

## Chlamydospore Formation in Sporangium-free Liquid Cultures of *Phytophthora parasitica*

Peter H. Tsao

Professor, Department of Plant Pathology,  
University of California, Riverside 92502.

### ABSTRACT

*Phytophthora parasitica* produced abundant chlamydospores when mycelial mats grown at 25 C in a liquid medium were submerged in deep water and subsequently incubated for 2 to 3 weeks at 15 to 18 C. Chlamydospore production was poor if incubation temperature was 24 C or above. Formation of sporangia was prevented due to reduced aeration in submerged cultures. *Phytopathology* 61: 1412-1413.

*Additional key word:* sporulation.

Most *Phytophthora* species produce chlamydospores (7) which play an important role in the survival of these fungi in nature. In our work with citrus isolates of *Phytophthora parasitica* Dast. [*P. nicotianae* var. *parasitica* (Dast.) Waterhouse (6)], studies on the physiological, soil ecological, and ultrastructural aspects of chlamydospores had been hampered because ordinary culturing procedures with either solid or liquid media produced a mixture of chlamydospores and sporangia. This paper reports a method for mass production of chlamydospores in axenic liquid cultures devoid of sporangia. A brief account has appeared earlier (3).

Based on Klebs' Principles and the knowledge of the importance of aeration in sporangium formation, several preliminary experiments were conducted which showed that the fungus produced only chlamydospores in liquid cultures when the vegetative mycelium was depleted of nutrients and aeration. The fungus was subjected first to an optimum presporulation growth phase in a suitable liquid medium, followed by a low-temperature sporulation phase in which the spent medium had been diluted with a large volume of water. Due to the lack of adequate aeration, the submerged mycelial mat converted the assimilated nutrients into chlamydospores instead of sporangia.

The procedures finally evolved were as follows: Inoculum plugs of 7-mm diam were taken from the edge of a 5- to 10-day-old culture grown on cornmeal agar (Difco, 17 g/liter) and each placed in a 12-oz glass prescription bottle (volume, ca. 350 ml) containing 25 ml of a liquid medium. The medium, similar to that used previously for sporangium production (1), was a modification of the clear V8-CaCO<sub>3</sub> broth medium reported by Mircetich et al. (2) for the production of *P. cinnamomi* chlamydospores. It consisted of filtered Campbell's V-8 juice, 100 ml; filtered 2% CaCO<sub>3</sub>, 100 ml; and deionized water, 800 ml. After incubation at 25 C in the dark for 22-24 hr, each bottle was shaken

vigorously by hand (30 short strokes) to fragment the hyphae which had grown out from the inoculum plug. Care was taken to resuspend the hyphal fragments adhering to the bottle wall by slowly rotating the bottle. The bottles were then incubated horizontally as stationary cultures at 25 C for 6 days, at which time the mycelium (free of any spore forms) had covered most of the medium surface. One hundred ml of sterile deionized water was added to each bottle, and the bottles were further incubated, but vertically and at 18 C. Usually within a few seconds or minutes, the mycelial mat sank to the bottom of the liquid (50 mm in depth in the bottle). Occasionally, a mycelial mat with entrapped air would float on the medium surface for considerable duration, and gentle shaking of the bottle for several minutes was necessary to facilitate sinking. After 2 to 3 weeks, abundant chlamydospores (about 5 X 10<sup>5</sup> to 1 X 10<sup>6</sup> spores/bottle) were formed on the submerged mycelial mats. Formation of sporangia was prevented in deep water.

For harvesting the chlamydospores, the spore-bearing mycelial mats from several bottles were pooled (with inoculum plugs removed), filtered and washed repeatedly on a Büchner funnel, and blended for 3 min in 150 ml of water in a Sorvall Omni-Mixer (setting of 8) surrounded by cold water to prevent overheating. The lighter mycelial fragments and heavier chlamydospores in the resultant suspension were partially segregated by centrifuging the suspension twice for 15 sec each in a clinical centrifuge (400 g at the end of 15 sec). Approximately 30-35% of the chlamydospores were lost during centrifugation. The chlamydospore suspensions at the bottom of centrifuge tubes were pooled and washed by centrifugation (3 min, 1,500 g) 3 times in sterile deionized water before use.

The viability of the harvested chlamydospores was determined by using 60 µg/ml of rose bengal solution (2). With chlamydospores harvested from 10 separate experiments, the viability ranged from 83 to 93% (average 87%). The germination of viable spores in diluted (10%) V8-CaCO<sub>3</sub> broth medium in microbeakers at 25 C ranged from 67 to 99% (average 90%) after 16 to 18 hr.

The postgrowth incubation at 18 C was critical (Fig. 1-A). Few chlamydospores were produced at 27 or 30 C, which are within the optimum temperature range for vegetative growth of the fungus. Maximum chlamydospore production at 3 weeks after the addition of water to the liquid medium was at 15-18 C. Chlamydospores produced at 15 C, however, were generally smaller than those produced at 18 C. All later experiments, therefore, were conducted at the postgrowth incubation temperature of 18 C.

Few or no chlamydospores were produced prior to the addition of water to the medium and incubation at 18 C (Fig. 1-B). The production of chlamydospores progressively increased and attained high concentrations at 2-3 weeks.

Microscopic examinations of chlamydospore suspensions showed they were usually free of sporangia or other spore forms. Occasionally 1-2% of

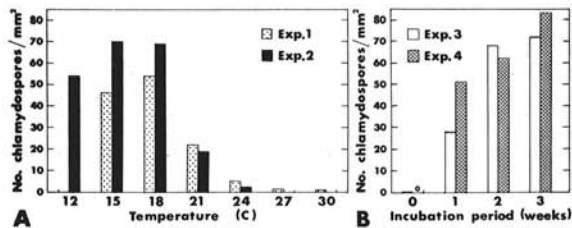


Fig. 1. Formation of chlamydospores of *Phytophthora parasitica* (isolate T131) in submerged liquid cultures. The data were averages of three replicate bottles. Mycelial mats were poured to individual petri plates and the number of chlamydospores was counted from each replicate under a stereoscopic microscope at X 100 magnification from 10 fields (each about 5.7 mm<sup>2</sup>) selected in a standardized manner. The figures in the graphs were converted from average numbers of chlamydospores/field. A) Effect of postgrowth incubation temperature (exp. 1, 15 to 30 C; exp. 2, 12-24 C) on chlamydospore formation 3 weeks after the addition of water to the liquid medium. The incubation temperature for the presporulation growth phase was 25 C for all treatments. B) Chlamydospore formation at 18 C at weekly intervals after the addition of water to the liquid medium. Three replicate bottles were harvested each week. One week of mycelial growth had occurred at zero week.

the propagules were observed to be sporangia. The formation of excessive numbers of sporangia in some culture bottles was in part due to (i) floating of air-entrapped mycelial mats on the medium surface for considerable durations; and (ii) sporulation from the mycelial growth emanated from hyphal fragments left on the bottle wall before the addition of water to the bottle. Caution should be taken to prevent these from occurring.

Chlamydospore production was equally high, or even higher, if 1-week-old mycelial mats were first washed aseptically to remove the medium, then incubated in deep water in the culture bottles. This procedure, however, caused substantial loss of mycelium and increased chances of contamination for the subsequent sporulation phase. Consequently, it was not adopted in our routine procedure.

This method of incubating submerged cultures at low temperature has been used successfully in over 70 trials during the past 5 years to produce large

quantities of chlamydospores of isolate T131 of *P. parasitica* for various physiological and soil ecological studies (4, 5). The method was applicable also to isolates T64 and T89 of *P. parasitica*. In an experiment in which isolates T64, T89, and T131 were used, the numbers of chlamydospores/mm<sup>2</sup> produced in submerged cultures after 2 weeks at 18 C were 55, 97, and 77, respectively. *Phytophthora palmivora* Butl. (papaya isolate) also produced chlamydospores with this method (W. H. Ko, personal communication). *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, however, failed to produce chlamydospores at this postgrowth incubation temperature. *Phytophthora capsici* Leonian, which is not known to produce chlamydospores, also failed to produce chlamydospores with this method. Suitable incubation conditions for postgrowth sporulation phase are apparently different for various species of *Phytophthora*, and need to be specifically devised for individual species.

#### LITERATURE CITED

- MENYONGA, J. M., & P. H. TSAO. 1966. Production of zoospore suspensions of *Phytophthora parasitica*. *Phytopathology* 56:359-360.
- MIRCETICH, S. M., G. A. ZENTMYER, & J. B. KENDRICK, JR. 1968. Physiology of germination of chlamydospores of *Phytophthora cinnamomi*. *Phytopathology* 58:666-671.
- TSAO, P. H. 1967. Production of chlamydospores of *Phytophthora parasitica* in liquid culture. *Ann. Phytopathol. Soc. Japan* 33:73 (Abstr.).
- TSAO, P. H. 1969. Studies on the saprophytic behavior of *Phytophthora parasitica* in soil, p. 1221-1230. In H. D. Chapman [ed.]. *First Intern. Citrus Symp. Proc. Univ. Calif., Riverside*.
- TSAO, P. H. 1970. Applications of the vital fluorescent labeling technique with brighteners to studies of saprophytic behavior of *Phytophthora* in soil. *Soil Biol. Biochem.* 2:247-256.
- WATERHOUSE, GRACE M. 1963. Key to the species of *Phytophthora* de Bary. *Mycol. Papers* 92, Commonwealth Mycol. Inst., Kew., England. 22 p.
- WATERHOUSE, GRACE M. 1970. The genus *Phytophthora* de Bary, diagnoses (or descriptions) and figures from the original papers [2nd ed.]. *Mycol. Papers* 122, Commonwealth Mycol. Inst., Kew, England. 59 p.