Increased Chlamydospore Production by *Phytophthora cinnamomi* Using Sterols and Near-Ultraviolet Light

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ABSTRACT

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A regimen was developed using near-ultraviolet light, temperatures between 23 and 28 C, and a natural medium (V8 agar) supplemented with β-sitosterol for consistently producing high yields of chlamydospores of *Phytophthora cinnamomi*. Addition of β-sitosterol to V8 agar at 10–40 μg/ml stimulated chlamydospore production in cultures incubated in the light but not in those incubated in the dark. In the dark, growth (dry weight) was stimulated by sterol at 20 and 40 μg/ml; in the light, however, no stimulation was detected with sterol up to 200 μg/ml. Chlamydospores produced on V8 agar plus sterol were more abundant in cultures illuminated with 100, 200, or 400 μW cm^{-2} light in the 310–420 nm (near-UV) region than in the 470–610 or 550–725 nm region at the same intensities or in the dark. Chlamydospore production comparable to that in 310–420 nm light was obtained with Blacklight Blue fluorescent lamps, which emit predominantly near-UV light. Optimum intensity of Blacklight Blue light for sporulation was 100–200 μW cm^{-2}.

Additional key words: soil fungi.

Chlamydospores are considered the most persistent propagules of *Phytophthora cinnamomi* Rands commonly found in soil (7). Chlamydospores are routinely produced in our laboratory for soil infestation studies, but we frequently have great variations in yields. A routine method for consistent production of large quantities of chlamydospores was deemed desirable.

Although the effect of nutrients on *Phytophthora* spp. has been studied, little quantitative information is available on how they affect chlamydospore production by *P. cinnamomi*. Sterol is required for chlamydospore production by *P. cinnamomi* (6), and it stimulates growth and sporulation of some Phycomycetes when added to certain natural media (1,3). Similarly, light is important to reproduction in *Phytophthora* spp. (9), but little has been published about its effect on chlamydospore production by *P. cinnamomi* (14). Recent studies showed that monochromatic light in the blue and far-red regions stimulated oospore germination, and near-ultraviolet (near-UV) light stimulated sporangium production by *P. cinnamomi* (12,15).

This investigation outlines a regimen using a natural medium (V8 agar) supplemented with β-sitosterol, near-UV illumination, and incubation at an optimum temperature to produce high yields of chlamydospores of *P. cinnamomi*. A preliminary report has been published (13).

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MATERIALS AND METHODS

Isolates. *P. cinnamomi* isolate Pc 10 (A^2 mating type) was used except where noted. The isolate was from the crown of a wilted rhododendron from a Rhode Island nursery, and after such characteristics as ability to form sporangia and chlamydospores and pathogenicity to rhododendron were considered, Pc 10 was selected as representative of 25 isolates of the species collected from rhododendrons in Rhode Island over a 3-yr period. *P. cinnamomi* isolates from Oregon (#536, B. Moore, Oregon State University, Corvallis 97331) and Ohio (#544, H. Hoitink, Ohio Agricultural Research and Development Center, Wooster 44691), both from diseased rhododendrons, were also tested for stimulation of chlamydospores with near-UV light.

Media formulation, preparation, and seeding. V8 sterol agar was used because it supports formation of chlamydospores and rapid hyphal growth of *P. cinnamomi*. V8 agar was prepared as described by Mircetich et al (11), except the juice was clarified by centrifugation and vacuum filtration through Whatman glass fiber paper (GF/A). V8 sterol agar contained 100 ml of clarified V8 juice, 0.1 g of $CaCO_3$, 17 g of Bacto agar, 0.02 g of β-sitosterol dissolved in

10 ml of hot 95% ethanol, and 890 ml of distilled water. No sitosterol or ethanol (solvent) was added to V8 agar in sterol controls, and only ethanol was added in solvent controls. Medium was autoclaved 16 min at 121 C, and 10-ml aliquots were pipetted into 6-cm diameter plastic petri dishes. The agar was seeded by placing an inverted 5-mm diameter disk of V8 sterol agar cut from the periphery of a 5- to 9-day-old colony of *P. cinnamomi* in the center of each dish.

Clarified V8 juice and agar were tested for inherent sterol. Samples were subjected to continuous Soxhlet extraction with anhydrous ether, spotted on silica gel thin-layer plates with sterol standards, and developed in ethylether:benzene:ethanol:acetic acid (40:50:2:0.2) and ethyl ether:hexane (6:94). The sterol was then made visible by applying ferric chloride and heating to 100 C. No sterol was detected in the juice or agar components of the medium, although esterified forms of sterol or amounts of sterol (0.08 μg/ml or less) may be present without being detected by this method.

Temperature control and measurement. Cultures were incubated on a temperature gradient apparatus, similar to that described by Leach (8). An aluminum sheet 54 cm wide, 130 cm long, and 2.5 cm thick, with thermostatically controlled hot water and coolant running through pipes embedded along opposite edges, produced rows of isothermal lines that permitted the replication of cultures at a number of temperatures. The temperature of the medium or of glycerin in plastic petri dishes was continuously monitored with a multipoint thermocouple recorder. Variation of incubation temperatures during the course of any temperature study never exceeded $\pm$0.5 C. Desiccation of culture media was minimized by placing open containers of water in the chamber enclosing the gradient apparatus. Cultures incubated in the dark were shielded from illumination by baffles that excluded light but allowed air circulation comparable to the circulation around illuminated cultures. In studies with different wavelengths of light, petri dishes were incubated on the aluminum sheet without temperature control. The aluminum sheet apparently was an effective heat sink, because no differences in temperature of the medium among various incident light treatments were detected by thermocouples inserted through the walls of closed petri dishes.

Illuminators, filters, and measurement of light. Sources of incident light capable of illumination from near-UV to infrared were used: conventional 40-W Cool White fluorescent tubes (F40 CW; spectral emission range, 320–725 nm; peak, 575 nm), 40-W Blacklight Blue fluorescent tubes (F40 BLB; range, 310–420 nm; peak, 355 nm), and 150-W incandescent bulbs (range, 300–2,500 nm; peak, 1,000 nm). The emission data were obtained from the Large Lamp Department, General Electric Co., Cleveland, OH 44101. Barrier filters were used to select specific regions of the spectrum from the incident light. Four filters were used: (i) Corning c.s. 7-54 with transmission ranges of 230–420 and 665–2,750 nm, peak transmission at 310–340 and 720 nm, and T MAX 50 (wavelength at which transmission is equal to 50% of peak transmission) of 250, 390, 690, and 800 nm; (ii) Kodak 58 with transmission ranges of 470–610 and $\geq$ 700 nm, peak transmission at 520 and $\geq$ 800 nm, and T MAX 50 of 505, 560, and 730 nm; (iii) Kodak 22 with a transmission range of $\geq$ 550 nm, peak transmission at $\geq$ 610 nm, and T MAX 50 of 565 nm; and (iv) Corning c.s. 7-56 with a transmission range of $\geq$ 840 nm, peak transmission at $\geq$ 1,180 nm, and T MAX 50 of 970 nm. Transmission data were obtained from the brochures "Corning Glass Color Filters" (Corning Glass Works, Corning, NY 14830) and "Kodak Filters" (Eastman Kodak Co., Rochester, NY 14604).

Filters were placed directly on lids of seeded plastic petri dishes and fitted with collars designed to prevent nonfiltered light from entering the sides of the dishes. Near-UV and visible light intensities reaching the colonies were measured with a pyranometer equipped with a fused silica cell (supra-sil-W, Eppley Laboratories, Newport, RI 02840). Light in the infrared region was measured with a pyranometer cell (PY 346-7501) on a radiometer (model LI-170 combination Quantum/Radiometer/Photometer, Lambda Instruments, Lincoln, NE 68504). Light intensity at the colony surface was adjusted by altering the distance from the lamps and by applying layers of gauze between the lamps and the petri dishes. In all light treatments, cultures were illuminated continuously throughout the period of incubation.

Since light intensities in the near-UV and visible range were determined with equipment not normally available, we devised a means of approximating these values that may prove useful to other investigators. Incident light from Blacklight Blue fluorescent lamps that passed through a Corning c.s. 7-54 filter was measured simultaneously with the pyranometer/fused silica cell apparatus and a Weston Photometer (model 603, Weston Electrical Instrument Corp., Newark, NJ 07101). The photocell of the Weston meter was in a quartz envelope; quartz detects light at wavelengths of 310–350 nm better than glass. The special ultraviolet-sensitive pyranometer indicated an intensity of 200 μW cm^{-2}, and the photometer indicated an illuminance of 129 lux (12 ft-c). Since manufacturer's data for the Weston meter/quartz cell show approximately 20% efficiency at 325 nm (the midpoint of the trans-

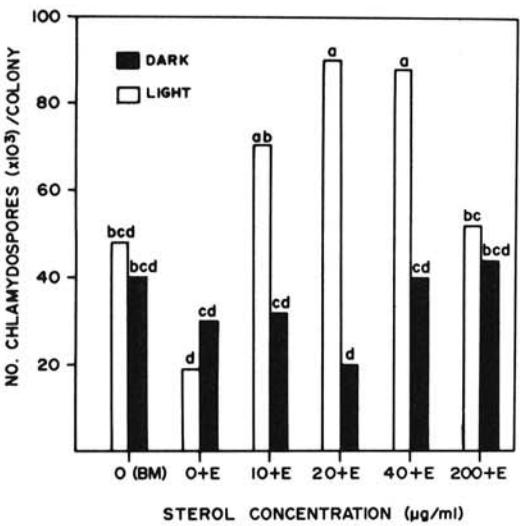

Fig. 1. Chlamydospore production by *Phytophthora cinnamomi* as influenced by the addition of ethanol (E) and β-sitosterol to V8 agar basal medium (BM). Cultures were incubated in the light (200 μW cm^{-2} near-UV) or in the dark. Bars having the same letters are not significantly different (P = 0.05).

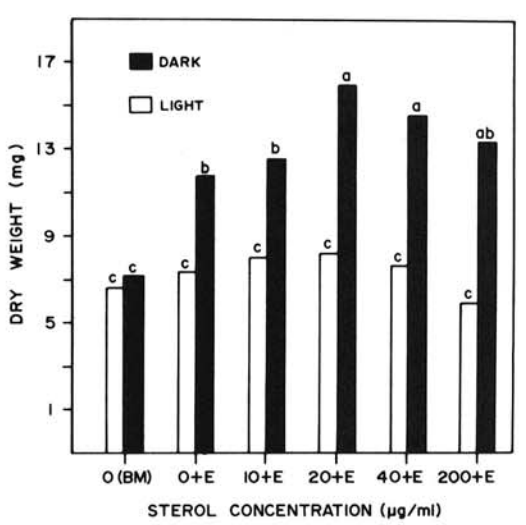

Fig. 2. Growth, measured as dry weight, of *Phytophthora cinnamomi* as influenced by the addition of ethanol (E) and β-sitosterol to V8 agar basal medium (BM). Cultures were incubated in the light (200 μW cm^{-2} near-UV) or in the dark. Bars having the same letters are not significantly different (P = 0.05).

mission peak of near-UV filter c.s. 7-54), a corrected reading of 646 lux (60 ft-c) is considered equivalent to 200 μW cm^{-2} under the described conditions.

Sporulation and growth measurements. The incubation period for each experiment was 7–10 days, at which time all cultures were fixed by flooding with FAA (48 ml of formaldehyde, 40 ml of acetic acid, 352 ml of 95% ethanol, and 360 ml of distilled water). Each culture was comminuted to a homogeneous suspension in a Virtis homogenizer (Virtis Co., Gardiner, NY 12525), transferred to a volumetric flask, and adjusted to 100 ml. Immediately after agitation, 0.1-ml samples were pipetted onto depression slides and the number of chlamydospores in each sample was determined microscopically. At least three 0.1-ml samples were examined from each culture, and three to five replicate cultures were sampled from each treatment.

Growth was measured as dry weight by heating comminuted cultures in a water bath at 90 C, vacuum-filtering the fungus and melted agar through tared Whatman 2 paper, and washing the melted agar through with a measured quantity of water heated to 90 C. Filter papers were oven-dried at 70 C to a constant weight. Average dry weight was determined for three to five replicate cultures from each treatment. Each experiment was performed at least twice. While chlamydospore numbers varied among illumination experiments, the relationship between dark controls and illumination treatments was consistent throughout duplicate experiments. Data were subjected to analysis of variance and, when appropriate, Duncan's multiple range test.

RESULTS

Effect of light and sterol interaction on chlamydospore production. Preliminary experiments showed that V8 agar containing β-sitosterol supported two to three times the amount of chlamydospore production by *P. cinnamomi* than V8 agar without sterol, but only when cultures were incubated in the light. Highest spore production occurred in cultures illuminated with 200 μW cm^{-2} near-UV light on media amended with 10, 20, and 40 μg/ml sterol, with fewer spores at 0 and 200 μg/ml sterol (Fig. 1). When cultures were incubated in the dark, none of the sterol concentrations increased spore production over that occurring on unamended V8 agar.

Further studies showed there is a broad optimum sterol concentration; no significant differences were found in chlamydospore production at sterol concentrations of 10, 15, 20, 30, and 40 μg/ml.

Growth, measured as dry weight, was not influenced by sterol at any concentration tested in V8 agar when cultures were grown in the light; in the dark, significantly more growth was obtained in all the sterol treatments and in the solvent control than on unamended V8 agar (Fig. 2). The culture medium used in all subsequent experiments was V8 agar amended with β-sitosterol at 20 μg/ml.

Quality and intensity of light stimulating chlamydospore production. Various light sources and Corning and Kodak filters were used to determine whether the stimulatory effect on sporulation by light was associated with a particular wavelength. Cultures illuminated with light at 100 μW cm^{-2} between 470 and 610 nm and between 550 and 725 nm and the dark control produced moderate numbers of chlamydospores (Fig. 3). A higher number of chlamydospores was found in cultures incubated under a mixture of unfiltered Cool White and Blacklight Blue fluorescent light, while even more spores were produced on cultures illuminated with 310–420 nm light. The latter range is predominantly near-UV. Similar results were obtained in experiments using the same spectral ranges but with light intensity increased to 200 μW cm^{-2} (Fig. 4). Lowest numbers of chlamydospores were obtained in cultures incubated in the dark and in light with wavelengths of 470–610 and 550–725 nm. Spore production was several times greater under light at wavelengths of 310–420 nm and from unfiltered, combined Cool White and Blacklight Blue lamps.

The apparent inability of light with wavelengths longer than near-UV to stimulate chlamydospore production may be due to the lower energy levels at these longer wavelengths; possibly, this is compensated for by higher intensities. Chlamydospore production was compared in cultures incubated under 200 and 400 μW cm^{-2} light intensities at the same ranges of wavelengths tested previously. There were no significant differences in the chlamydospore production of cultures incubated in the dark and those incubated in the light at wavelengths of 470–610 or 550–725 nm at either intensity (Fig. 5). In the event that the lack of light activity at longer wavelengths was due to exclusion of infrared radiation by our selective filters, chlamydospore production under $>$ 550 nm, $>$ 840 nm, and unfiltered incandescent light, each at 200 and 2,000 μW cm^{-2}, was compared to chlamydospore production in the dark. There were no consistent differences among any of the treatments.

Chlamydospore production was examined at different intensities of unfiltered Blacklight Blue fluorescent light, which is predominantly near-UV. More than twice the number of chlamydospores

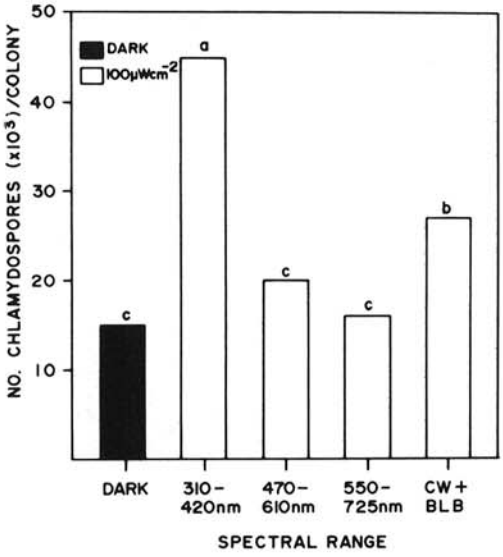

Fig. 3. Influence of light quality on chlamydospore production by *Phytophthora cinnamomi*. Spectral ranges tested were 310–420, 470–610, and 550–725 nm. Light from a combination of Cool White (CW) and Blacklight Blue (BLB) lamps was included for comparison. The light intensity for all treatments, except the dark, was 100 μW cm^{-2}. Bars having the same letters are not significantly different (P = 0.05).

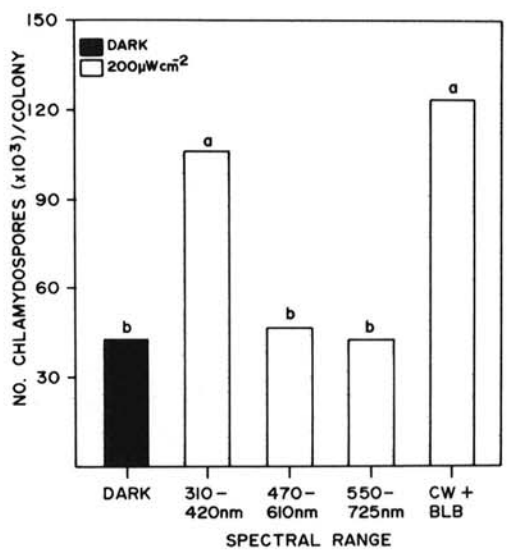

Fig. 4. Influence of light quality on chlamydospore production by *Phytophthora cinnamomi*. Spectral ranges of 310–420, 470–610, and 550–725 nm were tested at 200 μW cm^{-2}. Light from a combination of Cool White (CW) and Blacklight Blue (BLB) lamps, adjusted to 200 μW cm^{-2}, was included for comparison. Bars having the same letters are not significantly different (P = 0.05).

were produced on cultures incubated under 100 or 200 μW cm^{-2} light than on cultures incubated in the dark. The amount of chlamydospore production neared zero as light intensity approached 1,600 μW cm^{-2} (Fig. 6).

In contrast to the stimulatory effect of light on chlamydospore production, experiments with various qualities and intensities of light showed inconsistent and statistically nonsignificant differences in growth.

Light may influence chlamydospore production by affecting some component of the culture medium rather than by acting directly on the fungus. There was no increase in sporulation when petri dishes of unseeded V8 agar were exposed to 200, 650, or 1,250 μW cm^{-2} near-UV light for 7 days, subsequently seeded, and incubated in the dark, compared with cultures grown on medium stored in the dark before seeding and incubated in the dark.

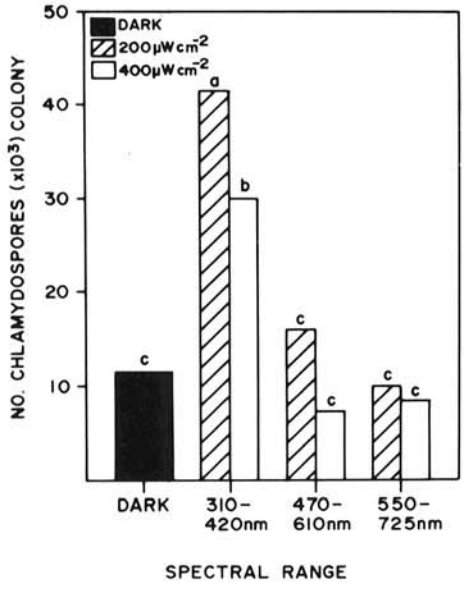

Fig. 5. Chlamydospore production by *Phytophthora cinnamomi*, as influenced by three spectral ranges of light, tested at light intensities of 200 and 400 μW cm^{-2}. Bars having the same letters are not significantly different ($P = 0.05$).

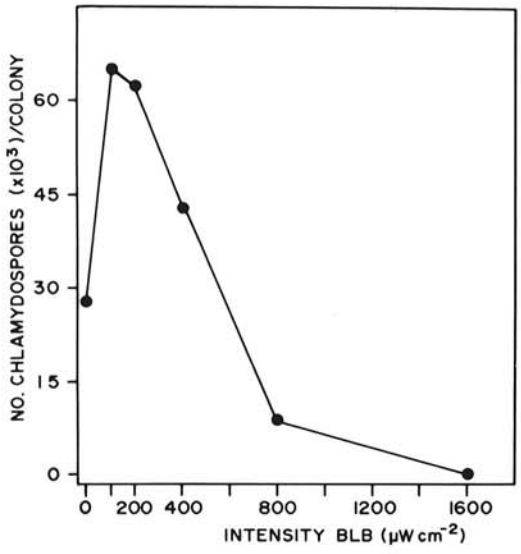

Fig. 6. Effect of various intensities of Blacklight Blue light (BLB), predominantly near-UV, on chlamydospore production by *Phytophthora cinnamomi*.

Effect of incubation temperature on chlamydospore production. The range of and optimum temperature for chlamydospore production were determined with near-UV light at 200 μW cm^{-2} and in the dark. Numerous chlamydospores developed on illuminated cultures incubated between 23 and 28 C, with maximum sporulation occurring at approximately 25 C (Fig. 7). Few chlamydospores developed at $\leq$ 18 C and none at $\geq$ 30.5 C. Compared with that in illuminated cultures, chlamydospore production in the dark was sparse, with a poorly defined optimum at approximately 21 C. The optimum temperature range for growth was approximately 26–30 C, with more growth occurring on colonies incubated in the dark (Fig. 8).

Growth and chlamydospore production were determined after various periods of incubation at 25 and 28.5 C in the light. At 25 C, the optimum for chlamydospore formation, highest numbers of chlamydospores occurred in 7–10 days, whereas chlamydospore production peaked at a much lower level in 7 days at 28.5 C. Growth curves were similar at both temperatures (Fig. 9).

Comparison with other isolates. The isolates from Oregon and Ohio showed responses similar to those of Pc 10. Growth (dry

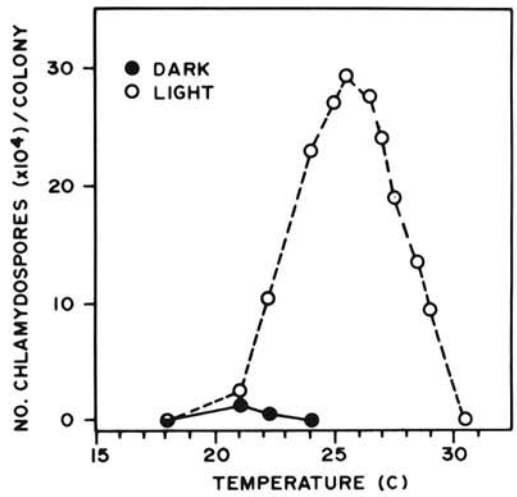

Fig. 7. Effect of incubation temperature on chlamydospore production by *Phytophthora cinnamomi*. Cultures were incubated in the light (200 μW cm^{-2} near-UV) or in the dark on V8 sterol agar.

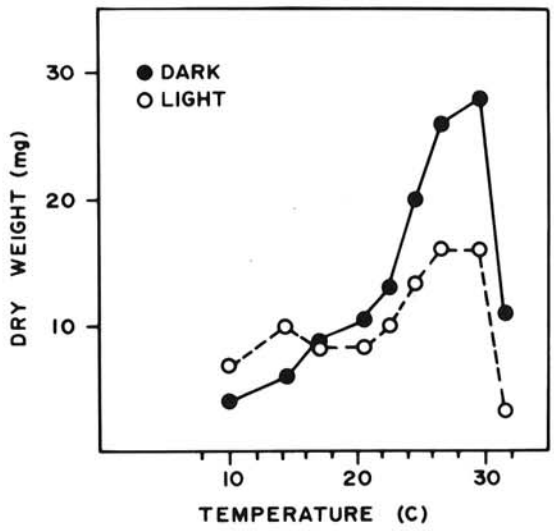

Fig. 8. Effect of incubation temperature on growth of *Phytophthora cinnamomi*. Cultures were incubated in the light (200 μW cm^{-2} near-UV) or in the dark on V8 sterol agar.

weight) of the two contributed isolates at various temperatures was nearly identical to that of Pc 10. Temperature optima for chlamydospore production were slightly lower than those for Pc 10 (23 C for the isolate from Oregon and 24 C for the one from Ohio). Chlamydospore production by all three isolates was stimulated threefold by near-UV light.

DISCUSSION

One major reason for variation in chlamydospore production is composition of the medium, especially the concentration of sterol. Another is temperature of incubation, which is optimal for chlamydospore production in the range of 23–28 C. Of special interest is that near-UV light (but not light with longer wavelengths) stimulates chlamydospore production by *P. cinnamomi* cultured on a suitable medium with adequate sterol. Near-UV light suppresses growth of *P. cinnamomi* slightly but consistently. Since sunlight and lamps commonly used for room illumination (fluorescent, incandescent) emit some light in the near-UV range, the practice of incubating cultures in various locations in the laboratory may result in inconsistent chlamydospore production among batches.

Merz and Vickers (10) found that inhibition by light of oospore production by four *Phytophthora* spp. was partially overcome by addition of sterol to the medium. Hendrix (5) concluded that light and sterol apparently were independent stimuli of sporangium induction in two *Phytophthora* spp. In our study, β-sitosterol resulted in increased chlamydospore production in the light but not in the dark. Likewise, near-UV light stimulated chlamydospore production only when the medium was supplemented with sterol. The site of this apparent interaction between light and sterol is probably in the fungus rather than in the medium, since chlamydospore production was not stimulated by culture on sterol-amended medium that had been pretreated with near-UV light. Growth (dry weight) of *P. cinnamomi* was stimulated by sterol only in the dark in our studies, whereas Hendrix (4) found that growth (colony diameter) of this species in the dark on glucose-nitrate agar or glucose-peptone agar was the same whether or not cholesterol (20 μg/ml) was present. This difference in results may reflect the method of measuring growth (dry weight vs. colony diameter), the basal media (synthetic vs. natural), or the choice of sterol solvent or sterol (β-sitosterol vs. cholesterol), although with regard to the latter possibility there is evidence to the contrary (2). Our selection of V8 agar was based on an initial goal to mass-produce quantities of mycelium and chlamydospores for inoculum. Support of chlamydospore formation and good growth was characteristic of V8 agar but not of synthetic media, and deficiencies of synthetic media in components other than sterol may hinder evaluation of the effect of sterol. The benefit to oospore production of adding sterol to V8 broth has been reported for several *Pythium* spp. (1).

There was no correlation between chlamydospore production and growth at any of the sterol concentrations or in the light quality and intensity studies. Similarly, there was no clear relationship between growth and chlamydospore production at various incubation temperatures; more chlamydospores formed at 25 C than at 28.5 C, whereas growth (dry weight) was similar at both temperatures.

Responses to light, temperature, and sterol by isolates of *P. cinnamomi* from Oregon and Ohio were similar to these by Pc 10. The isolates may have similar origins, since all three were from cultivated rhododendron, which is commercially transported throughout the country. Before generalizations can be made about the effect of near-UV light on *P. cinnamomi*, more isolates from diverse hosts should be examined.

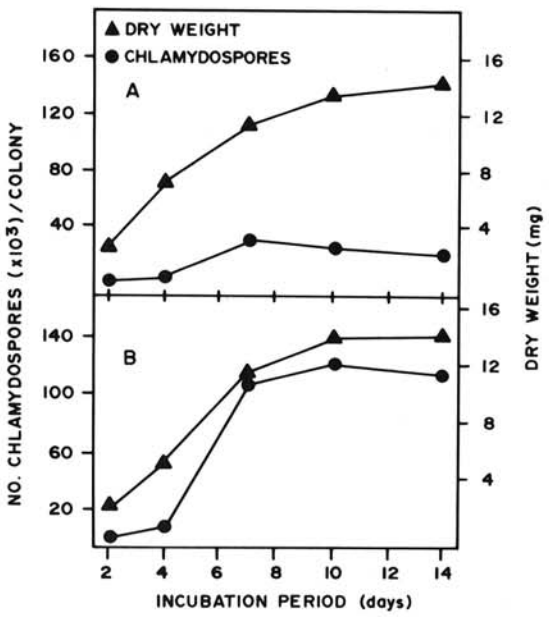

Fig. 9. Growth (dry weight) of and chlamydospore production by *Phytophthora cinnamomi* after various periods of incubation under 200 μW cm^{-2} near-UV light at **A**, 28.5 C and **B**, 25 C.

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