Production of Chlamydospores by Phytophthora palmivora in Culture Media

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Journal Series Paper No. 1482, Hawaii Agricultural Experiment Station. Supported in part by the W-38 Western Regional Research Project.

We thank M. Aragaki for assistance and the use of laboratory facilities.

Accepted for publication 1 November 1972.

ABSTRACT

More than 70% of the chlamydospores produced by *Phytophthora palmivora* in a papaya juice medium had thick walls. However, only thin-walled chlamydospores were produced by the same fungus using the submerged culture method described by Tsao for *P. parasitica*. Thick-walled chlamydospores germinated poorly, whereas almost all thin-walled chlamydospores germinated in water, on V-8 juice agar, and on Noble water agar. After exposure to 46 C for 5 min, the proportion of thick- and thin-walled chlamydospores still capable of germinating on Bacto water agar was 85 and 15%, respectively. Thick-walled chlamydospores produced in papaya juice medium were morphologically and physiologically similar to those produced in naturally infected papaya fruits.

Phytopathology 63:559-562

Additional key words: Carica papaya.

Phytophthora palmivora Butler, previously referred to as *Phytophthora parasitica* Dast., attacks fruits and trunks of papaya (*Carica papaya* L.) during rainy periods and causes root rot and death of papaya seedlings in replant fields (2, 4, 5). This fungus produces numerous sporangia on the surface of lesions and produces chlamydospores inside diseased tissues. To study survival of a fungus in soil, it is desirable to produce large quantities of spores which are similar to those produced in nature. This paper reports a method for inducing production of *P. palmivora* chlamydospores which are morphologically and physiologically similar to those produced in naturally infected papaya tissues.

MATERIALS AND METHODS.—*Source of cultures.*—A single zoospore isolate (18F-2P) of *P. palmivora* used in this study was obtained from an infected papaya root. Other isolates of *P. palmivora* were: P-152, P-162, and P-170 from papaya; and P-179, P-198, and P-199 from orchid. The isolates of *P. parasitica* were P-174 from papaya, and P-111 from citrus. All isolates of *P. parasitica* and *P. palmivora*, except 18F-2P, were obtained from M. Aragaki. Isolates were maintained on V-8 juice agar (per liter: 200 ml V-8 juice, 1 g $CaCO_3$, 20 g agar).

Chlamydospore production.—A submerged culture method described by Tsao (6) was used to produce chlamydospores of *P. palmivora*. V-8 juice supplemented with 0.1% $CaCO_3$ was clarified by centrifugation at 12,000 *g* for 10 min. Twenty-five ml of 20% clear V-8 juice broth in a 250-ml flask was autoclaved and inoculated with five discs (6-mm diam) from a 4-day-old culture. After incubation for 5 days at 22 C, the V-8 juice medium was replaced aseptically by 100 ml of sterile distilled water, and the submerged culture was further incubated for 4 weeks at 16 C in the dark.

To test chlamydospore formation in fruit and vegetable juice media, commercially prepared guava, passion fruit, orange, papaya, and V-8 juices supplemented with 0.1% $CaCO_3$ were centrifuged, diluted with the same amount of water, autoclaved, and inoculated as described above. Cultures in juice media were incubated at 22 C for 4 weeks.

Thick-, intermediate-, and thin-walled chlamydospores were defined as those spherical spores with wall thickness greater than 1.5 μ, between 1.5 μ and 1 μ, and less than 1 μ, respectively. The relative numbers of sporangia and each type of chlamydospore were determined by counting 300 spores per flask with three replicates per treatment.

To study chlamydospore formation in papaya fruits, mature fruits were washed in running tap water and placed in plastic containers with four or five fruits per container. Three agar discs from a 5-day-old culture were placed on each fruit at the distal, mid, and proximal points. After incubation for 48 hr, the covers of the containers were removed and replaced by four layers of cheesecloth. The infected tissue was examined microscopically after 2 weeks.

Germination of chlamydospores.—Suspensions of thick- and thin-walled chlamydospores were prepared by teasing apart the washed mycelial mat in a small petri dish containing ca. 20 ml of sterile distilled water. The suspension was washed six to eight times by sedimentation in HCl-washed test tubes to remove excess nutrients, mycelial fragments, and damaged spores. The undamaged spores settled first. The spore suspension was adjusted to ca. 10 spores per field at X100 magnification. Germination in water was tested by adding 1 ml of spore suspension to 1 ml of sterile distilled water in a small glass cell (22-mm diam). Germination on agar media was tested by inoculating 20% V-8 juice agar, 2% Bacto water agar and 2% Noble water agar with ca. 1 ml of spore suspension. After incubation for 24 and 48 hr at 28 C, 50 to 100

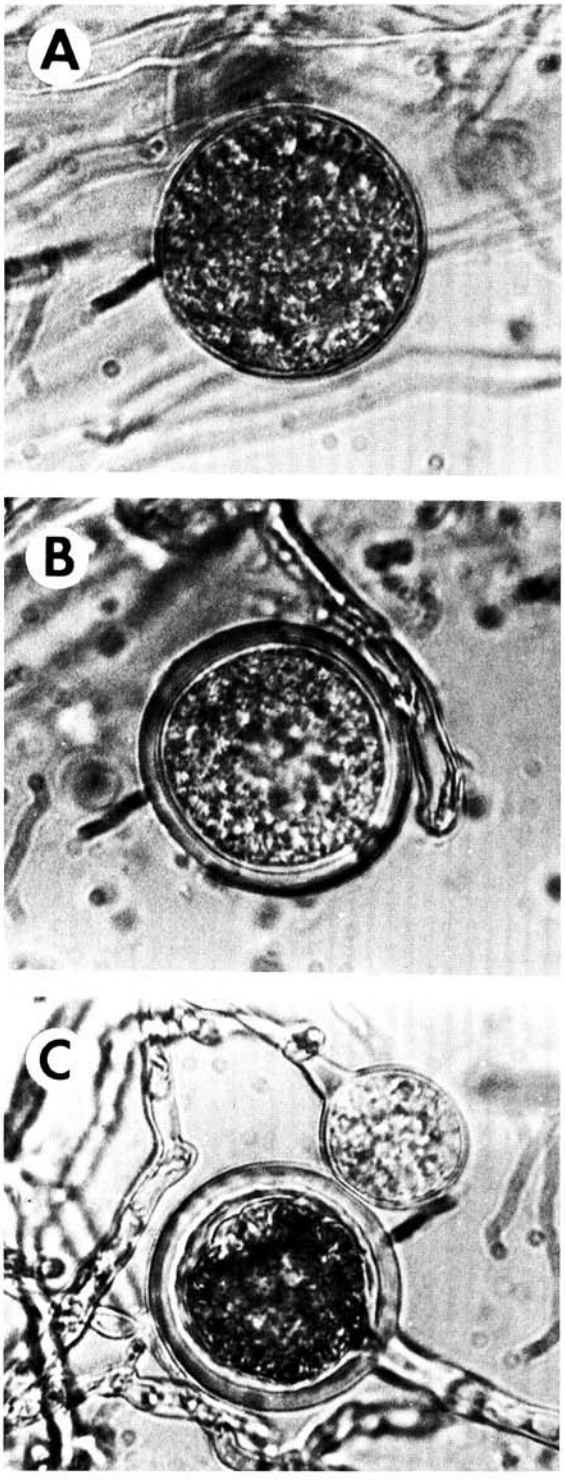

Fig. 1. Chlamydospores of *Phytophthora palmivora*. **A)** A thin-walled chlamydospore produced by the submerged culture method (× 1,500). **B)** A thick-walled chlamydospore obtained from a naturally infected papaya fruit (× 1,500). **C)** A thick-walled chlamydospore with an immature one produced in the papaya juice medium (× 1,500).

spores were counted per plate with three replicates per treatment.

Tolerance of chlamydospores to high temperature was tested by heating 7 ml of a suspension of thick- or thin-walled chlamydospores in a test tube at 46 C for 5 min. Percentage germination of treated spores was tested on Bacto water agar. The control consisted of untreated spores plated on this medium.

A suspension of thick-walled chlamydospores obtained from a naturally infected papaya fruit was prepared by macerating the internal tissue of the lesion in sterile distilled water. The mixture was filtered through a layer of tissue paper to remove aggregates of hyphae and cellular debris. Spores were then washed by sedimentation, heat-treated, and tested for germination as previously described.

RESULTS.—*Chlamydospore production.—Phytophthora palmivora* (18F-2P) produced abundant thin-walled chlamydospores using the submerged culture method described by Tsao (6) for *P. parasitica* (Fig. 1-A). In naturally infected papaya fruits as many as 90% of the chlamydospores had thick walls (3) (Fig. 1-B). Thus, various fruit juice media were tested for their ability to stimulate *P. palmivora* to produce thick-walled chlamydospores. *Phytophthora palmivora* produced abundant chlamydospores, 23% of which were thick-walled, in papaya juice medium (Table 1, Fig. 1-C), and only 6% of spores produced in this medium were sporangia. Passion fruit juice medium also stimulated formation of thick-walled chlamydospores and inhibited sporangium formation. However, the total spore production was much less than in the papaya juice medium. Thick-walled chlamydospores were not produced in guava, V-8, and orange juice media.

Phytophthora palmivora was grown in 1, 20, 50 and 100% papaya juice medium to determine the effect of papaya juice concentration on sporulation of this fungus. The concentration of papaya juice used was inversely correlated with percent sporangia and was directly correlated with percent thick-walled chlamydospores produced by *P. palmivora* (Table 2). No thick-walled chlamydospores were produced in 1 and 20% papaya juice media. However, in 50 and 100% papaya juice media, respectively, 21 and 74% of spores produced were thick-walled chlamydospores.

The other three papaya isolates and three orchid isolates of *P. palmivora* produced thick-walled chlamydospores in papaya fruits and in papaya juice media. Some isolates produced more thick-walled chlamydospores in 50% than in 100% papaya juice medium. *Phytophthora parasitica* isolates P-111 and P-174 produced no chlamydospores in either papaya fruit or papaya juice media.

Germination of chlamydospores.—Germination of thick- and thin-walled chlamydospores of *P. palmivora* on agar media and in water is shown in Table 3. Thick-walled chlamydospores germinated poorly, but the thin-walled chlamydospores germinated completely in distilled water, on V-8 juice agar and Noble water agar. Both types of chlamydospores germinated completely on Bacto

TABLE 1. Effect of fruit and vegetable juice media on sporulation of *Phytophthora palmivora*

Medium[a]	Total No. of spores[b]	Ratio of spores			
			Thickness of chlamydospore wall		
		Sporangia (%)	Thin (<1μ) (%)	Intermediate (1μ-1.5μ) (%)	Thick (>1.5μ) (%)
Guava	++	23	76	1	0
V-8	+++	26	72	2	0
Orange	+++	47	52	1	0
Passion fruit	+	7	11	64	18
Papaya	+++	6	13	58	23

[a] Clear nutrient broth containing 50% of each kind of juice and 0.1% $CaCO_3$.
[b] + = moderate; ++ = abundant; +++ = extremely abundant.

TABLE 2. Effect of concentration of papaya juice on sporulation of *Phytophthora palmivora*

Concentration of papaya juice[a] (%)	Ratio of spores			
		Thickness of chlamydospore wall		
	Sporangia (%)	Thin (<1μ) (%)	Intermediate (1μ-1.5μ) (%)	Thick (>1.5μ) (%)
1	48	52	0	0
20	21	75	4	0
50	9	9	61	21
100	1	1	24	74

[a] All the papaya juice media contained clear papaya juice diluted with various amounts of distilled water as indicated, and 0.1% $CaCO_3$.

water agar. On V-8 juice agar both thick- and thin-walled chlamydospores germinated by producing germ tubes which continued to grow and formed mycelial mats; whereas, in water and on water agar chlamydospores germinated by producing short germ tubes, each with a sporangium at the tip. Similar to thick-walled chlamydospores produced in the papaya juice medium, thick-walled chlamydospores obtained from naturally infected papaya fruits germinated completely on Bacto water agar, but poorly in distilled water, on V-8 juice agar and on Noble water agar.

After exposure to 46 C for 5 min, only 15% of the thin-walled chlamydospores were able to germinate on Bacto water agar. However, about 85% of the thick-walled chlamydospores obtained from diseased papaya fruits or produced in papaya juice medium were able to germinate after exposure to the same temperature for the same period.

DISCUSSION.—Observations of naturally infected papaya fruits revealed that as many as 90% of the chlamydospores produced by *P. palmivora* were thick-walled (3). More than 70% of the chlamydospores produced by *P. palmivora* grown in a papaya juice medium were thick-walled and were similar to those obtained in nature. However, only thin-walled chlamydospores were produced using the submerged culture method described by Tsao (6) for *P. parasitica*.

Besides the morphological difference, thick-walled chlamydospores differed from thin-walled chlamydospores in that (i) thick-walled

TABLE 3. Germination of thick- and thin-walled chlamydospores of *Phytophthora palmivora* on agar media and in water

Type of chlamydospores	Percent germination[a]			
	V-8 juice agar (%)	Bacto water agar (%)	Noble water agar (%)	Water (%)
Thick-walled	5	90	1	17
Thin-walled	95	95	90	82

[a] Percent germination was determined by counting 50 to 100 spores in each of three replicates.

chlamydospores germinated poorly, but the thin-walled chlamydospore germination was nearly 100% in distilled water, on V-8 juice agar and Noble water agar; and (ii) thick-walled chlamydospores had a higher tolerance to high temperature than thin-walled chlamydospores. This indicates that chlamydospores produced by some artificial methods differ both morphologically and physiologically from those produced in nature. Hendrix & Kuhlman (1) also reported that chlamydospores of *Phytophthora cinnamomi* produced in culture had a thinner wall than those recovered from naturally infested soil.

Our results suggest that Noble agar does not contain sufficient nutrients for germination of thick-walled chlamydospores of *P. palmivora*, whereas nutrients in the Bacto agar stimulate these spores to germinate by production of a sporangium on the tip of each germ tube. On V-8 juice medium, 5% of the thick-walled chlamydospores germinated by producing germ tubes which continued to grow. Substances in the V-8 juice agar are apparently inhibitory to germination of thick-walled chlamydospores with production of sporangia, and weakly stimulatory to germination by means of mycelial growth.

All isolates of *P. palmivora* which produced thick-walled chlamydospores in infected papaya fruits formed the same type of spores in papaya juice medium. No chlamydospores were produced by *P. parasitica* in either infected papaya fruits or papaya juice medium. These results indicate that extracts of host tissue which support chlamydospore formation of a fungus may be good materials for production of the same type of spores by the same fungus in an artificial medium.

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