival it would be difficult to select auxotrophic mutants. Selection of mutants was based on rate of growth and morphology of the colony, two factors which in our study were not always correlated with mutation. The grading of mutants was done after the survivors had been transferred 3 times in complete media, increasing the possibility of reversion to the wild type. He also irradiated the zoospores while motile, a condition which appears to be less suitable for obtaining auxotrophic mutants, as will be discussed later. Galindo's (6) attempt to obtain auxotrophic mutants of P. drechsleri by Buddenhagen's technique probably failed for the same reasons.

Clarke & Robertson (5) attempted to isolate auxotrophic mutants of P. infestans. Although their method appears suitable for this purpose, the use of P. infestans is not recommended since the physiology of this fungus is not well established. Many isolates of this fungus are not able to grow on synthetic media. These workers also reported that some survivors behaved as nongrowers on MM when the inoculum was smaller than 10 mm diam, but the same condition was noted with the wild type. Their negative results were not surprising since the behavior of the wild type was inconsistent, as indicated by the fact that 85% of the zoospores failed to grow on a complete medium due to unknown factors, and sporangia or mycelium from the wild type also failed to grow on MM. Therefore, a survivor that behaved as a nongrower could be a wild type and not a mutant.

The problems in obtaining mutants can be divided into two major categories: (i) induction of mutants; (ii) isolation of mutants.

Induction.—The establishment of the optimal dosage for production of mutants by ultraviolet irradiation is of primary importance. This is difficult because the optimum dosage depends on: (a) age of cells, (b) conidial spores, etc.; however, one of the most important factors is the "sensitivity period". Witkin (20) concluded that the sensitivity period is closely related to protein synthesis and DNA replication during the first postirradiation
cell division. It is proposed that the sensitivity period may be a limiting factor in obtaining auxotrophic mutants of *Phytophthora*. When zoospores were irradiated while motile, no auxotrophic mutants were found among more than 1,500 survivors studied by Buddenhagen (2), Clarke & Robertson (5), and us: 300, 542, and 685, respectively. But when zoospores were irradiated after encystment, the yield of auxotrophic mutants was about 1% among the slow growers tested. Therefore, it appears that the nucleus of the motile zoospore is not in the sensitivity period but instead is in a resting state. During encystment, the nucleus may begin coding for enzymes and other substances required for germination.

The differences in response to ultraviolet irradiation of motile and encysted zoospores were also evident in the survival curves. With swimming zoospores, a linear response was obtained, while with encysted zoospores the curve was sigmoidal when plotted on a log scale.

Buddenhagen (2) stated, "Motility of zoospores immediately after irradiation was not affected by dosages which resulted in complete lethality or even by dosages three times those which resulted in 99% lethality". We confirmed this in tests, using twice the dosage that killed 99.8% of the zoospores. Thus, motility of zoospores appears to be independent of nuclear condition.

It is interesting to note that shaking of zoospores for 1 min caused a rapid encystment, while high dosages of ultraviolet had no effect.

The sensitivity of zoospores appeared to vary at different stages of germination. When zoospores were irradiated at 25 min after encystment, the yield of survivors was lower than when irradiated at 5 min after encystment. Since the same time of irradiation (3 min) was used in both cases, this difference might be explained on the basis of differences of sensitivity at different stages of germination. The fact that the auxotrophic mutants were isolated from zoospores irradiated at 5-15 min after encystment indicates that this is the best time for ultraviolet treatments.

Isolation of mutants.—There are several methods for isolation of mutants, each with some advantages and limitations depending upon the organism under study (3, 11). In the case of *Phytophthora*, the rescue method had some disadvantages. When the irradiated zoospores were plated on complete media, the fast growers produced a profuse mycelium which overgrew the slow growers. When MM was used, and zoospores with short germ tubes were recovered, a high proportion of the isolated spores failed to grow in CoM. Partially enriched media (0.1 CoM) appeared to be the most suit-
able for isolating mutants. The limited amount of nutrients permitted the growth of auxotrophic mutants and reduced the growth rate of prototrophs. Thus, slow growers could be recovered after 4-5 days of incubation. No auxotrophic mutants were recovered by the rescue method.

The filter technique was the most selective method because prototrophic survivors are eliminated by repeated filtration. One of the problems encountered was that zoospores became attached to the bottom of the petri dish during incubation in liquid MM, and in the process of dislodging them many were damaged. When the suspension was shaken to prevent the attachment, the zoospores agglutinated. The problem was partially solved by shaking the zoospore suspension for short time periods (5-10 min) at intervals of 3-4 hr during the first 24 hr. The suspension was filtered only once after 2-3 days of incubation. Since ultraviolet irradiation causes a delay in growth, many prototrophs may have passed through the filter, resulting in a low yield of mutants among the slow-growing survivors. Therefore, it is suggested that in future work more frequent filtration and a longer time of filtration should be tried. Despite these problems, this technique appeared more promising than the rescue method. All of the auxotrophic mutants obtained were recovered by the filtration method.

Auxotrophic mutants.—Auxotrophic mutants were isolated in pairs (two methionine and two tryptophan), which may mean that these loci were more mutable and/or that these mutants were selected for by the method of isolation. In this study, methionine-dependent zoospores survived up to 8 days on MM. Fontecorvo et al. (13) reported that 60% of the mutants obtained by the filter technique responded to methionine, cystine, and thiosulfate. P50S-6 responded only to methionine and cystine; which is not unusual since these kinds of mutants have been reported previously in other fungi and in bacteria.

Some workers have attempted to correlate changes in nutritional requirements with changes in pathogenicity. Wilde (19), using induced pathogenic mutants of P. infestans, found no correlation between these characteristics. Our studies with P. capsici confirmed the lack of correlation, since the auxotrophic mutant P50S-6 was as pathogenic as the wild type. Passage of the mutant through leaf tissue did not affect its nutritional requirements.

In contrast to the results of Clarke & Robertson (5), the size of the inoculum disc had no effect on the growth of our mutants in MM, MM + methionine, or MM + tryptophan. P50S-6 as mycelium or zoospore (smallest unit) grew if methionine was present. The presence of some sparse hyphae in MM when large inocula of P50S-6 were used may be the result of translocation of nutrients from the disc rather than adaptation or reversion. When discs containing these hyphae were transferred again to MM they failed to grow. The fact that some reversion occurred with large inocula might be explained by the fact that the probability of reversion is increased because of the larger number of nuclei and nutrients present in large discs.

The role of tryptophan in oospore and sporangium formation as well as in the morphology of the mycelium deserves more investigation. The response of both mutants to different concn of tryptophan indicates that it could be acting as a precursor for a hormone-like substance required for formation of oospores and sporangia. The response of some strains of P. capsici which failed to form oospores when mated with A1 or A2 types but formed them when tryptophan was added supports this hypothesis. When tryptophan was added to the medium, two of the six strains tested produced 2 to 3 times more oospores when mated with A2 type than in those matings without tryptophan.

Failure to obtain biochemical mutants in Phytophthora has been one of the bases for Sansome's (16) proposal of gametangial meiosis. The fact that auxotrophic mutants could be isolated from P. capsici and P. drechsleri strongly supports the commonly accepted theory of Raper (14) that the vegetative stage in this organism is haploid rather than diploid. Our results are not in accord with the interpretations of Buddenhagen (2), Clarke & Robertson (5), and Sansome (16), and suggest that the previous failures to isolate nutritionally deficient mutants were due to methodology rather than to the plodding of the Phytophthora nucleus.

LITERATURE CITED

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