AN ABSTRACT OF THE THESIS OF

LARRY ENGLANDER (Name of student) for the DOCTOR OF PHILOSOPHY (Degree)

in Plant Pathology (Major) presented on September 19, 1972 (Date)

Title: GROWTH AND SPORULATION OF PHYTOPHTHORA LATERALIS IN VITRO AS INFLUENCED BY THE CHEMICAL AND PHYSICAL ENVIRONMENT

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Abstract approved: Lewis F. Roth

Phytophthora lateralis Tucker and Milbrath, causal agent of a serious fungus root rot of Chamaecyparis lawsoniana, has seriously damaged natural stands in southwestern Oregon forests and affected ornamentals throughout the Pacific Northwest. Progress with an effective control program in the field has been limited by lack of critical knowledge of fungal biology. Knowledge is needed of reliable means of producing the various spore stages to be used, for example, in inoculation studies or survival capacity studies.

Where culture is in liquid media, agitation enhances vegetative growth. Gentle agitation results in normal growth morphology and in dry weight production as great as that of vigorously shaken cultures. Increase in fungus growth of shaken cultures in contrast to still cultures is independent of oxygen concentration. pH influences

P. lateralis by favoring growth in the more acidic range from 4.5 to 5.5 and inhibiting growth in the range from 6.5 to 7.0. The reaction of the medium from still cultures becomes slightly more acidic as the cultures develop, whereas medium from shaken cultures becomes much less acidic or even slightly basic with growth.

P. lateralis was found to have a partial growth requirement for calcium. The effect occurs only when β-sitosterol, which is not required for growth, acts synergistically with the calcium (10^{-3} M). The fungus does however require sterols (β-sitosterol, cholesterol) for production of chlamydospores and sporangia.

Sporangial production, but not growth, is greatly stimulated by illumination (190 foot-candles, supplied by a combination of cool white and near-ultraviolet 40 watt fluorescent lamps) but only when colonies are grown in a medium containing sterols. Between temperatures of 10 and 25 C more sporangia form in cultures illuminated for 12 hrs each day than form with continuous illumination. Illumination, even at relatively low light intensity (25 foot-candles) inhibits chlamydospore production by two-thirds. Higher light intensities cause correspondingly greater reduction.

The optimum temperature for chlamydospore production is about 24 C; fewer spores form at lower temperatures and these are notably smaller and have thinner walls. The optimum temperature for chlamydospore production is the same as the maximum

temperature for growth. Best sporangial production occurs at 14 to 15 C. Neither sporangial or chlamydospore production is stimulated by diurnal fluctuations of temperature.

Detached chlamydospores germinate readily over a wide range of temperatures, with good germination between 17 and 23 C. A dormancy period is not required. In nutrient media germ tubes develop into colonies while in deionized water functional sporangia form. The optimum temperature for zoospore discharge by sporangia is 12 to 13 C. More zoospores are released in the dark than in the light.

Growth and Sporulation of *Phytophthora lateralis* *in vitro* as Influenced by the Chemical and Physical Environment

by

Larry Englander

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1973

APPROVED:

Redacted for Privacy

Professor of Botany and Plant Pathology
in charge of major

Redacted for Privacy

Chairman of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented Sept 19, 1972

Typed by Mary Jo Stratton for Larry Englander

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Lewis F. Roth for his guidance, patience and counsel during the course of this work. A special note of appreciation is due Dr. Charles M. Leach for his encouragement and helpful discussions, and generosity with respect to use of specialized equipment, upon both of which this work depended. I wish to thank Dr. Malcolm E. Corden, Dr. Edward J. Trione and Dr. Chester E. Horner for their willing advice and the use of their equipment. I wish to thank the members of my graduate committee.

My most grateful appreciation is extended to my wife, Carol, for her understanding and encouragement, and for invaluable aid in the preparation of this manuscript. Finally, I would like to thank the staff and graduate students of the Botany and Plant Pathology Department who have made my work and life at Oregon State University a most pleasant and rewarding experience.

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GROWTH AND SPORULATION OF *PHYTOPHTHORA LATERALIS IN VITRO* AS INFLUENCED BY THE CHEMICAL AND PHYSICAL ENVIRONMENT

INTRODUCTION

Chamaecyparis lawsoniana (Murr.) Parl., commonly known as Port-Orford-cedar or Lawson cypress, is a component of forest stands mainly within an irregular strip 30 miles wide extending along the coast of southern Oregon and northern California. It is often found in low, wet areas near the coast in mixed stands with Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.). In the adjacent mountains cedar is sometimes abundant in the Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) forest. Port-Orford-cedar logs have increased in value disproportionately faster than those of the other soft woods. This is largely due to increased demand in the overseas market. Characteristically, the wood is highly resistant to decay and to attack by insects. It is readily worked, and is attractive in appearance. The cut lumber gives of a pleasant scent. Until the late 1950's, and the recognized danger from root rot, this species was widely used in reforestation programs.

The beauty of Lawson cypress as an ornamental is attested by the large number of horticultural varieties in the ornamental nursery industry. Widespread transport of these popular ornamentals has been implicated in introducing *Phytophthora lateralis* Tucker and Milbrath

into the areas where cedar occurs naturally, thereby threatening the perpetuation of the tree as a forest species (Roth et al., 1957).

History of this fungus may be briefly summarized as follows. During the late 1930's, a root rot appeared affecting ornamental Chamaecyparis in coastal cities of Washington and Oregon and in the Willamette Valley of Oregon. The causal agent was found to be an undescribed fungus which, in 1942, was designated Phytophthora lateralis by Tucker and Milbrath. They were able to isolate the fungus only from Chamaecyparis species, and inoculation studies confirmed the host specificity of P. lateralis for Chamaecyparis. By 1950, the fungus was recognized as prevalent on ornamental Chamaecyparis in western Washington and British Columbia. It seemed inevitable that the coastal area of natural cedar would become affected.

In 1952, the disease was discovered in Coos Bay, Oregon, marking the first record of P. lateralis in the native Port-Orford-cedar area (Roth et al., 1957). The next few years witnessed an epiphytotic of cedar root rot, with localized intense outbreaks along the western edge of the natural range. Disease spread followed waterways and roads. Logging, urban development and road work presumably helped spread the fungus by moving infested soil. Once the fungus became established in an area, exposed, nearby cedars perished in a few years. While the rate of disease spread has somewhat diminished after these first years, the disease is continuing an irregular advance into cedar areas not previously damaged.

In the original description of P. lateralis, Tucker and Milbrath (1942) reported the distinctively slow growth rate and high-temperature sensitivity along with morphological features of sporangia and chlamydospores. Trione (1957) discovered the sexual stage of the fungus during studies on biology and pathogenicity. He reported occasional aerial infection and emphasized the importance of sporangia and zoospores in both above- and below-ground dissemination. He postulated that chlamydospores are an oversummering stage of the fungus in the native cedar habitat with its distinctively dry summers.

The extremely limited range of the principal host has restricted geographic distribution of the disease and economic loss. The disease is regarded as of regional concern and consequently has received very little study. Nevertheless, the high economic value and aesthetic importance of existing Port-Orford-cedar, and the potential loss of this tree as a forest species, warrants that efforts be made towards control of the root rot disease. This investigation of Phytophthora lateralis was undertaken to fill in some of the deficiencies in our basic knowledge, particularly in relation to recent discoveries of nutritional requirements of other Phycomycetes.

Basic information on fungal development is critically needed in support of applied studies. Work on dissemination and infection requires the ability to produce quantities of sporangia, and to achieve simultaneous liberation of large numbers of zoospores. Studies of

persistence require the ability to produce quantities of mature chlamydospores at will. Concurrent with these requirements is the need to produce substantial amounts of hyphae supporting the various spore stages.

LITERATURE REVIEW

Literature concerning Phytophthora lateralis is scarce. This results from the limited host range of the fungus (restricted to members of the genus Chamaecyparis) and the limited range over which the principal host occurs naturally as a forest species. Contrarily, the literature on other Phythiaceae is voluminous, and some of it can provide background for concepts and theories on growth and sporulation biology of P. lateralis. Material on other fungi is reviewed when it provides the reader with understanding of the status of knowledge in specific relevant fields.

Form and function of the hyphal and spore stages were, of course, detailed by Tucker and Milbrath (1942) in the report of P. lateralis as a new species of Phytophthora and as the causal agent of a root rot of Chamaecyparis species in the northwestern United States.

> Hyphae continuous when young, becoming septate with age, usually smooth, but sometimes gnarled or tuberous. Sporangia none on agar media but developing fairly abundantly on washed mycelial mats transferred from 7-day pea broth cultures (20° C.) to sterile distilled water and incubated 7 days at 20° C.; sporangia borne sympodially on sporangiophores resembling vegetative hyphae, mostly ovate, obovate or obpyriform, occasionally elongate, hyaline to lemon yellow, nonpapillate, with apical refringent plug very thin and often indistinguishable, 26-60 x 12-20 micra, averaging about 36 x 15 micra. Zoospores fully differentiated within the sporangium, biciliate, reniform in motile stage, spherical in non-motile phase, 10-12 micra in diameter. Chlamydospores abundant in agar cultures and liquid media, usually subspherical to spherical, occasionally ovate to irregular, contents densely and often coarsely granular, lemon yellow to light brown; wall

> usually thin, sometimes thick (6-7 micra); terminal or intercalary, the latter developing as lateral swellings of the hyphae, often appearing sessile at maturity, 20-77 micra in diameter, averaging about 40 micra; germination by germ tubes. Oogonia, antheridia and oospores unknown (Tucker and Milbrath, 1942, p. 97).

Trione (1957) discovered the sexual stage and determined that the species is homothallic, oospores being produced by colonies of single zoospore origin when grown on certain media containing plant tissue or plant extracts. Oogonia are terminal, and the antheridia paragynous. Germination is by a single germ tube terminating in a sporangium, however chlamydospores as well as oospores of P. lateralis rarely germinate. A dormant period of at least 1 week at 3 C is a prerequisite for germination of chlamydospores, and oospores require low temperatures for several months. Chlamydospores germinate with several tubes per spore. Trione (1957, 1959) found that the sporangia usually remain firmly attached to the sporangiophores, unlike many phytophthoras. Sporangia may germinate either directly or indirectly, depending on environmental conditions. Direct germination is characterized by the emergence of one or more germ tubes, indirect germination by discharge of motile zoospores from the sporangium.

Chlamydospores, which are asexual survival structures, are formed by at least 10 species of Phytophthora (Zentmyer and Erwin, 1970). These hold certain features in common such as shape (generally globose), variable wall thickness, and germination by one or many

tubes (either producing a sporangium or developing into a mycelium) (Zentmyer and Erwin, 1970). Chlamydospores are considered a primary means of survival for Phytophthora in plant tissue and soil (Dastur, 1913; Hendrix and Kuhlman, 1965; Holdaway and Tsao, 1971; Mircetich and Zentmyer, 1966; Trione, 1957, 1959; Trujillo and Hine, 1965; Zentmyer and Mircetich, 1966).

Many species of Phytophthora, including P. lateralis, form oospores, resulting from fusion of antheridium and oogonium. Both homothallic and heterothallic species occur. Generally, oospores are spherical and thick walled. In addition to affording a species the opportunity for recombination of genetic material, for some species oospores are believed to be responsible for the extended survival in the absence of a host (Zentmyer and Erwin, 1970).

Shake culture stimulates growth of several phytophthoras. Frequently the benefits of shake over still culture are attributed to increased aeration without presenting experimental evidence (Hendrix et al., 1966; Kennedy and Erwin, 1961). In a recent review on environmental influences on fungi, Hawker (1966, p. 454) states that aeration may affect fungal development by changing the concentration of oxygen, carbon dioxide, ammonia and other gases.

Several reports show that growth may be limited by the oxygen concentration in the atmosphere in which cultures are incubated. Phytophthora cactorum grew better at an oxygen concentration of 21

percent than at lower oxygen levels, although some growth occurred at 0. 2 percent oxygen (Covey, 1970). Mitchell and Zentmyer (1970) studied growth of four species of Phytophthora growing at different levels of oxygen. They found that growth was reduced on solid media at oxygen concentrations below 5 percent, while growth was reduced in liquid media at concentrations below 20 percent. These results indicate that increased oxygen concentration may be an important factor in the growth stimulation found in shake cultures.

Carbon dioxide from fungus respiration may accumulate to inhibitory levels in still, liquid culture media (Hawker, 1966). Mitchell and Zentmyer (1970) found that growth in liquid and solid media of several phytophthoras was reduced when carbon dioxide concentrations exceeded 5 percent.

Accumulation of ammonia in poorly aerated culture media has been implicated in inhibition of growth of many fungi (Hawker, 1966). Leal et al. (1970) showed that several species of Phytophthora released ammonia when cultured on a medium with an amino acid nitrogen source. They found that the increasingly basic reaction of the medium with growth was caused by the release of ammonia by the fungus. Ammonia concentrations as low as 17 ppm killed P. cinnamomi mycelium (Gilpatrick, 1969).

Benefits of shake culture may derive from causes other than aeration. Burkholder and Sinnot (1945) found that many fungi

developed spherical colonies in shake culture, as compared with the mats produced in still culture. They hypothesized that the colony morphology of shaken cultures was caused either by constant contact with the sides of the flask, or by the deficiency of orienting factors, such as gravity, on colonies in shake culture. Thus, increased growth in shake culture may result from colony extension in three dimensions, rather than two dimensional growth as with colony mats in still culture (Emerson, 1950).

Historically, Phytophthora nutrition in culture has rested heavily on certain natural media such as oatmeal and pea media, but the active substances accountable for superiority of these media was not known until less than a decade ago. Possibly the delayed discovery of the steroid nature of the stimulatory substances was due to the report in 1937 by Leonian and Lilly that an unidentified fraction extracted from garden peas, stimulatory to Phycomycete reproduction, was associated with the sterol fraction but was not itself a sterol. In 1964 several workers independently discovered that sterols are required for sporulation of Phycomycetes. Haskins et al. observed that a species of Pythium produced sexual structures when grown on cellophane on the surface of an agar medium, but not when cultured directly on the medium. These workers first determined that reproduction was stimulated by the fatty acids which serve as a plasticiser in cellophane. Upon purification they learned that sterol contaminants of the fatty

acids were responsible (Haskins et al., 1964). Hendrix (1964) found that growth and sporulation of a Pythium species and a Phytophthora species, in a synthetic medium, was stimulated by the addition of cholesterol to the medium. Leal et al. (1964) determined that a sterol was responsible for the activity of an ether extract of oatmeal in stimulating sexual reproduction in six species of Phytophthora. Elliot et al. (1964), investigating the component of oatmeal agar which allowed Phytophthora cactorum to produce oospores, found that sterols were responsible.

The inability of Pythiaceae to synthesize sterols and the sterol requirement for sporulation have both been demonstrated frequently (Elliot et al., 1964; Hendrix, 1966; McCorkindale et al., 1969).

In the past 8 years numerous reports have been made of the effects of sterols on fungus growth and sporulation. The selection of a particular sterol has been the fancy of each investigator. Sterols of higher plants have been chosen for studies on the basis of their natural availability to Phycomycetes. Fieser and Fieser (1959, p. 352) stated that sitosterols are the "most abundant and widely distributed of plant sterols," and Heftmann (1963, p. 229) reported that the "most widely distributed sterols in higher plants are the C_{29} sterols, stigmasterol, β-sitosterol, and γ-sitosterol." Oats and peas (both of which are very stimulatory to Phycomycete reproduction) contain β-sitosterol, stigmasterol and isofucosterol (Elliot et al., 1964, 1966;

Leal et al., 1964). The sterol fraction in roots of plants in the Cruciferae are mainly β-sitosterol (Knights, 1970). Ergosterol and cholesterol have been studied, as these substances are found to occur naturally in certain fungi (McCorkindale et al., 1969). Fortunately, a number of sterols elicit identical responses among the Phycomycetes. A sterol is active as a fungal stimulant if all of the following criteria are met: A 3 β-hydroxyl group, a double bond between carbon 5 and 6, a methyl group at C-10 and C-13, and a C-17 side chain of 8 to 10 carbons (Elliot et al., 1966; Harnish, 1968; Hendrix, 1970; Lilly, 1966).

The fate of sterols in the fungus cell is not clearly understood. Exogenous sterols were incorporated mostly into the protoplasmic membrane of a species of Pythium (Sietsma and Haskins, 1968). Child et al. (1969b) found that, when in distilled water, cells of this species which had been formed previously in medium with sterol were more retentive of cellular nucleotide, nitrogen and protein, and less retentive of carbohydrates than cells formed in medium without sterol.

Sterols stimulate the growth of some species of Pythiaceae and have no effect on others. Schlosser and Gottlieb (1968) reported dry weights of six species of Pythium to be increased between 65 and 100 percent by cholesterol. Linear growth of four of six species of Pythium was stimulated by cholesterol on at least one synthetic medium, while among 12 species of Phytophthora only P. lateralis,

P. cinnamomi and P. palmivora were not stimulated by the sterol (Hendrix, 1965). The vegetative growth rates of Phytophthora cactorum and P. palmivora were increased by β-sitosterol (Barnett et al., 1966). P. cinnamomi also was stimulated vegetatively by β-sitosterol as well as cholesterol, ergosterol, and stigmasterol (Chee and Turner, 1965). Linear growth of P. cactorum was most stimulated by β-sitosterol and cholesterol among 42 sterols and related substances tested (Elliot et al., 1966). Dry weight also increased when cholesterol was added to sterol free medium (Elliot, 1972). Cholesterol stimulated dry weight growth of P. parasitica var. nicotianae more than did β-sitosterol (Hendrix et al., 1969). Linear growth of P. megasperma var. sojae and P. parasitica var. nicotianae was doubled when cholesterol or phytosterol was added to the synthetic sterol free medium. β-sitosterol stimulated growth of P. parasitica var. nicotianae but not P. megasperma var. sojae (Hendrix, 1964). Erwin et al. (1968), however, reported stimulation of P. megasperma var. sojae on a β-sitosterol amended natural medium.

Generally, when dry weights were measured with time, the differences between weights of sterol grown and sterol free cultures decreased with time. Thus, growth is initially stimulated, but the final amount of growth is similar to that in sterol free media (Barnett et al., 1966; Child et al., 1969a; Schlosser and Gottlieb, 1968).

Apparently all species of Phytophthora require sterols for

formation of sporangia. P. lateralis formed occasional sporangia on a synthetic agar medium with sterol, but in sterol free medium only occasional hyphal swellings developed (Hendrix, 1965). In a synthetic medium with sterol, P. parasitica var. nicotianae produced normal appearing sporangia but in the absence of sterol only small abnormally shaped spores formed (Hendrix, 1964). P. cactorum produced sporangia only when sterol was included in the synthetic liquid medium (Elliot, 1972).

Chlamydospore formation is dependent on sterols also. Hendrix (1965, p. 793) commented that "Phytophthora cinnamomi and, to a lesser extent, P. lateralis formed chlamydospores in response to sterol." He believed that the knotty vesicular structures which P. cinnamomi produced on sterol free synthetic media develop into chlamydospores when sterols are present. Phytophthora parasitica var. nicotianae produced numerous large chlamydospores in media with sterols, but only small chlamydospores in sterol free medium (Hendrix, 1964).

The sterol requirement for sexual reproduction by Phytophthora and Pythium species has been established (Child et al., 1969a; Child and Haskins, 1971; Elliot, 1972; Elliot et al., 1966; Erwin et al., 1968; Harnish, 1968; Hendrix, 1964, 1965; Hunter et al., 1965; Leal et al., 1964).

A homothallic species of Pythium produced oospores in proportion

to the sterol concentration up to 100 mcg/ml, the highest concentration tested (Child et al., 1969a). Individuals of a single mating type of reportedly heterothallic strains of Pythium formed antheridia, oogonia and oospores in the presence of sterols, however, the compatible mating type from each pair failed to sporulate (Child and Haskins, 1971). Sterols stimulated heterothallic species of Phytophthora to produce oospores, but only in paired cultures with both mating types (Hendrix, 1965; Hunter et al., 1965). A number of homothallic species did not form oospores unless the medium contained sterol (Hendrix, 1965; Hunter et al., 1965).

Calcium nutrition of fungi has been studied repeatedly but the existence of an absolute requirement for calcium has not been settled. One complicating factor is the difficulty in elminating calcium contamination in control media. Calcium requirements have been suspected from differences in growth on agar and liquid media with identical constituents except agar (Elliot, 1972). Agar contains calcium. The exact amount has been variously reported as 0.13 percent and 0.60 percent by weight (Englander and Corden, 1971; Miller, 1956). This may account for the lack of growth stimulation by calcium when six calcium salts and no calcium were added singly to agar medium on which P. cinnamomi was grown (Chee and Newhook, 1965).

In 1948, Steinberg used a liquid medium containing highly purified chemicals to demonstrate that representatives of certain genera

grew equally well with or without calcium (Aspergillus, Fusarium), while others (Rhizoctonia, Pythium) grew poorly in a calcium free medium. Of subsequent reports concerning growth of Phycomycetes, Hodgson (1958) suggests that Phytophthora infestans in synthetic liquid medium was not stimulated by calcium while an absolute calcium requirement has been shown for P. fragariae (Davies, 1959) and P. parasitica var. nicotianae. The latter fungus required calcium only in a medium with nitrate as the nitrogen source (Hendrix and Guttman, 1970; Hendrix et al., 1969). Other species of Phytophthora with a partial requirement for calcium include P. cactorum (Elliot, 1972), P. megasperma (Erwin, 1968; Kennedy and Erwin, 1961), P. cinnamomi (Erwin, 1968), P. cryptogea, P. drechsleri, P. parasitica and P. boehmeriae (Erwin and Katznelson, 1961).

Sporangial formation was stimulated in three species of Pythium when hyphae were transferred to a calcium salt solution (Yang and Mitchell, 1965). Chen and Zentmyer (1969) found that when a relatively high concentration (10^{-2} M) of calcium was present, formation and differentiation of Phytophthora cinnamomi sporangia was stimulated.

Formation of oogonia by several species of Pythium also was stimulated by calcium, and calcium was required for maturation to oospores (Lenney and Klemmer, 1966; Yang and Mitchell, 1965). Phytophthora cactorum produced a few oogonia in calcium free media containing sterol, but abundant oogonia and oospores formed only when calcium was included in this medium (Elliot, 1972).

The source of calcium in most of the experiments reported above was readily soluble calcium chloride. Other calcium salts produced similar effects. There is ample evidence that growth stimulation is associated with the calcium ion, and not with calcium salt anions such as the hydroxide, oxide, phosphate, nitrate, chloride, carbonate, or sulfate (Erwin, 1968; Erwin and Katznelson, 1961; Hendrix et al., 1969).

In addition to the effects of specific nutrients such as sterol and calcium, the overall nutrient concentration in the medium has been related to the ability of certain phytophthoras to sporulate. In some species of Phytophthora, a period of nutrient deprivation by leaching the hyphae in water, or by incubation in an inorganic salt solution, or a soil extract solution is a prerequisite for formation of sporangia and chlamydospores. Other species are able to sporulate in nutrient media. Occasionally reports of requirements of a particular species are in conflict.

Removal of the mycelium from nutrient medium was unnecessary for sporangial formation by P. palmovora and P. capsici (Hendrix, 1967), or P. citrophthora (Schiffman-Nadel and Cohen, 1966). In this vein, Leonian (1925) studied 53 strains of Phytophthora and found that only six strains produced more sporangia after transfer to distilled water than when left in the culture medium. Sporangia of P. lateralis were not observed on nutrient media by Tucker and Milbrath

(1942), but Trione (1957) frequently found sporangia on culture media low in nutrients.

Sporangia of P. cinnamomi (Chen and Zentmyer, 1970), P. megasperma (Kennedy and Erwin, 1961), or P. parasitica (Tsao, 1969) failed to form on mycelium left in nutrient media. Ayers and Zentmyer (1971) found that glucose, over a wide range of concentrations, inhibited sporangial production by P. cinnamomi in aseptic conditions, whereas in the presence of bacteria the nutrient suppression of sporulation reversed. They believe reversal was due to the sugar utilization by the bacteria. Bacteria, notably pseudomonads, and non sterile soil extracts stimulated sporangial formation by P. cinnamomi and P. parasitica var. nicotianae (Ayers and Zeytmyer, 1971; Wills, 1954). Most studies found that sporangial inducing properties of bacterial culture filtrates or soil extracts were lost after filter sterilization or autoclaving (Chee and Newhook, 1966; Mehrlich, 1935). Several reports show that certain species produced more sporangia when the nutrient medium was replaced with tap or distilled water (P. citrophthora, Fawcett and Klotz, 1934; P. parasitica, Tsao, 1969; P. lateralis, Trione, 1957; Tucker and Milbrath, 1942). When sporangial production was compared in organic salt solutions and in water, the following species produced more spores in the salt solutions: P. cinnamomi (Chen and Zentmyer, 1969, 1970); P. parasitica var. nicotianae (Wills, 1954; Gooding and Lucas, 1959) and P. megasperma (Kennedy and Erwin, 1961).

Chlamydospores of P. cinnamomi were produced abundantly in culture media rich in nutrients, especially V8 juice agar (Zentmyer, 1959; Chen and Zentmyer, 1969). P. lateralis produced chlamydospores in most media which supported growth, and production was best in rich media (Trione, 1957). However, P. parasitica did not form chlamydospores if nutrients were continually available; chlamydospores were produced in submerged culture in very dilute liquid medium or in water (Tsao, 1969, 1971). P. palmivora, in submerged culture, produced thin-walled chlamydospores. Most chlamydospores of this species which formed on papaya fruit or in old cultures growing in a papaya medium had thick walls (Kadooka and Ko, 1971).

The nutrient concentration of the germination medium affects the germination morphology of sporangia and chlamydospores of several species of Phytophthora. Sporangia of P. lateralis (Trione, 1957), P. parasitica (Tsao, 1969), P. cinnamomi (Chen and Zentmyer, 1970) and an isolate reported as P. parasiticia (Aragaki et al., 1967) but later considered as P. palmivora (Tokunaga and Bartnicki-Garcia, 1971) germinated indirectly in water, directly when the nutrient level of the medium was high.

In tap water or solutions of low nutrition, chlamydospores of P. cinnamomi (Mircetich et al., 1968; Zentmyer, 1959) and P. parasitica (Dastur, 1913; Tsao, 1969) germinated by single or multiple germ tubes terminating in sporangia. In deionized or demineralized water,

chlamydospores of P. parasitica (Tsao, 1969) and P. cinnamomi (Mircetich et al., 1968) germinated poorly or not at all. In rich media, chlamydospores of P. parasitica (Tsao, 1969) and P. cinnamomi (Mircetich et al., 1968) germinated by one to many germ tubes to form a mycelium. Germination medium differentially affected germination of thick- and thin-walled chlamydospores of P. palmivora. On water agar a high percentage of spores of both types germinated, whereas on V8 agar only thin-walled spores germinated (Kadooka and Ko, 1971).

Temperature has been studied more extensively perhaps than other physical factors naturally acting on a fungus. Valuable information is often derived from knowledge of temperature optima and the limits for fungus growth, sporulation, germination, infection and survival. Most work has been with constant incubation temperatures but a few studies on fungal growth and sporulation have been with cyclic alternating temperatures (Burgess and Griffin, 1968; Hyre and Ettinger, 1969; Smith, 1964).

Typically, fungi grow at steadily increasing rates as temperature increases up to the optimum. As temperature is raised above the optimum, a rapid decline in growth rate often is observed (Deverall, 1965). The optimum temperature for growth of Phytophthora lateralis is lower than optima for most other phytophthoras (Roncadori, 1965). Tucker and Milbrath (1942) reported the optimum for growth on solid media to be 20 C, with growth of all isolates restricted at 25 C, and no

growth at 30 C. After 8 days at 30 C the fungus was dead (Tucker and Milbrath, 1942). Trione (1957) observed linear growth of P. lateralis to be best at 15 to 20 C; dry weight growth was greatest at 20 C.

Torgeson (1953) reported that washed hyphae of P. lateralis produced sporangia at 20 C, but not at 15, 25, or 30 C. Trione (1957) found that this species produced most sporangia at 15 and 20 C, but, with extended incubation, some sporangia formed at 5 and 25 C. Cedar twigs infected with P. lateralis incubated in an inorganic salt solution at seven temperatures between 3 and 30 C produced the highest numbers of sporangia at 15 C, with fewer at 10 and 20 C (Trione, 1957, 1959).

Temperature greatly influences the mode of sporangial germination. Certain temperature phenomena apply to sporangia germination of a number of phytophthoras, although the specific temperatures may vary with the species. High temperature encourages direct germination (Aragaki et al., 1967; Harvey, 1954), whereas a sharp change in temperature, usually accomplished by a brief chilling followed by return to a moderate temperature, stimulates zoospore formation and discharge (Chen and Zentmyer, 1970; Tsao, 1969). Trione (1957) reported that P. lateralis sporangia germinated directly at temperatures above 25 C if free water was present, and at temperatures above 20 C in the absence of free water. Indirect germination occurred between 5 and 25 C, with 15 C as the optimum (Trione, 1957).

P. lateralis produced most chlamydospores at temperatures between 15 and 25 C (Trione, 1957). Reports vary concerning the optimum temperature for chlamydospore formation by P. parasitica; Dastur (1913) found that excessive heat caused cultures to form many chlamydospores, but Tsao (1971) reported the spores were produced mainly at 15 to 18 C, with very few forming at 27 or 30 C. There is little information on the temperature requirements for chlamydospore germination. Chlamydospores of P. lateralis were rarely seen to germinate, and then only after at least a week at 3 C (Trione, 1957). Mircetich et al. (1968) found that temperature was not critical for germination of P. cinnamomi chlamydospores. Percentage germination was high between 18 and 30 C.

Illumination has stimulated, depressed or otherwise affected many fungal activities, such as growth (quantitative and tropic responses), pigmentation, sporulation, spore discharge and spore germination. The literature on fungal photophenomena has been recently reviewed (Carlile, 1970; Leach, 1971). Considerations in illumination methodology are discussed by Leach (1971). Light in the near ultraviolet and blue regions was shown to be most active in stimulating sporulation of fungi, as determined by action spectra (Leach, 1971). Lilly (1966) reviewed illumination effects on the genus Phytophthora. Sexual reproduction of phytophthoras is, as a rule, inhibited by light, while sporangial formation is either stimulated or

not affected. There appear to be no reports of light affecting mycelial growth of a Phycomycete. Morphology of Phytophthora palmivora and P. hevea sporangia was influenced sufficiently by illumination that, according to the commonly accepted taxonomic treatment (Waterhouse, 1963), they might be classified as other species, depending on whether cultures were illuminated during sporulation (Brasier, 1969a; Hendrix, 1967).

Trione (1957) found that P. lateralis produced sporangia equally well in light or darkness. Other species of Phytophthora in which asexual sporulation is not affected by presence or absence of light are: P. parasitica var. nicotianae (Wills, 1954; Gooding and Lucas, 1959), P. cactorum, P. citrophthora, P. colocasiae, P. cryptogea and P. megasperma (Aragaki and Hine, 1963).

P. cinnamomi produced sporangia equally well in darkness and in alternating light and darkness, but fewer spores formed in continuous light (Zentmyer, 1959; Zentmyer and Marshall, 1959). Sporangial production by P. hibernalis and P. syringae was less on illuminated colonies than on dark incubated colonies (Harnish, 1965).

Illumination stimulated sporangial production by P. palmivora, P. hevea (Brasier, 1969a), P. parasitica (Dastur, 1913), P. citrophthora (Fawcett and Klotz, 1934; Schiffman-Nadel and Cohen, 1966), P. capsici, P. cactorum (Harnish, 1965), and an isolate reported as P. parasitica in the illumination study (Aragaki and Hine, 1963) but later considered P. palmivora (Tokunaga and Bartnicki-Garcia, 1971).

One study on asexual sporulation showed a quantitative influence of light intensity and photoperiod *per se* (Hyre and Ettinger, 1967), while another stated these factors to have no effect (Hendrix, 1967).

The lack of publications concerning the effects of light on chlamydospore formation was noted in a recent review on reproduction of *Phytophthora* (Zentmyer and Erwin, 1970). Light apparently did not affect chlamydospore production by *P. cinnamomi*, but *P. palmivora* produced about four times as many chlamydospores when illuminated (Zentmyer and Erwin, 1970).

The inhibition by light of oogonial formation and maturation has been well documented for many species of *Phytophthora*. In continuous light, few or no oospores form on *P. palmivora*, *P. hevea*, *P. sojae*, *P. megasperma*, *P. cactorum*, *P. capsici*, *P. erythroseptica*, *P. hibernalis*, *P. himalayensis*, *P. ilicis*, *P. drechsleri* and *P. infestans* (Brasier, 1969a, b; Harnish, 1965; Klisiewicz, 1970; Romero and Gallegly, 1963; Savage, 1966). However, several species produced oospores under conditions of alternating light and darkness (Harnish, 1965; Savage, 1966).

In contrast to its inhibitory role in oospore formation, light is a major factor in stimulating oospore germination. In the dark, little or no germination occurred from oospores of *P. megasperma* var. *sojae*, *P. palmivora*, *P. infestans* (Berg and Gallegly, 1966), *P. hevea*, *P. erythroseptica* or *P. cactorum* (Berg and Gallegly, 1966; Leal and

Gomez-Miranda, 1965), whereas light, especially the blue wavelengths, stimulated germination in all the above species. There is evidence of a relationship between exposure to light of developing oogonia and their subsequent germination requirement for light. P. infestans formed a reduced number of oogonia when illuminated. However, these oogonia, after fusion, germinated readily without forming thick walls characteristic of oospores. Similar dark produced structures did not germinate (Romero and Gallegly, 1963). P. drechsleri oospores which had been previously exposed to light were not affected by light or darkness during germination. However, oospores produced in darkness germinated poorly in the dark, but were stimulated to germinate by illumination. An exposure as short as 10 minutes (580 foot candles) was effective (Klisiewicz, 1970).

GENERAL METHODS AND MATERIALS

Media Formulae and Preparation

Culture media used in this study were of the following kinds and characteristics.

Corn Meal Agar. As used here CMA was the dehydrated commercial Bacto corn meal agar which, when rehydrated with distilled water and then autoclaved, resulted in a 1.5 percent agar medium with a pH of 6.0 (Difco Laboratories, Inc., Detroit, Michigan). *Phytophthora lateralis* grows adequately on this medium, although hyphal growth is never dense and sporulation is sparse.

V8 Juice. Clarification of V8 juice (Campbell Soup Co., Camden, New Jersey) is essential because the commercial product contains particulate matter which, even when diluted to 10 percent, interferes with *in situ* spore counting in poured plates. Clarification by filtration is inadequate. A convenient method for producing a medium allowing good growth and sporulation consists of centrifuging V8 juice in 50 ml nalgene tubes at 1000 x g for 10 min (in a swinging bucket head), decanting and passing the supernatant through glass fiber paper (Whatman GF/A). The resulting clear fluid is included in V8 media at the rate of 10 percent of final volume.

V8-(Calcium) Formulations. Initially, V8 medium was prepared similarly to that used by Mircetich *et al.* (1968) for *P. cinnamomi*

culture. In their medium, several grams of calcium carbonate are added to each liter of distilled water intended for use as a diluent for the V8 juice. After a period of time the undissolved precipitate is filtered off and the calcium carbonate solution combined with the V8 juice. Due to the fact that the actual calcium concentration in this medium is not known, the medium is designated here with the word calcium enclosed in parentheses, for example V8-(calcium) broth.

V8-Calcium Formulations. It was established in an experiment reported later that calcium stimulates growth of P. lateralis. In all calcium-containing media used in this research, the optimum calcium concentration, 10^{-3} M, was included. These media are designated without the parentheses, i. e., V8-calcium broth. The chemical routinely used was reagent grade $CaCO_3$, low in alkalis (Matheson, Coleman and Bell, New Jersey).

Sterol Dissolution and Incorporation in Media. The relative insolubility of sterols in aqueous media creates a problem of achieving uniform distribution of sterol particles throughout media in which sterols are used. A method employed by some workers involves addition to each empty test tube or petri dish equivalent quantities of sterol dissolved in an organic solvent such as ether. This is followed by addition of the medium and the inoculum after the solvent has evaporated. I found this procedure time consuming, conducive to contamination, and ineffective in distributing the sterol. Internal surface

characteristics of the culture containers, upon addition of aqueous media, resulted either in the sterol adhering to the surface on which it was initially precipitated or in its becoming dislodged, sometimes to float to the surface in large pieces.

A suitable method was found of incorporating the sterols into media prior to dispensing. Stock solutions were prepared by dissolving 40 mg sterol in 20 ml of 95 percent ethanol previously heated to 70-75 C. Addition while hot of 10 ml of this solution to 1000 ml of hot or cold medium resulted in only a slight milky appearance. This method, adding the hot stock solution just prior to autoclaving the medium, was used in all experiments. If cooled stock solution is added, flocculation results.

The effectiveness of sterol dispersion in media was analyzed colorimetrically. In a series of tests, sterol in ethanol was added in varying amounts to distilled water. Both autoclaved and non autoclaved mixtures were tested. The mixtures were poured into upright pieces of tygon tubing, 48 cm long and 2.5 cm inside diameter, which had been sealed at the bottom end. After standing at room temperature for periods up to 5 hrs, the tubes were placed with minimum disturbance in a deep freeze overnight. In the morning the frozen cylinder was removed and, with a solvent-cleaned hacksaw blade, was cut into eight segments, each approximately 5 cm long. Each segment was placed in a test tube and allowed to melt. After measuring liquid

volumes, water was evaporated under vacuum. The sterol in each tube was redissolved in 1.5 ml chloroform and comparisons were made with standard amounts of sterol in chloroform. A color reagent composed of three parts H_2SO_4 added to 57 parts cooled acetic anhydride was added to each tube at the rate of 3 ml per tube. Color was allowed to develop for 1 hr in the dark in a water bath at 22 C. Percent transmission at 520 mμ was then determined spectrophotometrically using a Spectronic 20 (Bausch and Lomb spectrophotometer). From the standard curve, equivalent mg sterol per ml medium was computed for each fraction. Results showed no significant differences in sterol concentration among different levels in the tubes.

Preliminary tests showed no apparent differences between cholesterol and β-sitosterol in their effect on growth or sporulation of Phytophthora lateralis. The latter sterol was chosen for routine use, since it was considered a more prevalent plant constituent than cholesterol. Prior to autoclaving, final concentrations of β-sitosterol and 95 percent ethanol in culture media were 20 ppm and 10^4 ppm, respectively. The β-sitosterol was in crystalline form, originating from soybeans (Sigma Chemical Co., St. Louis, Missouri).

Synthetic Medium (Trione). The synthetic medium used here was that which Trione (1957) developed for P. lateralis, except that it was sterilized by passage through a millipore filter. The formula, per liter of distilled water, follows: glucose, 40 g; DL-asparagine, 4.97 g;

Hoagland's minor element solution, 1 ml; Fe as EDTA salt, 0.12 mg; $MgSO_4$, 2 mM; $CaSO_4$, 0.05 mM; KH_2PO_4, 10 mM; thiamine, 1.0 mg. The medium was adjusted to pH 6.0 with NaOH.

Synthetic Medium (Hendrix et al.). This medium, used in an experiment on calcium effects on growth, was that reported by Hendrix et al. (1969) for P. parasitica. Sterlization was by passage through a millipore filter and sterol was added in an ethanol solution. The formula, per liter of distilled water, is: glucose, 21.6 g; DL-asparagine, 5.8 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; thiamine, 0.002 g; $FeCl_3$, 0.1 mg; $CuSO_4 \cdot 5H_2O$, 0.1 mg; $MnCl_2 \cdot 4H_2O$, 0.01 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.01 mg; β-sitosterol, 20 mg; 95 percent ethanol, 10 ml.

Synthetic Medium (Erwin et al.). This medium also was used for growth studies with calcium. It was similar, as used here, to the medium reported by Erwin and Katznelson (1961) except that the components were dissolved in 90 percent of the final liquid volume and filter sterilized. When used, sterol was added to the sterile medium. Various calcium concentrations (as $CaCl_2 \cdot 2H_2O$) were made ten times the final concentration and autoclaved. Four and one-half ml of inoculated basal medium combined with 0.5 ml calcium solution resulted in final concentrations per liter as follows: sucrose, 15 g; L-asparagine, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; KH_2PO_4, 0.43 g; K_2HPO_4, 0.30 g; thiamine HCl, 50 μg; minor elements providing 1 ppm

of Zn, 0.02 ppm Cu, Mo and Mn; 20 mg β-sitosterol in some treatments, and, depending on the treatment requirements, from 0 to 0.6 M $CaCl_2 \cdot 2H_2O$. Initial pH of all treatment media was 4.5.

Verification of Pathogenicity and Stock Culture Maintenance

Pathogenicity of P. lateralis isolates was ascertained prior to any studies reported in this thesis. Inoculum representing each isolate was used to infest the soil in several flats containing rooted cuttings of Chamaecyparis lawsoniana. In the greenhouse, first indications of disease were expressed in a few plants 3 months after inoculation, namely, off-green color and slight wilting of the foliage. In 6 months, symptom expression was prevalent throughout flats inoculated with six of the isolates. At this time isolations were made from the basal stem of recently afflicted plants. After microscopic verification that subcultures were P. lateralis, these isolates were considered pathogenic, and were assigned new stock culture numbers, PL 11, 12, 14, 17, 20 and 21.

Preliminary tests showed very little variation in growth habit and sporulation ability among the isolates. Isolate PL 11 was considered representative and was used in all experiments except as noted.

Duplicate sets of stock cultures were transferred at 2 to 6 month intervals to fresh corn meal agar and V8-calcium-sterol agar slants in

screw cap test tubes. Initially incubated at 20 to 22 C for 2 weeks, cultures were then examined macroscopically for contaminants. If normal growth was observed, tubes were stored at 10 to 18 C until subcultured.

Inoculating and Dispensing Media

Agar media in petri dishes and linear growth tubes were inoculated by means of a 5 mm diameter disc removed from the edges of actively growing colonies of P. lateralis, generally on corn meal agar. The inoculum discs were cut with a sterile cork borer, removed with a microspatula, inverted and placed firmly on the surface of the fresh medium.

Inoculum discs proved unsatisfactory to inoculate liquid media. Hyphal fragments often became dislodged from the disc at the time of inoculation, greatly increasing the initial growth potential and introducing an undesirable source of variation in growth within treatments. Liquid media were inoculated by adding homogeneous suspensions of fungal matter to the autoclaved, cooled medium before dispensing to dishes or tubes. Thus, in one step each culture container received both media and inoculum, time was saved, contamination was reduced, and greater uniformity was achieved. Fungal suspensions serving as inoculum were prepared by transferring colonies from three or four liquid cultures in petri dishes or test tubes to one or several petri

dishes containing sterile distilled water to remove unassimilated nutrients, and then to a sterile 68 mm diameter by 160 mm long glass jar containing 100 ml distilled water. Using an adapter ring to mount the lid and cutting blade assembly, the jar was attached to a Servall Omnimixer (model 0. -115, Ivan Sorvall, Inc., Norwalk, Connecticutt) which was operated at 50 percent maximum voltage for three periods, each of 5 sec duration, with sufficient time between periods to allow the suspension to stop spinning. This suspension was added to liquid medium at the rates of 1.0 to 2.5 percent final volume. The medium in each case was formulated to account for this additional volume.

A repetitive pipetting apparatus (Aupette, Clay-Adams, Inc., New York) was used to dispense equal volumes of medium to culture containers. Frequent agitation was applied to the bulk medium during the dispensing process to prevent settling of hyphae and nutrients.

Description of Culture Containers

Petri Dishes. Glass petri dishes employed were the standard 90 mm pyrex culture dishes. Plastic petri dishes were of the disposable type, with the bottom section 55 mm in diameter and 12 mm deep (Falcon Plastics, Oxnard, California). Depths of 5, 7 and 10 ml medium in these latter dishes measured approximately 3, 4 and 5 mm, respectively. This plastic transmitted greater than 90 percent monochromatic light at wavelengths above 360 mμ, greater than 80 percent

above 327 mμ, and greater than 50 percent at 300 mμ. Plastic dishes were used in all experiments employing temperature gradient plates as described later. The small diameter and flat base made these dishes particularly suitable for this purpose.

Culture Tubes. Pyrex tubes 150 mm long and 16 mm in diameter (20 ml capacity) were used for agitated cultures and in stationary controls in experiments involving the rotating tube method of culture. Tubes used for culture in all other experiments were 200 mm long and 25 mm in diameter (75 ml capacity). The larger diameter tubes supported more growth in still cultures than occurred with smaller tubes. Clusters of four of these larger tubes also were conveniently attachable to the finger clamps for wrist action shaking. Linear growth tubes were 200 by 25 mm culture tubes with a transverse crease about 10 mm deep about 65 mm from the open end. When the tube was placed horizontally with this crease side down, a trough of uniform depth and width was formed. Solidification of 10 ml of molten agar medium in this trough provided a 15 mm strip of medium with a uniform cross sectional profile for hyphal extension.

Tubes containing cultures aerated with forced air were capped with autoclavable compressible form plugs (Identi-plugs, Gaymar Industries, Inc., Buffalo, New York), which allowed air pressure relief from within the tubes and excluded contaminants. In all other

instances, tubes were capped with polypropylene closures of appropriate diameter (Bacti-Capalls).

Depression Slides. Twenty-five by 75 mm glass depression slides with a well volume of exactly 0. 1 ml were used for studies of chlamydospore germination. Careful placement of a 22 mm^2 cover slip over a well containing 0. 1 ml of aqueous spore suspension sealed the mount without trapping air bubbles or expressing liquid from beneath the cover slip. High humidity was maintained in incubation areas to minimize loss of moisture.

Kinetic Culture Equipment

The Burrell Wrist Action Laboratory Shaker (model DD, Burrell Corp., Pittsburgh, Pennsylvania) with 16 finger clamps on the side arms was used to shake liquid cultures of P. lateralis in 200 by 25 mm tubes. It was necessary to remove the motor housing and provide circulated air to this area, as previous experiments showed heat buildup around the housing caused inhibition of growth in culture tubes adjacent to the motor. A setting of half the maximum amplitude was found to provide uniform motion to the medium in tubes at any position on the shaker. Fungal matter produced with this shaker consisted of atypical, small, smooth, very dense, hollow balls which, upon drying at 75 C, became extremely hard.

In contrast, a large, spherical colony formed in each 150 by 16

mm culture tube on the other kinetic device employed, viz. a Roller-drum tissue culture apparatus (New Brunswick Scientific Co., New Brunswick, New Jersey). Colonies on this apparatus had a fluffy appearance, with hyphae radiating in all directions throughout most of the medium. In this device the tubes are held in a perforated disc at a constant angle (about 60° was selected) and the entire disc is rotated. Thus, tubes are rotated about their long axes, while the medium remains nearly still. The colonies were gently and continuously tumbled in the medium by this action. Two models representing different rotation speeds (1 rpm and 1/5 rpm) were used, with no apparent differences in the nature or quantity of fungus produced.

Temperature Gradient Plate Design

The temperature gradient equipment used in these studies was developed by C. M. Leach.[1] Aluminum plates measuring 60 by 122 by 1.6 cm were fitted along a pair of opposite edges with copper tubing (Figure 1). Water from a heated reservoir was continuously pumped through the tubing on one edge of each plate and water from a refrigerated reservoir was circulated through the other. Water temperatures were thermostatically controlled. By choosing appropriate water temperatures, a constant near-linear gradient within a

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[1] A more complete description of design of this equipment may be found in Leach (1967).

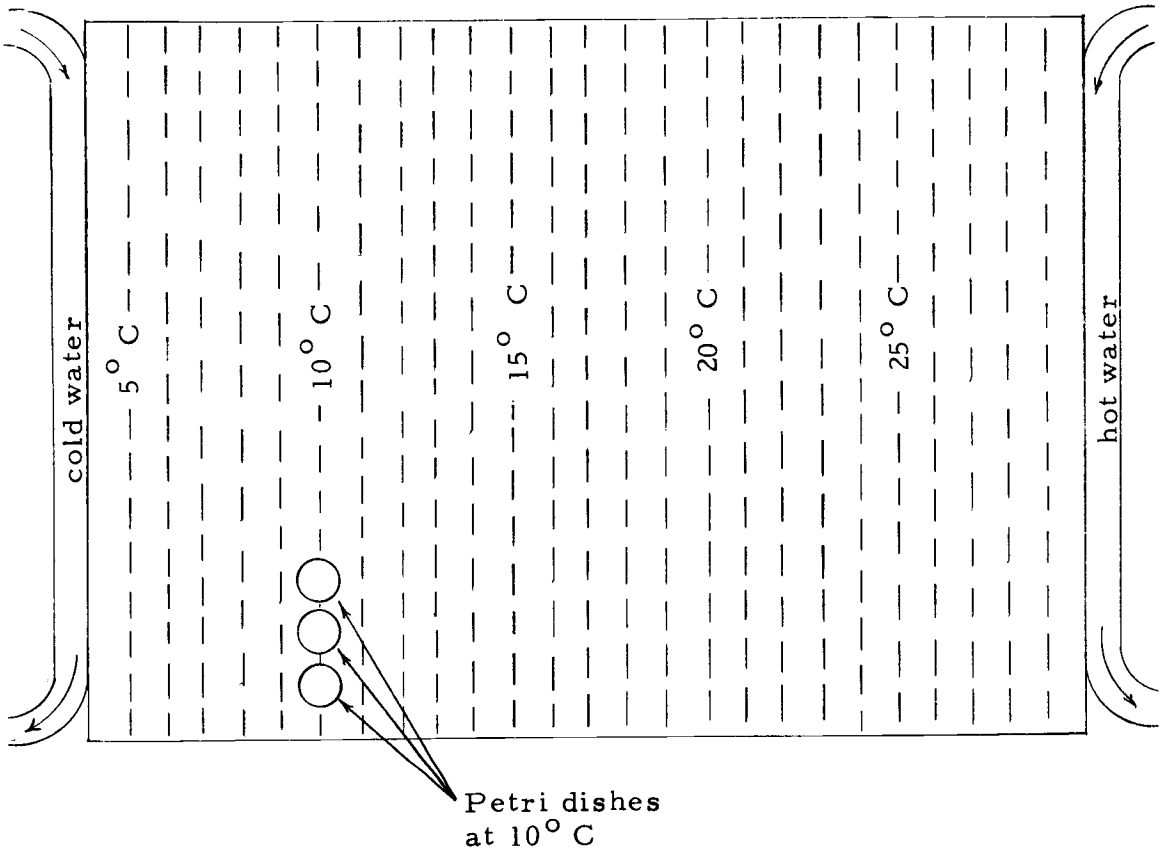

Figure 1. Physical layout of components of a temperature gradient plate.

desired temperature range was established along each plate.

Identical temperatures were found along any single line on the plate parallel to the edges receiving heat or cold applications. Thus, eight replicate petri dishes could be placed along an isothermal line. At a particular location on a gradient plate under constant conditions, temperature varied less than ± 0.5 C over the course of an experiment. Temperatures under alternating illumination conditions fluctuated to a greater degree, sometimes as much as ± 1.5 C, but were typically ± 1.0 C.

In one study of the effect of light intensity on chlamydospore production, temperature was not a variable. A gradient plate apparatus was used to maintain uniform temperature between treatments by circulating water at a specified temperature through both edges of the plate. This uniform temperature plate served as a heat sink, absorbing radiant heat from the light incident on the medium and aluminum plate surface.

Several plates were modified so that copper tubes were fitted along all four edges (Figure 2). By means of solenoids controlled by time clocks, at 12 hr intervals hot water circulation was switched from one edge (Figure 2A) to an adjacent edge (Figure 2B), while cooled water was simultaneously rerouted to the edge opposite the heated one. This allowed a large number of day-night fluctuating temperature regimes to be tested. Since each spot on the plate

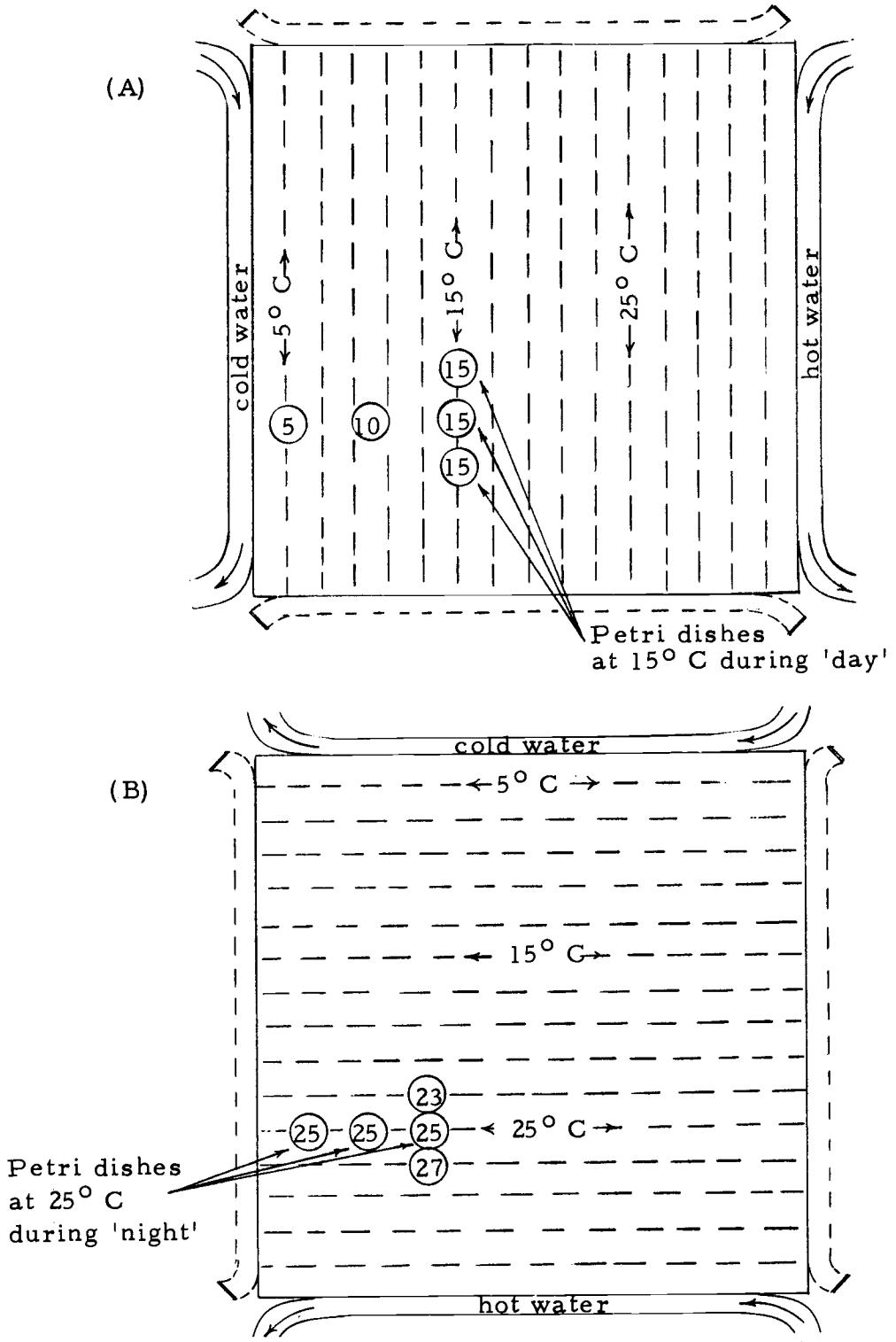

Figure 2. An alternating temperature gradient plate illustrating (A) gradient of decreasing temperatures from right to left of the plate for the first 12 hrs daily, and (B) gradient of decreasing temperatures from front to rear of the plate for the remaining 12 hrs daily.

received a unique temperature program, replications were not possible. These plates are referred to hereafter as alternating gradient plates.

Measurements of Temperatures on Gradient Plates

Temperatures were recorded to the nearest 0. 1 C twice daily by means of thermocouples in conjunction with a temperature potentiometer (Leeds and Northrup Co., Philadelphia, Pennsylvania). Readings were made just prior to the end of each 12 hr illumination or dark period to record the widest variation due to the heating effect from the lamps. A series of thermocouples were permanently attached along the direction of the gradient on each plate (Leach, 1967). On the alternating gradient plates, thermocouples were arranged along two perpendicular lines.

Illumination of Cultures on the Plates

Illumination in all gradient plate experiments was supplied by an equal number of cool white and near UV (BLB) 40 watt fluorescent tubes. Light intensity at the level of the medium was 190 foot candles unless stated otherwise. Illumination levels of individual treatments on the uniform temperature plates are described in the Results section. Measurements were made through a plastic petri dish lid with a

Weston foot candle meter (model 756) with quartz window.

Methods of Evaluating Growth

Dry weight determinations were made on colonies grown in liquid media. The contents of each culture container were placed on a tared Whatman #1 filter paper in a Buchner funnel under vacuum. Unassimilated nutrients were washed from the fungal matter by pulling a measured amount of water through the filter paper and the fungus. Filter papers containing fungus were dried to constant weight at 75 C in a forced air oven, cooled in a desiccator and weighed.

Colonies on agar culture media were measured either as colony diameters or in the linear growth tubes as linear distance of development from the inoculation point.

Methods of Evaluating Sporulation

Due to the large number of colonies in each test and the time consuming nature of spore counting, a means was devised to stop fungus development, after the prescribed incubation period was completed, in all cultures at the same moment. Two ml of Formalin-Acetic acid-Alcohol (FAA) added to liquid media or flooded over agar media effectively killed and fixed hyphae, chlamydospores and sporangia, preserving these until microscopic examination was done at a later time. The formulation of the FAA solution was: 360 ml

distilled water, 352 ml 95 percent ethanol, 40 ml acetic acid, and 48 ml formaldehyde.

Cultures in liquid media were prepared for spore counting as follows: Colonies from each dish were placed in an 8 ml capacity Servall Omnimixer can containing 5 ml distilled water. The can and an adapter were connected to the Omnimixer, which was operated for three 10-sec periods at 70 percent maximum voltage. The resultant suspension of spores and hyphal fragments was diluted to 250 ml and, during agitation, two or four 0.1 ml samples were placed in depression slides. Sporangia and chlamydospores were counted microscopically in the entire 0.1 ml sample, averaged with the other samples from the same culture, then multiplied by the dilution factor to allow computation of total numbers of sporangia and chlamydospores per dish.

Cultures on solid media were first microscopically scanned to determine the uniformity of sporulation, and a radius line with sporulation typical of the colony was selected. Spores were counted, using an ocular counting grid, in 10 or 20 microscope fields (each 0.5 mm^2) spaced uniformly along the radius so that no preference was given to hyphae of a particular age.

RESULTS

Mycelial Growth

On media generally used for Phycomycete culture (i. e., corn meal agar, water agar), Phytophthora lateralis is among the slowest growing Phytophthora species. This fact has greatly hindered studies of many kinds which require substantial amounts of inoculum. Experiments described in this section on the effects on growth of mechanical agitation, nutrition and temperature, show the conditions under which the growth rates may be stimulated.

Kinetic Studies in Liquid Culture Media

Acceleration of growth of many fungi may be accomplished by moving or stirring the medium of liquid cultures. Growth of P. lateralis, measured as dry weight, was compared after cultures were subjected to several kinetic regimes.

Ten ml of V8-(calcium)-sterol broth previously inoculated with fungus was pipetted into each of a number of sterile 75 ml culture tubes. In one comparison equal numbers of tubes were placed on a Burrell wrist action shaker and in a stationary tube rack. Determinations of fungal dry weight were made at approximately three-day intervals for 21 days (Table 1). In a concurrent experiment, groups of six inoculated tubes were transferred after 3, 6, 9, 12, 15 and 18 days

Table 1. Growth of Phytophthora lateralis expressed as mycelial dry weight, and final pH of the V8-(calcium)-sterol broth after various incubation periods in still or shake culture.

Incubation period (days)	Still culture		Shake culture	
	Dry weight (mg)	Final acidity (pH)	Dry weight (mg)	Final acidity (pH)
3	0.2[a]	4.5	0.4[a]	4.5
6	3.8	4.2	7.7	4.6
9	3.9	4.2	35.5	5.2
12	5.7	4.2	33.8	6.1
15	5.5	4.2	27.2[b]	6.5
19	7.9	4.2	--	-
21	7.3	4.1	45.5	7.3

[a] Average of six replications unless individually indicated.

[b] Two replications.

from still cultures to a Burrell shaker where they remained to complete a total incubation period of 21 days (Table 2). Preliminary stationary culture was introduced to determine the need for an agitation-free recovery period for the inoculum fragments prior to agitation. This need for adjustment has been shown for certain other Phycomycetes (Erwin and Katznelson, 1961). All cultures were incubated at room temperature (20 to 22 C).

Tables 1 and 2 show that shaking greatly stimulated growth of P. lateralis and that a brief still period prior to shaking was beneficial. Growth produced on the wrist action shaker was distinctly atypical if not abnormal. Colonies consisted of numerous, small, extremely dense, hollow spheres. Hyphae in these balls were compressed so greatly that individual filaments could not be distinguished microscopically. The spherical cells of which the spheres were primarily composed may in fact have been rudimentary chlamydospores.

In seeking to combine the growth increasing characteristics of shake cultures with conditions favoring a more typical hyphal growth habit, cultures were grown on the Roller-drum apparatus, described earlier, which agitated the medium much more gently than did the wrist action shaker. The effectiveness of roller agitation in increasing growth over that occurring in still culture and its favorable comparison with wrist shaken cultures is shown in Tables 5 and 6. Colonies in media on the Roll er-drum apparatus were composed of morphologically typical hyphae.

Table 2. Growth of Phytophthora lateralis expressed as mycelial dry weight, and final pH of the V8-(calcium)-sterol broth after 21 days of culture in which various periods of still culture preceded shake culture.

Still/shake (days)	Dry weight (mg)	Final acidity (pH)
21/ 0	7.3[a]	4.1
18/ 3	6.0	4.2
15/ 6	21.5	5.4
12/ 9	31.4	5.5
9/12	39.9	6.2
6/15	47.5	7.1
3/18	51.0	7.2
0/21	45.5	7.3

[a] Average of six replications.

The preceding experiment with shaken cultures led to the belief that growth benefits derived from culture agitation have not been fully identified as to cause. Accordingly, the following experiment was designed to compare growth in a highly aerated medium, and in both moving and still media with a limited oxygen supply.

V8-calcium-sterol broth was dispensed at the rate of 15 ml per 75 ml culture tube. A 50 mg pad of glass wool was submerged beneath the surface of the medium in each tube. This wool was included to provide a stable matrix for colony development below the surface strata of medium which was agitated by the stream of air bubbles. The tubes were autoclaved, cooled, and inoculated in the following manner. A 3 mm diameter disc of inoculum was placed at the bottom of each tube, beneath the glass wool, using a glass tube fitted with a rubber bulb for discharging the inoculum discs. Culture tubes to receive forced aeration were prepared as follows: a capillary pipette was inserted through a porous polyurethane foam plug in the neck of each culture tube. The narrow diameter tip of the pipette was adjusted to 2 to 3 mm below the surface of the medium. The other end of the pipette, projecting out of the tube, was connected to a pressurized manifold. A millipore in-line filter maintained sterility of the air. Temperature of air entering the cultures was stabilized by passage through a condenser with water at about 20 C continuously flowing in the jacket. Air flow into each tube was adjusted by means of

relief and constriction valves in the supply tubing so that a steady stream of bubbles was discharged just below the surface of the medium. Culture tubes intended for the two limited aeration treatments (stationary culture or rotating culture) were capped with polypropylene closures and sealed with plastic electrical tape. One group of these tubes was placed on the 1 rpm Roller-drum apparatus while others were kept under stationary culture conditions throughout the incubation period.

Dry weights of fungal matter were determined from four or five replicate cultures from each treatment 10 and 17 days after inoculation. These data are shown in Table 3. On the 17th day, before removal of the tube contents for drying, the amount of dissolved oxygen in the medium was determined with a GME oxygraph (Gilson Medical Electronics). Using a syringe flushed with nitrogen gas, 2-ml samples of medium were withdrawn from the bottom 5 mm of each tube and placed in the oxygen electrode chamber which had been purged with nitrogen gas. Readings from the culture medium (average of two or three readings per replicate) were converted to percentages of the maximum dissolved oxygen the medium could contain, determined as follows. Samples of the 'used' culture medium were aerated by repeated squirting from a syringe into a beaker until no change occurred in percent oxygen, and the average percent dissolved oxygen determined from these was considered as 100 percent. (Similar

Table 3. Comparative growth of Phytophthora lateralis under liquid culture conditions testing the influence of oxygen concentration on dry weight production. Table values are means of either four or five replicate cultures.

Culture treatment	10 days incubation		17 days incubation		
	Dry weight (mg)	Final acidity (pH)	Dry weight (mg)	Final acidity (pH)	O_2 conc. (percent)
Sealed stationary	8.0	4.5	7.6	4.4	23
Sealed rotated	29.4	5.3	35.2	5.8	26
Aerated by bubbling	11.0	4.4	21.1	4.6	59

maximum dissolved oxygen concentrations were obtained from uninoculated medium held 17 days at room temperature. The oxygen concentration in fresh medium was not measured.) Treatment means appear in the table.

After 17 days of culture, there was more than twice as much dissolved oxygen in the culture media receiving bubbled air as in media from the other treatments, yet far more growth occurred in media rotated continuously where oxygen was relatively limited. The sealed still and rotated culture tubes initially contained the same volume of oxygen, and similar final concentrations of dissolved oxygen were measured after 17 days of culture, yet the rotated cultures developed more than four times as much growth as the still cultures. These results show that the stimulatory nature of moving culture media is not dependent on increased oxygen exchange.

Temperature Requirements

General observations in the laboratory showed that higher ambient temperatures during summer were associated with erratic or slow growth rates of P. lateralis. These temperatures were only a few degrees higher than the optimum (20 C) reported in the literature (Trione, 1957). The effect of temperature on P. lateralis was studied especially to determine optima and maxima for hyphal growth.

Linear growth tubes in incubators were used. These tubes, in

this case containing corn meal agar, confine colony extension to a narrow, 15 cm long band of medium of uniform width and depth. Each culture tube was inoculated by placing a disc of P. lateralis hyphae at one end of the medium strip. Four tubes were placed in each incubator at temperatures of 10, 15, 20, 22.5, 24.5, 27.5 and 30 C. Linear colony extension was measured every three days for 21 days and again after 35 days (Table 4).

Maximum growth occurred at 24.5 and 22.5 C, with slightly less growth at 20 C. At 10 C there was a 12-day lag before growth could be detected. The fungus did not grow at 27.5 or 30 C. Later experiments with V8 broth and agar media in studies of temperature effects on sporulation employed incubation temperatures at closer intervals (1 and 2 degrees C). The optimum for growth, measured as dry weight, colony diameter or colony area, was confirmed as 22 to 24 C, and no growth occurred at 26 C. Incubation temperatures of 20 to 22 C were used for subsequent culture of P. lateralis. Despite the good growth at slightly higher temperatures, these were not adopted generally for culture due to the proximity to the lethal temperature.

Hydrogen Ion Concentration of Culture Media

Preceding experiments have shown an increase in pH of the medium of shake cultures with time and fungal growth. The final near-neutral to basic reaction (Tables 1 and 2) may relate to the stimulation

of growth reported in shaken cultures. Contrarily, it could partially counteract the benefits of shake culture, thus slowing subsequent growth. The effect of initial pH on fungal growth in still and shake cultures was examined as follows.

Batches of sterile V8-(calcium)-sterol broth were adjusted with 1 N NaOH to pH values of 4.5, 5.5, 6.5 and 7.0 and were inoculated with a hyphal suspension. In a first experiment, 10 ml aliquots from each pH batch were pipetted into 24 culture tubes (75 ml size). After a 3-day still incubation, 12 of these tubes were placed on a Burrell wrist action shaker and 12 were retained still. Growth, as dry weight, was measured after 5, 10 and 15 days in four cultures from each pH in each group, shaken and still. Means appear in Table 5.

In a concurrent experiment, 5 ml aliquots from each batch of the above adjusted, inoculated media were pipetted separately into 24 culture tubes (20 ml size). Twelve were incubated from the beginning in a 1 rpm Roller-drum apparatus and 12 were kept still. Growth, as dry weight, after 4, 8, 12 and 16 days of incubation was measured in each of three cultures from each pH in both rolled and still groups (Table 6).

In shaken cultures of media with low initial pH (4.5 or 5.5) the amount of growth correlated with a rapid increase in pH, irrespective of the form of agitation. In contrast with this, pH tended to decrease slightly with increasing growth in stationary cultures. Lack of good

Table 4. Linear growth of Phytophthora lateralis with temperature on corn meal agar in growth tubes.

Temperature (°C)	Incubation period (days)							
	3	6	9	12	15	18	21	35
	(mm)	(mm)	(mm)	(mm)	(mm)	(mm)	(mm)	(mm)
10.0	0 [a/]	0	0	1.2	2.5	3.5	5.0	13.0
15.0	0	2.8	5.8	8.2	10.8	13.2	16.0	27.5
20.0	1.8	7.0	10.5	16.2	22.0	27.0	32.0	55.2
22.5	2.8	9.5	14.8	20.8	27.2	31.5	38.5	62.5
24.5	2.8	9.2	14.2	21.2	27.3	32.7	38.7	65.3
27.5	0	0	0	0	0	0	0	0
30.0	0	0	0	0	0	0	0	0

[a/] Average of four cultures.

Table 5. Mycelial dry weight of Phytophthora lateralis with time in still and shaken culture at four initial levels of pH. Final pH values appear in parentheses.

Initial acidity (pH)	Stationary culture			Wrist shaken culture		
	Incubation period (days)			Incubation period (days)		
	5 (mg)	10 (mg)	15 (mg)	5 (mg)	10 (mg)	15 (mg)
4.5	1.2 [a/] (4.6)	3.7 (4.3)	5.5 (4.2)	13.0 (4.7)	30.7 (7.1)	31.0 (7.7)
5.5	1.5 (5.1)	5.0 (4.5)	5.2 (4.3)	8.8 (5.3)	31.0 (7.5)	29.7 (7.5)
6.5	0.2 (6.3)	0 (6.2)	1.5 (5.4)	0 (6.3)	0.3 (6.1)	0.1 (6.1)
7.0	0.8 (6.8)	0.1 (6.9)	1.8 (6.7)	0.3 (6.9)	0 (6.9)	0.8 (6.7)

[a/] Average of four cultures.

Table 6. Mycelial dry weight of Phytophthora lateralis with time in still and in rotated culture at four initial levels of pH. Final pH values appear in parentheses.

Initial acidity (pH)	Stationary culture				Rotating tube culture			
	Incubation period (days)				Incubation period (days)			
	4 (mg)	8 (mg)	12 (mg)	16 (mg)	4 (mg)	8 (mg)	12 (mg)	16 (mg)
4.5	1.6 [a] (4.5)	0.8 (4.4)	2.1 (4.3)	1.6 (4.1)	1.6 (4.5)	9.1 (4.5)	13.6 (6.1)	13.6 (6.2)
5.5	0.1 (5.2)	1.3 (4.4)	2.8 (4.4)	2.0 (4.2)	0.8 (5.2)	4.3 (4.5)	12.5 (5.6)	13.1 (6.5)
6.5	0 (6.2)	0.6 (6.2)	0.6 (6.1)	2.0 (5.9)	0.8 (6.2)	0.1 (6.2)	0.1 (6.2)	2.8 (6.3)
7.0	0.6 (6.8)	0 (6.7)	0.1 (6.7)	0.5 (6.7)	1.0 (6.8)	0 (6.6)	0.1 (6.7)	0 (6.6)

[a] Average of three cultures.

growth in cultures initially at pH 6.5 and 7.0 suggests that when cultures having low initial pH support rapid growth, the rise in pH accompanying this activity becomes inhibitory.

Effect of β-Sitosterol and Ethanol

Hendrix (1965) compared linear growth of 11 species of Phytophthora on synthetic media with and without cholesterol. He found that growth of P. lateralis was not stimulated by the sterol. I compared growth of P. lateralis on V8-calcium broth media containing β-sitosterol in concentrations of 1, 2, 10, 20, 100 and 200 ppm. Sterol was added, prior to autoclaving, as a solution in a quantity of 95 percent ethanol equal to 1 percent of the final volume of the medium. Two control media were included; one contained ethanol without sterol, and the other contained no ethanol or sterol. Dry weight determinations were made every 2 days for 14 days (Table 7).

Maximum dry weight was reached between the 8th and 10th day. The presence of ethanol in the medium, with or without sterol, substantially increased fungal growth. The data show no clear-cut stimulation of growth by sterol.

Effect of Calcium

Calcium has been shown to stimulate growth of at least eight species of Phytophthora (Christie, 1958; Davies, 1959; Erwin and

Table 7. Mycelial dry weight of Phytophthora lateralis with time in V8-calcium broth containing seven concentrations of β-sitosterol.

Sterol (ppm)	Ethanol	Incubation period (days) 2 (mg)	4 (mg)	6 (mg)	8 (mg)	10 (mg)	12 (mg)	14 (mg)
0	-	0.7[a]	23.6	24.8	22.3	22.5	21.8	21.1
0	+	0.7	24.7	34.1	40.1	41.8	40.3	38.7
1	+	3.1	27.8	35.2	41.7	41.1	40.2	37.3
2	+	0.7	24.7	36.3	42.1	40.8	40.0	37.6
10	+	0.8	30.2	33.8	40.6	39.0	38.3	36.5
20	+	0.7	29.0	33.3	44.2	48.2	47.5	44.7
100	+	1.3	26.0	33.6	42.7	45.2	43.6	40.2
200	+	2.0	25.1	34.3	43.2	45.8	46.1	42.8

[a] Average of four cultures.

Katznelson, 1961; Lopatecki and Newton, 1956). P. lateralis was not included in these tests. Two experiments were run here examining the effect of calcium on growth of P. lateralis. Synthetic media were selected to allow the formulation of media with known amounts of calcium.

In the first test, batches of synthetic glucose-asparagine basal medium (Hendrix et al., 1969) were prepared with concentrations of calcium ($CaCl_2 \cdot 2H_2O$) from 10^{-2} to 10^{-6} M, and no calcium. Inoculated calcium-adjusted media were pipetted into glass petri dishes, 10 ml per dish. Growth was measured as dry weight from each of four cultures from each calcium concentration after 24 days incubation (Table 8).

Concentrations of calcium greater than 10^{-4} M greatly increased the growth of P. lateralis.

In another experiment, growth on a sucrose-asparagine synthetic basal medium (Erwin and Katznelson, 1961) was compared at calcium ($CaCl_2 \cdot 2H_2O$) concentrations from 6×10^{-1} to 10^{-4} M. Media with and without β-sitosterol dissolved in 95 percent ethanol (2 mg and 1 ml/100 ml medium, respectively) were included at each calcium concentration tested. Culture tubes (20 ml size) containing 5 ml of inoculated media were incubated at room temperature in a Roller-drum apparatus. Mycelial dry weights were determined after 12, 18 and 24 days.

Table 8. The effect of calcium on mycelial dry weight of Phytophthora lateralis on a synthetic medium.

Concentration of calcium (Molar)	Dry weight (mg)
0	2.7[a]
10^{-6}	2.2
$5x10^{-6}$	3.2
10^{-5}	6.0
$5x10^{-5}$	7.1
10^{-4}	5.0
$5x10^{-4}$	22.6
10^{-3}	28.6
$5x10^{-3}$	26.1

[a] Average of four cultures.

Figure 3 shows 10^{-3} M calcium to be the optimum concentration for growth in this medium. When compared with mycelial dry weights in comparable calcium-deficient controls, 10^{-3} M calcium stimulated growth threefold in the presence of sterol and twofold in its absence. Final pH measurements taken at the time of dry weight determination indicate that the differential effect of the various calcium concentrations on growth was not due to pH or buffering. There was no correlation between dry weight growth and pH. The synergistic interaction of calcium with sterol and/or ethanol is evident.

Production and Germination of Chlamydospores

It would appear that the dry summers characteristic of the Pacific coastal region constitute a deleterious period in the life cycle of P. lateralis. It has been postulated that chlamydospores are important in the oversummering survival of the fungus (Trione, 1959). Little is known about the physical and chemical requirements for formation or germination of chlamydospores by P. lateralis. These processes were therefore studied in vitro, including: the specification of a suitable basal medium, evaluation of the influence of sterol, and the effect of light and temperature.

Chlamydospore Production

Selection of a Medium. Different media were inoculated with

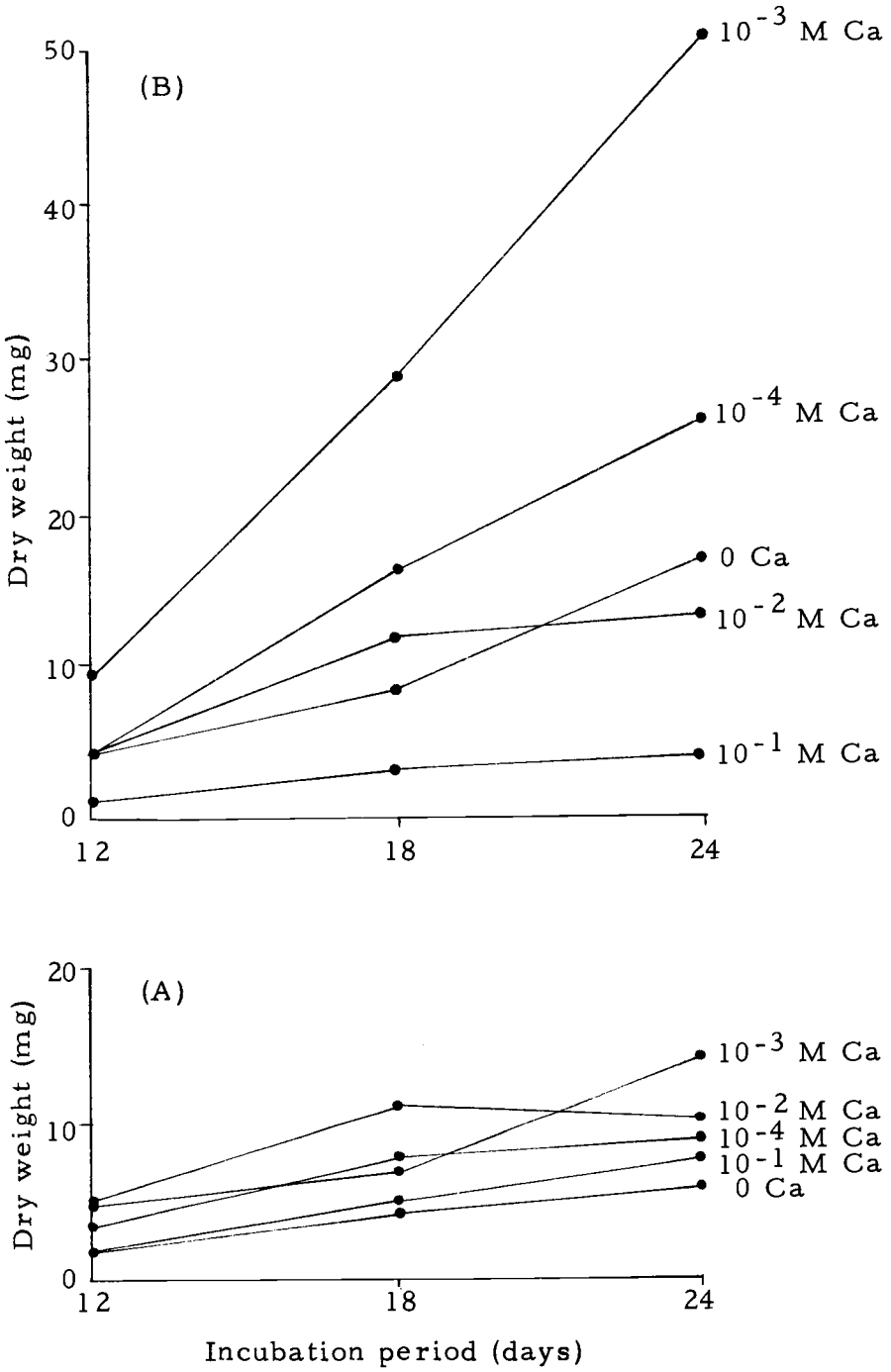

Figure 3. The effect of five levels of calcium on mycelial dry weight of Phytophthora lateralis in a synthetic glucose-asparagine liquid medium (A) with β-sitosterol, and (B) without β-sitosterol.

P. lateralis, and after several weeks, were examined for chlamydospores. Broth and agar media containing 10 percent commercial V8 juice were judged superior to corn meal agar, water agar, pea broth, and potato dextrose agar or broth in their ability to support growth and to mature chlamydospores. V8 broth made with distilled water or tap water supported similar fungus growth and chlamydospore production. Since the constituents in tap water may vary from time to time, distilled or deionized water was thenceforth used in the preparation of all media.

Culture media containing a 10 percent suspension of unfiltered commercial V8 juice contains substantial particulate matter which interferes with in situ microscopic examination and hinders removal of unassimilated nutrients from fungus material prior to plant inoculation or soil infestation. An experiment was run to learn the extent to which desirable qualities of V8 juice medium are lost in clarification of the juice. Treatments consisted of (1) no clarification, (2) vacuum filtration through four layers of cheesecloth, and (3) centrifugation for 10 min at 1000 x g. The V8 juice, V8 filtrate and V8 supernatant were added to distilled water to obtain a final concentration of 10 percent V8 juice. After the media were autoclaved and cooled, each of 20 petri dishes were poured with 15 ml of each medium and inoculated with bits of hyphae. Weekly microscopic examinations revealed that in 2 weeks there were slightly smaller colonies and slightly fewer chlamydospores

produced in the centrifuged V8 medium than in the other treatments, while after 3 weeks there were no differences among treatments. Several advantages of the highly clarified centrifuged V8 medium compensated for a slight lag period of growth and sporulation. Thus, in all V8 juice formulations the juice was centrifuged at 1000 x g for 10 min, and the supernatant passed through glass fiber paper (Whatman GF/A) to remove any particulate matter inadvertantly decanted from the pellet in the centrifuge cups.

Effect of Sterols

In the past decade a great deal of evidence has accumulated on stimulation of formation of reproductive structures by sterols in species of the Pythiaceae. There is some indication that chlamydospore formation is influenced by sterols (Hendrix, 1965).

In a preliminary test, cultures of P. lateralis grown in a glucose-asparagine synthetic liquid medium (Trione, 1957) with 50 ppm β-sitosterol produced large numbers of chlamydospores, while without sterol only small swellings on the hyphae developed. The effect of sterol in a natural medium was tested. More chlamydospores formed in V8 broth containing 50 ppm β-sitosterol or cholesterol than in the medium without sterol. In another test, V8 agar (2 percent agar) was prepared with and without 20 ppm β-sitosterol, and 15 petri dishes containing each medium were inoculated with P. lateralis.

After 1 month incubation in the dark at room temperature, colonies grown in the unamended V8 agar contained chlamydospores, but only embedded deep in the agar in the region of sparse growth near the edge of the petri dish, while cultures on V8 agar with 20 ppm sterol contained chlamydospores in high densities at all depths in the medium, throughout the colonies.

To determine the amount of sterol most effective in stimulating chlamydospore formation, batches of V8 broth media were prepared to contain 0, 0.02, 0.2, 2 and 20 ppm β-sitosterol. After 8 days, colonies in the medium with 20 ppm sterol contained large numbers of chlamydospores, while colonies in all other media contained no chlamydospores. To the unaided eye, all media supported similar amounts of growth.

Another experiment tested a wider range of sterol concentrations, and provided a longer incubation period. The sterol concentrations were 1, 2, 10, 20, 100 and 200 ppm, each added to V8-calcium broth in equal amounts (1 ml ethanol per 100 ml medium) of 95 percent ethanol. V8-calcium broth with ethanol reagent only, and V8-calcium broth only, were included as controls. Ten ml of inoculated medium was dispensed into each 9 cm diameter petri dish. Cultures were incubated in the dark at 20 C. Four cultures from each medium were examined microscopically every other day through the 14th day of incubation. An interaction between the sterol concentration and the minimum incubation period for chlamydospore development became

apparent. On the 6th day a small number of chlamydospores were found, and only in media containing 20 ppm sterol. By the 8th day many chlamydospores were present in the 20 ppm sterol medium, with nearly as many in the medium with 10 ppm sterol. On the 10th day, large numbers of chlamydospores were present in all media containing sterol, although in media with 100 or 200 ppm sterol there appeared to be great variation in the number of chlamydospores among colonies in any one dish. At this time a few chlamydospores were present in colonies in both control media. After 12 days incubation all media containing sterol had large numbers of chlamydospores at the peripheries of the colonies. Chlamydospores in media with 10 or 20 ppm sterol were uniformly distributed throughout the colonies.

After 19 days incubation, the media in the above cultures were replaced with distilled water. Cultures were incubated at 10 C for 1 week, then at about 20 C for 1 week under fluorescent room lights. Chlamydospores were counted in samples of two cultures from each initial medium. Dry weight of each culture was determined, and the number of chlamydospores per mg dry weight was computed. Data appear in Table 9. A sterol concentration of at least 20 ppm in V8 broth medium stimulated maximum chlamydospore production by P. lateralis. Ethanol apparently did not influence sporulation.

Later in this section it will be seen that chlamydospore production is temperature dependent, with greatest spore production

Table 9. Effect of β-sitosterol and ethanol on chlamydospore production by Phytophthora lateralis cultures incubated first in V8-calcium broth, then in distilled water.

Amendment		Chlamydospores/ culture (no.)	Chlamydospores/ mg. dry wt. (no.)
Sterol (ppm)	95% Ethanol (10^4 ppm)		
0	-	44,687[a/]	7,448[a/]
0	+	36,250	2,288
1	+	80,625	4,734
2	+	207,812	11,393
10	+	476,875	22,747
20	+	782,812	26,378
100	+	567,187	25,763
200	+	660,312	27,239

[a/] Average of two cultures, four samples per culture.

occurring at temperatures above 21 C (incubation temperature in the above experiment was below 21 C). A test was designed to demonstrate the influence of sterol on chlamydospore production at temperatures near the optimum. Cultures were incubated in the dark in V8-calcium broth, with and without 20 ppm β-sitosterol. After 11 days, chlamydospores were counted in samples of four cultures from each medium at each temperature. Partial data (yields at temperatures in the vicinity of the optimum) are presented in Table 10.

Effect of Temperature and Light

Cultures containing large quantities of chlamydospores frequently were required for soil infestation experiments. For this purpose, cultures were grown for 2 to 3 weeks in petri dishes under benchtop conditions. Temperature was generally between 18 to 22 C, and no attempt was made to control the amount of light on the cultures from the overhead fluorescent illumination. Consistent high yields of chlamydospores were rarely realized from cultures so incubated. Variability occurred between 'crops' as well as among cultures incubated at the same time. Occasionally, however, extremely high yields of chlamydospores were found. One such incident is described below.

One hundred petri dishes, each with 10 ml V8-calcium-sterol broth medium inoculated with fragmented hyphae, were incubated on a benchtop at about 20 C for 6 days, then at about 25 C for 5 days as

Table 10. Effect of temperature on chlamydospore production by Phytophthora lateralis in V8-calcium broth with and without 20 ppm β-sitosterol. Incubation was in darkness for 11 days.

Temperature (°C)	Chlamydospores	
	Sterol (no.)	No sterol (no.)
19.5	203[a]	11[a]
21.6	326	24
23.6	388	44
25.2	91	6

[a] Average of four cultures.

ambient temperature increased. Microscopic examination on the 5th day revealed no chlamydospores, but on the 8th day, numerous chlamydospores were found in all cultures. The spores were located mostly on or near hyphal tips, indicating recent formation. By the 11th day most chlamydospores were intercalary, due to further hyphal growth. These observations indicated that it was possible to produce large numbers of chlamydospores in a relatively short time. In light of the higher than normal ambient temperature (25 C) experienced by these high yielding cultures, and the fact that several previous endeavors to produce chlamydospores resulted in low or erratic yields (using the same medium but with culture taking place in an incubator at a constant 20 C), investigations were undertaken on the effects of temperature on chlamydospore formation. Also, since the colonies were subjected to various amounts of light during incubation, illumination treatments were included in these experiments.

Gradient plates were used to control temperatures between the selected hot and cold extremes. The gradients allowed selection of closely spaced incubation temperatures by placement of cultures at appropriate locations along the gradient. At each selected temperature four cultures were kept dark, while four others were illuminated by overhead fluorescent tubes. Light intensity at colony level was 190 foot candles. Figure 4 shows a diagrammatic representation of treatments in the experiment described below.

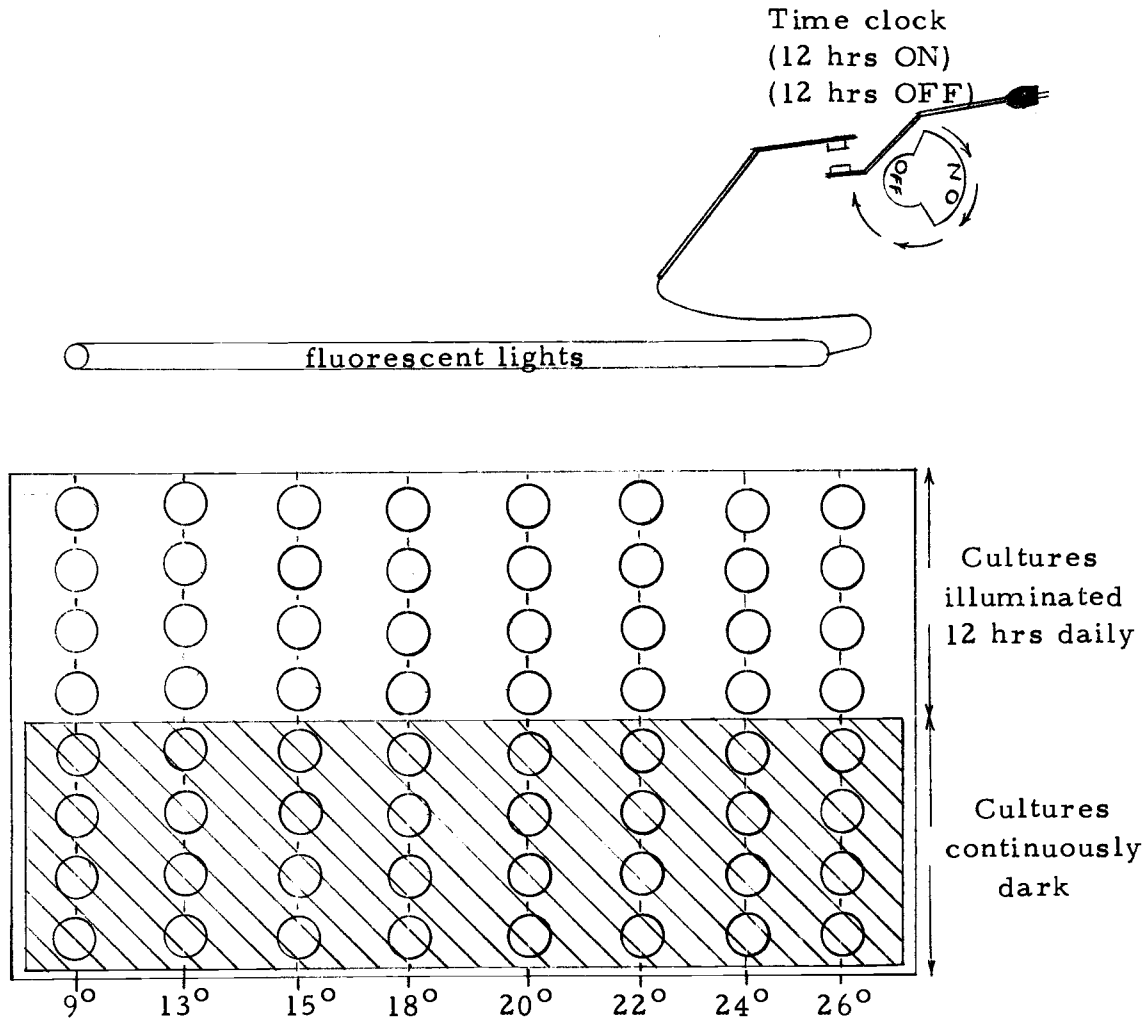

Figure 4. Temperature and illumination treatments of a representative experiment on a temperature gradient apparatus.

Plastic petri dishes containing 7 ml V8-calcium-sterol agar (1.7 percent agar) were each inoculated with a 5 mm diameter disc of P. lateralis hyphae. At each of eight temperatures four dishes were incubated in continuous darkness and four were illuminated 12 hrs daily by fluorescent lamps. After 11 days chlamydospores were counted. The optimum temperature for chlamydospore production by dark-incubated colonies was approximately 24 C (Table 11). Few chlamydospores formed at any temperature on illuminated colonies.

This experiment was repeated, using 5 ml of medium per petri dish rather than 7, and part of the cultures were illuminated continuously. Results after 11 days incubation appear in Table 12.

The optimum temperature for chlamydospore production on dark-incubated colonies was about 24 C, as in the previous experiment. Colonies illuminated continuously formed slightly fewer chlamydospores than those illuminated 12 hrs daily, and both produced many fewer than developed in continuous darkness.

A similar test compared temperature and light effects on chlamydospore production by P. lateralis in a liquid medium. Real or apparent differences in sporulation ability between liquid and solid medium culture may occur due to differences in light filtration and temperature conduction by the medium as well as to changed growth habit of the fungus. Plastic petri dishes containing 5 ml of inoculated V8-calcium-sterol broth were incubated at temperatures similar to

Table 11. Chlamydospore production by Phytophthora lateralis colonies grown in V8-calcium-sterol agar at different temperatures, in darkness and in alternating light-dark periods.

Continuous darkness		12 hrs light daily	
Temperature (°C)	Chlamydospores (no.)	Temperature (°C)	Chlamydospores (no.)
9.0	0[a]	9.3	0[a]
13.0	0	13.0	0
15.3	0	15.6	0
17.8	216	18.0	29
19.9	300	20.1	92
21.9	1,232	22.2	68
24.1	1,578	24.2	0[b]
26.0	0[b]	25.9	0[b]

[a] Average number of chlamydospores in one 10 mm^2 area, from each of four colonies.

[b] No growth; lethal temperature.

Table 12. Chlamydospore production by Phytophthora lateralis in V8-calcium-sterol agar at different temperatures either in darkness, in alternating light-dark periods or under continuous illumination.

Continuous darkness		12 hrs light daily		Continuous light
Temperature (°C)	Chlamydospores (no.)	Temperature (°C)	Chlamydospores (no.)	Chlamydospores (no.)
9.4	0[a/]	10.3	0[a/]	0[a/]
12.8	4	14.1	0	0
15.2	9	16.8	4	0
17.8	43	19.9	27	8
20.0	191	22.3	127	95
22.2	449	24.2	60	0
24.3	750	26.3	0	0
26.2	0	28.1	0	0

[a/] Average number of chlamydospores in a 10.0 mm^2 area, from each of four cultures.

those in the previous experiment. At each temperature four cultures were kept continuously dark and four cultures were illuminated 12 hrs daily. The average number of spores in samples from four cultures from each temperature and illumination treatment after 11 days appear in Table 13.

As with agar medium, the optimum temperature for chlamydospore production by dark-incubated cultures in broth is about 24 C, and a great many more spores formed on dark than on illuminated colonies.

In the previous tests, sporulation was examined on cultures incubated at constant temperatures. However, many natural fungal habitats (surface soil layers, above-ground plant parts) undergo seasonal and diurnal temperature fluctuations. A further experiment tested the influence of fluctuating temperatures on chlamydospore production. Alternating temperature gradient plates were employed. Cultures were incubated for 12 hrs at one temperature, then 12 hrs at a second, simulating day-night fluctuations. This cycle was repeated daily throughout the incubation period.

Sixty-four locations were selected on each of two alternating gradient plates, each location experiencing a unique day-night temperature regime. A plastic petri dish containing 10 ml inoculated V8-calcium-sterol agar (1.7 percent agar) was placed on each location. Cultures on one gradient plate were illuminated each day for 12 hrs

Table 13. Chlamydospore production by Phytophthora lateralis in V8-calcium-sterol broth at ten temperatures, in darkness and in alternating light-dark periods.

Continuous darkness		12 hrs light daily	
Temperature (°C)	Chlamydospores (no.)	Temperature (°C)	Chlamydospores (no.)
9.0	2[a]	8.6	0[a]
(10.8)[b]	2	(10.9)[b]	1
12.6	3	13.2	1
(13.8)[b]	9	(14.6)[b]	1
14.9	7	15.9	0
17.4	60	18.4	3
19.5	203	21.3	60
21.6	326	22.8	129
23.6	388	24.6	0
25.2	91	26.6	0

[a] Average of four cultures.

[b] Derived by interpolation from adjacent thermocouple-monitored locations.

coincident with the daytime half of the temperature regime. Cultures on the other gradient plate were in continuous darkness. Figure 5 illustrates the treatments.

Outlines of colonies were periodically traced on the bottoms of the petri dishes. After 14 days incubation, chlamydospores were counted on areas of the colony 5 to 9 days old. Data appear in Figure 6.

Although high and low numbers for spore production appear to be randomly distributed throughout the different temperature cycles, examination of the spore morphology information shows a distinct temperature-related pattern (Figure 6C). Chlamydospores which formed on illuminated colonies were small, lacking in pigmentation, and were thin-walled. However, large, thick-walled spores were produced by colonies incubated in continuous darkness when either day or night temperatures were near 26 C. Although the greatest number of chlamydospores were produced at a constant 26 C, these did not develop thick walls during the incubation period of this experiment. The apparent higher numbers of thick-walled chlamydospores under fluctuating temperatures (26 C to lower temperatures) as compared with a constant 26 C may be due to an optimum temperature at some point between 23 and 26 C. This hypothetical optimum temperature would be traversed twice daily during the temperature shift from day to night but would not be met at a constant 26 C. It cannot be concluded

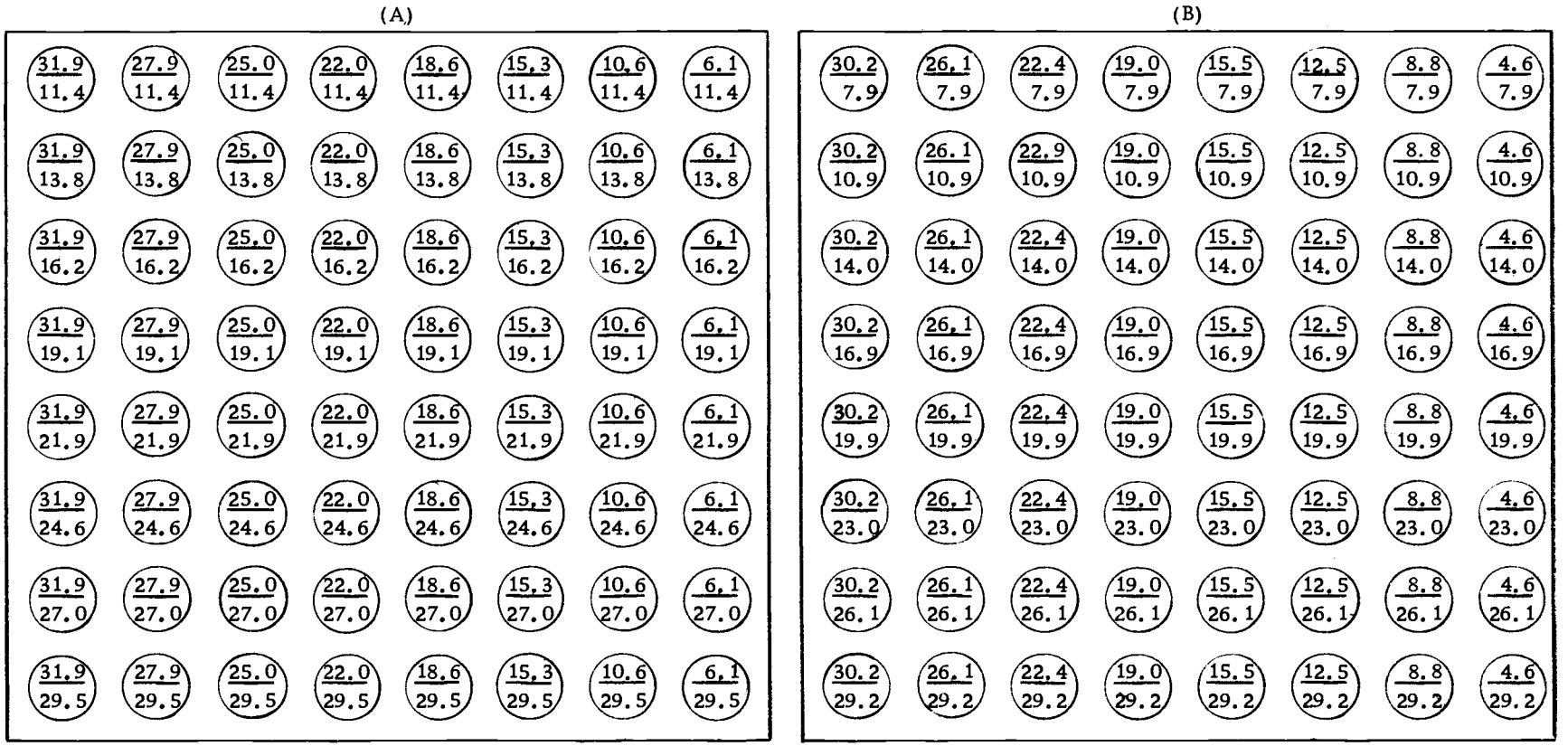

Figure 5. Temperature and illumination program on alternating gradient plates A and B. Each circle represents a single culture. Within the circle the numerator is the temperature at which the culture was incubated for the first 12 hrs daily; the denominator, the temperature during the remaining 12 hrs. Cultures on gradient plate A were illuminated during the first 12 hrs daily, kept dark the remaining 12 hrs. Cultures on gradient plate B were incubated in continuous darkness.

(A)

Night temperature (°C); dark	Day temperature (°C); illuminated							
	31.9	27.9	25.0	22.0	18.6	15.3	10.6	6.1
11.4	0[a]	0	19	124	29	10	44	0
13.8	0	0	5	42	5	7	24	0
16.2	0	0	12	20	10	0	37	0
19.1	0	0	8	7	5	2	26	4
21.9	0	0	5	49	4	0	26	9
24.6	0	0	0	99	27	48	81	20
27.0	0	0	0	26	13	46	0	0
29.5	0	0	0	0	0	0	0	0

(B)

Night temperature (°C); dark	Day temperature (°C); dark							
	30.2	26.1	22.4	19.0	15.5	12.5	8.8	4.6
7.9	0[a]	211	177	148	204	125	89	0
10.9	0	266	130	137	250	233	139	0
14.0	0	286	79	92	264	314	77	0
16.9	0	389	132	16	248	322	130	0
19.9	0	283	58	23	145	368	287	9
23.0	0	377	102	245	242	380	219	15
26.1	0	447	48	290	112	272	92	0
29.2	0	0	24	0	9	51	0	0

(C)

Day temperature (°C); dark							
30.2	26.1	22.4	19.0	15.5	12.5	8.8	4.6
	●●00	00o	00oo	0ooo	0ooo	ooo	
	●●00	00o	00o	0oo	00oo	ooo	
	●●00	00o	00o	00oo	00oo	ooo	
	●●00	00o	00o	00oo	00o	0oo	
	●●00	00o	00o	00oo	●0o	00oo	
	●●00	●000	●●o	00o	●0o	0oo	
	0000	●●o	●●o	●0o	●0o	00oo	
					oooo		

[a] Number of chlamydospores in ten microscopic fields, each field 0.5 mm^2.

[b] Relative proportion of large, thick-walled spores (●), large, thin-walled spores (0), and small spores (o) in each treatment.

Figure 6. Number of chlamydospores produced by Phytophthora lateralis colonies incubated at different day-night temperatures, illuminated as follows: (A) lighted 12 hrs daily, (B) continuous darkness. Morphology of dark grown spores at each temperature is shown in (C).

from these data that fluctuating temperatures are as important as high constant temperatures in stimulating formation of mature chlamydospores.

There are no reports in the phytophthora literature concerning inhibition of chlamydospore formation by light. In the preceding four experiments substantially fewer chlamydospores were produced on illuminated colonies than on colonies incubated in darkness, regardless of the incubation temperature or temperature cycle. This response may be peculiar to the light intensity in these experiments, 190 foot candles. The next experiment examines the number and morphology of chlamydospores produced under several light intensities. A 5.0 mm disc from the edge of a P. lateralis colony was placed in each 55 mm diameter plastic petri dish containing 5 ml V8-calcium-sterol agar. Layers of gauze were placed over three replicate cultures in each treatment to adjust light intensities to 200, 100, 50 and 25 foot candles, as measured through a petri dish cover with a Weston model 756 foot candle meter. Also, some dishes were covered with aluminum foil and served as dark controls. Continuous illumination was provided by two cool white and two near-UV (BLB) 40 watt fluorescent tubes. A uniform-temperature plate (described in the Methods section) on which the colonies were incubated maintained temperature at colony level between 24 and 25 C in all treatments.

After 14 days, chlamydospores in each colony were counted in

five microscopic fields (1.02 mm^2 each) located along the circumference of a circle having a 7 mm radius from the colony center. The greatest number of chlamydospores formed in colonies incubated in darkness (Table 14). These were mostly thick-walled, and appeared larger than those in other treatments. In comparison with these dark grown spores, chlamydospore numbers and dimensions were markedly reduced in colonies which had received 25 foot candles of light, and spore numbers and size decreased as light levels increased. No thick-walled spores were seen in colonies receiving illumination. Orange-brown chlamydospore pigmentation was most intense on dark reared colonies, was less on colonies grown under 25 to 50 foot candles, and was very pale or absent in spores formed under 100 or 200 foot candles of light.

Table 14. Chlamydospore production by Phytophthora lateralis colonies grown on V8-calcium-sterol agar at five light intensities.

Light intensity (foot candles)	Chlamydospores (no.)
0	152[a]
25	57
50	36
100	9
200	11

[a]Average number of spores in five 1.02 mm^2 microscope fields examined on each of three cultures.

Chlamydospore Germination

Preparation of Hypha-free Chlamydospores. It has been proposed that chlamydospores serve as resistant, oversummering propagules, forming in infected or infested host tissue in the late spring as moisture becomes limited and temperature approaches the upper limit for P. lateralis growth, and thereafter remaining dormant throughout the dry, warm summer to germinate at the onset of cool, wet autumn weather (Trione, 1959). The fact that chlamydospore production is stimulated by higher temperatures, up to about 24 C, as shown in the previous experiments is consistent with this proposal. Moreover, there is evidence that chlamydospores resist enzymatic lysis and mechanical disruption. When colonies containing chlamydospores were ingested by aquatic snails (Lymnaea auricularia), the subsequent snail feces primarily were composed of unbroken chlamydospores, many of which germinated shortly thereafter. This behavior led to investigation of requirements for chlamydospore germination. Studies included (1) determination of the need for an obligate, extended period of dormancy (Trione, 1957), (2) effect of exogenous nutrients, (3) effect of temperature, and (4) effect of light on germination.

First, a procedure was devised for the propagation and collection of large numbers of uninjured chlamydospores free of attached hyphae and detached hyphal fragments. Knowledge of the beneficial effects of

darkness, temperatures between 20 and 24 C, and culture in V8-calcium-sterol broth expedited culture of colonies with quantities of chlamydospores. Inoculation of media with a concentrated suspension of hyphal fragments was advantageous, causing numerous small colonies to form in each container, with a greater proportion of the fungus matter as chlamydospores than when a few large colonies comprised a culture.

The intercalary nature of the spores presented difficulties in their separation from hyphae; attached filaments, upon regrowth, could appear as germ tubes. After extensive tests of several blenders with different container sizes, liquid volume contents, blending speeds and blending times, the following method yielded the most uninjured chlamydospores with the fewest attached hyphae.

The large blade of a Servall Omnimixer was fitted with an adapter allowing attachment of a sterile glass jar (165 mm in height with standard Mason threads), containing 100 ml of sterile distilled water. Washed colonies from three or four petri dishes were placed in the jar, and the mixture was blended at 80 percent line voltage for four 5-sec periods. The suspension was rapidly poured through alcohol sterilized nylon filter cloth (Nitex; Tobler, Ernst and Traber, New York) with 80 μ mesh openings. Long hyphal filaments and clumps were trapped by the cloth. The small hyphal fragments and chlamydospores passing the filter were aseptically collected in a 125 ml flask. After 15 to 30

min, a layer of sound chlamydospores settled on the bottom of the flask, while most of the injured spores, spores with attached hyphae, and hyphal fragments remained in suspension. All liquid above the layer of settled spores was carefully drawn off with a capillary pipette connected to a vacuum pump. The procedures of suspension, settling and concentration were repeated one or two times to more effectively remove suspended fungal matter and to dilute nutrients as well as cell contents released in the spore separation process.

Effect of Incubation Medium, Temperature and Illumination.

Mircetich et al. (1968) reported that germination of chlamydospores of P. cinnamomi occurred over a wide temperature range, and the germination morphology was affected by the concentration of nutrients in the medium. I studied the germination ability and characteristics of P. lateralis chlamydospores in two solutions, at 11 temperatures, in continuous darkness and continuous light. One germination solution was half-strength V8-calcium-sterol broth plus 0.875 g L-asparagine per liter; the second germination solution was deionized water. Chlamydospore suspensions were diluted with sterile water to give 50,000 spores per ml. Equal volumes of the test medium and spore suspension were then mixed. A calibrated chromatography pipette was used to transfer 0.1 ml of this spore suspension into the sterile depression slides used as incubation chambers. One ml exactly filled the depression so that a cover slip displaced all visible air bubbles,

facilitating microscopic examination. For each germination solution, six depression slides were incubated at each of 11 temperatures between 7 and 31 C on a temperature gradient plate. Three slides at each temperature were covered to exclude light, while the other three were exposed to continuous illumination. After 24 to 48 hrs of incubation, 200 or more chlamydospores were examined on each slide, and the percent germination determined. The criterion for germination was a germ tube at least equal in length to the diameter of the chlamydospore. The type of germination including size and number of tubes per spore and presence of sporangia also was noted. Results are shown in Figure 7.

In nutrient solution, the optimum temperature (22 C) for germination of chlamydospores in the light was higher than in the dark (17 C), and more spores germinated in the light than in the dark at the respective optimum temperatures. In deionized water more spores germinated in the dark than in the light at corresponding temperatures, and this difference was most pronounced at the optimum temperature for germination in the dark (20 C).

Regardless of the germination solution, more germ tubes per chlamydospore formed at or near the optimum temperature for germination (average of five tubes per spore); most spores which germinated at suboptimal temperatures had one to three tubes. Sporangia formed, and zoospores were discharged, only on germ tubes of chlamydospores incubated in water in the dark between 14 and 21 C.

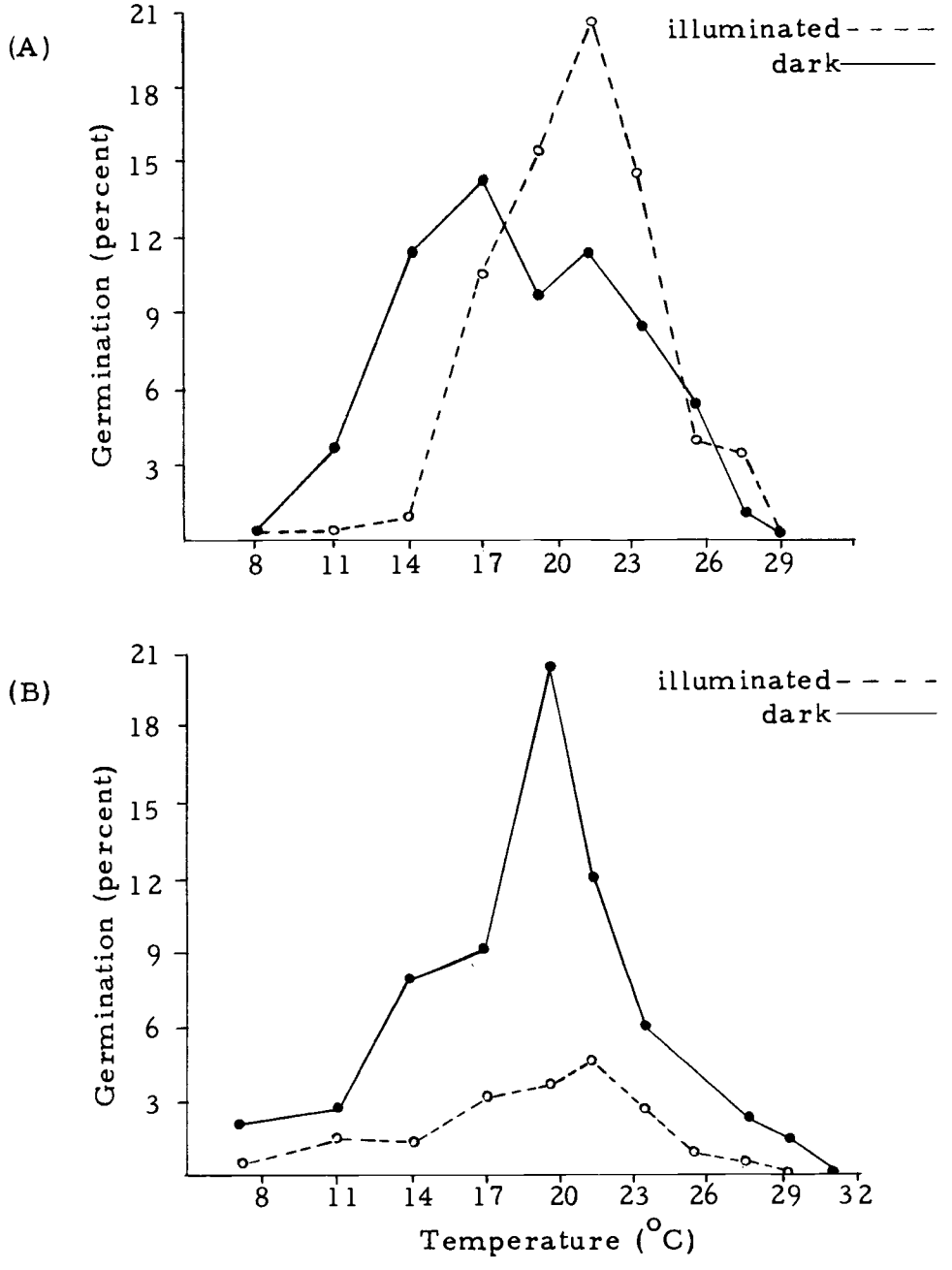

Figure 7. Germination of detached chlamydospores of Phytophthora lateralis in the light and in the dark at nine temperatures in (A) complete medium and (B) deionized water.

Sporangial Production and Discharge of Zoospores

Sporangia are considered to be important in the multiplication, dispersal and inoculation phases of the phytophthora root rot disease cycle. Large numbers of zoospores may be released from a single mature sporangium. These zoospores may be washed passively for some distance in streams and through soil, and by virtue of their motility may effect a limited movement to susceptible wounds or root tips. Evidence from other species of Phytophthora indicates that germinating zoospores are the principal, if not the only, propagules capable of infecting.

My early attempts to obtain sporangial production with P. lateralis gave very inconsistent results. An attempt to account for this variability was undertaken.

Sporangial Production

Effect of β-Sitosterol. The stimulatory effect of sterols on sporangial production of certain Phycomycetes has been firmly established during the last decade. In one of the few reports on this subject with reference to P. lateralis (Hendrix, 1965), sporangia occurred occasionally in cultures grown in a synthetic medium containing cholesterol, but only hyphal swellings developed in a similar medium lacking sterol.

I found sterols to have a more striking effect. Cultures were grown at 20 C in basal medium (Trione) both with 50 ppm β-sitosterol and without sterol. After 5 days the medium was replaced with sterile distilled water. Several days later, abundant sporangia were present on colonies from medium containing sterol while only hyphal swellings occurred on colonies in sterol-deficient medium. No zoospores were observed.

Sporangial formation also was compared in a natural medium with no sterol, or with 50 ppm cholesterol or β-sitosterol. After incubation for 5 days at 20 C in V8 broth or in V8 broth with sterol, cultures were transferred to distilled water and incubated for 2 days at 10 C. The fungus from V8 broth with no sterol formed chains of sporangia-like structures while cultures from media amended with either sterol formed numerous terminal sporangia and motile zoospores. No differences were apparent in the degree of sporulation or the morphology of sporangia produced when comparing fungus grown in media with cholesterol or β-sitosterol.

The effect of concentration of β-sitosterol on sporulation was tested. Sterol concentrations were 1, 2, 10, 20, 100 and 200 ppm. V8-calcium broth with ethanol reagent only, and V8-calcium broth only, were included as controls. With the exception of the latter medium, final concentration of 95 percent ethanol in all media was 1 percent. After the sterile media were inoculated with a hyphal

suspension, 10 ml aliquots were poured in 9 cm diameter petri dishes, and these were incubated in the dark at 20 C for 19 days. At that time the medium in each dish was replaced with sterile distilled water. The dishes were kept at 10 C for 1 week and then at about 20 C for 1 week under fluorescent room lights. Sporangia were counted in samples of the partially blended contents of two replicate cultures from each sterol concentration, as described in the Methods section. After fungal dry weights were determined, the number of sporangia per mg dry weight was computed. Data appear in Table 15. Distinctly higher numbers of sporangia formed when the basal medium contained at least 10 ppm sterol. Ethanol by itself somewhat suppressed the number of sporangia in sterol-deficient medium.

Sterol stimulation of sporangial production also was shown in an experiment testing the effect of temperature and illumination on sporangial formation (Table 16). On illuminated colonies incubated at temperatures near the optimum for sporangial production (13 to 16 C) approximately 10 times as many sporangia formed in V8 medium containing 20 ppm sterol than in V8 medium without sterol. Sterol had no influence on sporangial production in the dark.

Effect of Temperature and Illumination. Temperature dependence of sporangial formation probably is universal in fungi. Light has been reported to stimulate sporangial production in certain

Table 15. Effect of β-sitosterol and ethanol on sporangial production by Phytophthora lateralis cultures incubated first in V8-calcium broth, then in distilled water.

Amendment		Sporangia/ culture (no.)	Sporangia/ mg. dry wt. (no.)
Sterol (ppm)	95% Ethanol (10^4 ppm)		
0	-	5,625[a/]	837[a/]
0	+	937	58
1	+	8,437	497
2	+	7,500	407
10	+	53,125	2,526
20	+	41,250	1,384
100	+	40,937	1,863
200	+	39,062	1,610

[a/] Average of two cultures, four samples per culture.

Table 16. Sporangial production by Phytophthora lateralis cultures grown in V8-calcium-sterol broth at ten temperatures, in darkness and in alternating light and dark.

Continuous darkness			12 hrs light daily		
	Sporangia			Sporangia	
Temperature (° C)	+ Sterol (no.)	- Sterol (no.)	Temperature (° C)	+ Sterol (no.)	- Sterol (no.)
9.0	8[a]	1[a]	8.6	1[a]	0[a]
(10.8)[b]	54	15	(10.9)[b]	133	3
12.6	55	40	13.2	321	23
(13.8)[b]	62	70	(14.6)[b]	384	39
14.9	68	83	15.9	361	32
17.4	32	58	18.4	192	12
19.5	62	33	21.3	111	3
21.6	22	12	22.8	7	0
23.6	0	0	24.6	0	0
25.2	0	0	26.6	0	0

[a] Average of four replications.

[b] Derived by interpolation from adjacent thermocouple-monitored locations.

Phytophthora species, and to inhibit or have no effect on this process in others (Lilly, 1966). Observations in preliminary tests prompted study of the influence of temperature and light on sporangial production by P. lateralis.

Colonies grown 1 week in V8 broth were transferred to sterile distilled water and incubated at 10, 15 or 20 C. After 4 days microscopic examination showed the absence of sporangia on colonies at 20 C, while many sporangia had formed at 10 and 15 C. Some of these sporangia were devoid of contents and most likely had released zoospores. Large numbers of zoospores were observed in the water.

Most zoospores from cultures at 10 C were motile at the time of this first examination, while at 15 C there were fewer zoospores and half of these had encysted. About half of the sporangia at 15 C showed partly discharged protoplasmic masses, evidently abortive zoospores. After 13 days at 20 C many sporangia were present, some with a protoplasmic mass partially extruded, others completely empty. It is noteworthy that zoospores were observed at this temperature, although not in the high numbers occurring at lower temperatures. The highest numbers of both motile and germinating zoospores developed in the 10 C cultures.

Further tests were made on the temperature gradient plates employing several illumination levels at each temperature.

In the first test, plastic petri dishes containing 5 ml of inoculated

V8-calcium broth or V8-calcium-sterol broth were incubated at 10 temperatures and two light regimes (continuous darkness and 12 hrs illumination daily). In each medium four replicate cultures were grown at each temperature and light regime. A diagram of temperature and illumination treatments is shown in Figure 4. After 11 days sporangia were counted in samples from partially blended cultures. Treatment means of the four replications appear in Table 16. The highest yields of sporangia developed on cultures incubated between 13 and 16 C in the light. Temperature optima were not clearly defined on dark cultures. At temperatures between 13 and 16 C, sporangial numbers were approximately five to six times higher on illuminated cultures than on dark-incubated cultures. The effect of sterol is discussed in the previous section.

In a similar experiment, the effect of temperature and light was studied in agar culture. Plastic petri dishes with 5 ml of V8-calcium-sterol agar (1.7 percent agar) were each inoculated with a disc of P. lateralis hyphae. At each of eight temperatures, four colonies were incubated in continuous darkness, four in intermittent light (12 hrs illumination daily), and four were continuously illuminated. Sporangia were counted after 11 days (Table 17). The optimum temperature for sporangial production by colonies receiving intermittent light was 14 C. Even at optimum temperature for sporangial production under continuous illumination (10 C), colonies contained somewhat fewer spores than

Table 17. Sporangial production by Phytophthora lateralis on V8-calcium-sterol agar at different temperatures either in continuous darkness, alternating light and dark, or continuous light.

Continuous darkness		12 hrs light daily		Continuous light
Temperature (° C)	Sporangia (no.)	Temperature (° C)	Sporangia (no.)	Sporangia (no.)
9.4	9[a/]	10.3	189[a/]	192[a/]
12.8	6	14.1	267	119
15.2	5	16.8	91	45
17.8	22	19.9	55	15
20.0	76	22.3	0	0
22.2	4	24.2	0	0
24.3	0	26.3	0	0
26.2	0	28.1	0	0

[a/] Average number of sporangia in a 10.0 mm^2 area, from each of four cultures.

colonies under intermittent illumination at 14 C. Much lower numbers of spores were produced on dark grown colonies at the apparent optimum temperature (20 C).

While we have shown intermittent light to increase sporangial production over that in total darkness, fluctuating daily temperatures might be involved in the increase. This was studied by exposing cultures to alternating temperatures at 12 hr intervals. The first test was made before the benefits of sterol and of alternating light were known.

A temperature gradient plate providing 10 incubation temperatures between 15 and 30 C for the first 12 hrs each day later provided 12 hrs of 16 C throughout the plate. Plastic petri dishes containing V8 agar (lacking sterol) were inoculated and placed on the plate. After 13 days incubation, sporangia were counted in 9 to 15 microscope fields (each field 1.5 mm in diameter) on colonies from each temperature. Average numbers of sporangia per microscope field at each temperature are shown in Table 18. The relatively constant combination of 15/16 C yielded more sporangia than any other.

Another test used the wider range of diurnal temperature cycles obtainable from the alternating gradient plates and employed sterol in the medium.

Plastic petri dishes containing 10 ml inoculated V8-calcium-sterol agar (1.7 percent agar) were placed on 64 locations on each of

Table 18. The effect of alternating daily temperature on the number of sporangia produced by continuously illuminated colonies of Phytophthora lateralis growing on V8 agar.

Temperature		
A. M. (° C)	P. M. (° C)	Sporangia (no.)
15.0	16.0	88[a/]
18.0	16.0	63
20.0	16.0	43
22.0	16.0	27
24.0	16.0	10
25.0	16.0	12
25.5	16.0	8
26.5	16.0	0
28.0	16.0	0
30.0	16.0	0

[a/] Sporangia counted in a 1.5 mm diameter microscopic field, average of nine to fifteen fields per treatment.

two alternating gradient plates, each culture experiencing a unique day-night temperature cycle. Cultures on one gradient plate were illuminated each day for 12 hrs coincident with the 'day' temperature period. Cultures on the other gradient plate were incubated in continuous darkness. Figure 5 illustrates the treatments.

Outlines of colonies were periodically traced on the bottoms of the petri dishes. After 14 days, sporangia were counted on areas which were 5 to 9 days old. Data appear in Figure 8. Alternating temperature failed to stimulate sporangial production with either light routine.

Discharge of Zoospores

Effect of Temperature and Illumination. Indirect germination of sporangia of some species of Phytophthora is quite temperature dependent. A test was made to determine temperatures affecting discharge of zoospores of P. lateralis. The effect of light was also noted. Large numbers of sporangia were produced on cultures grown in V8-calcium-sterol broth for 8 days at 20 C under intermittent illumination. Zoospore discharge was induced by drawing off the medium and flooding the colonies with sterile deionized water. This was done three times within 4 hrs. Then 7.5 ml deionized water was pipetted into each glass petri dish containing the cultures, and these were incubated at different temperatures on a gradient plate. Half the cultures were

(A)

Night temperature (°C); dark	Day temperature (°C); illuminated							
	31.9	27.9	25.0	22.0	18.6	15.3	10.6	6.1
11.4	0[a]	0	0	11	27	7	0	0
13.8	0	0	2	81	42	106	2	2
16.2	0	0	0	134	333	126	2	0
19.1	0	0	1	57	261	86	6	0
21.9	0	0	3	4	103	77	11	0
24.6	0	0	0	0	40	25	0	0
27.0	0	0	0	0	0	2	0	0
29.5	0	0	0	0	0	0	0	0

(B)

Night temperature (°C); dark	Day temperature (°C); dark							
	30.2	26.1	22.4	19.0	15.5	12.5	8.8	4.6
7.9	0[a]	0	1	17	11	7	0	0
10.9	0	0	1	25	48	18	2	4
14.0	0	0	49	243	59	36	9	0
16.9	0	9	77	45	281	57	13	0
19.9	0	0	65	56	249	67	16	0
23.0	0	0	8	38	188	38	11	0
26.1	0	0	0	4	8	3	17	0
29.2	0	0	0	0	0	0	0	0

[a]Number of sporangia in ten microscopic fields, each field 0.5 mm^2

Figure 8. Sporangial production by Phytophthora lateralis incubated at different day-night temperature combinations in (A) intermittent light, and (B) continuous darkness.

continuously illuminated, half were kept dark. After 60 hrs zoospores were separated from the original cultures as follows. Each culture solution (containing zoospores) was poured into a clean dish. The mycelium was washed forcefully in a stream of water to dislodge additional spores trapped in the hyphae. This wash water was added to the dish with the zoospore suspension. Zoospores were killed by adding 1 ml of 1:1000 mercuric chloride to each dish. The killed zoospores uniformly precipitated on the bottom of the dish. Spores were counted in ten 1 mm^2 microscopic fields in each treatment. The results of these counts appear in Figure 9.

The highest numbers of zoospores were liberated from sporangia incubated between 12 and 14 C. Almost twice as many were discharged when incubation was in the dark. The temperature range over which zoospores were discharged is quite broad, indicating that these spores may be released at any temperature allowing sporangial formation.

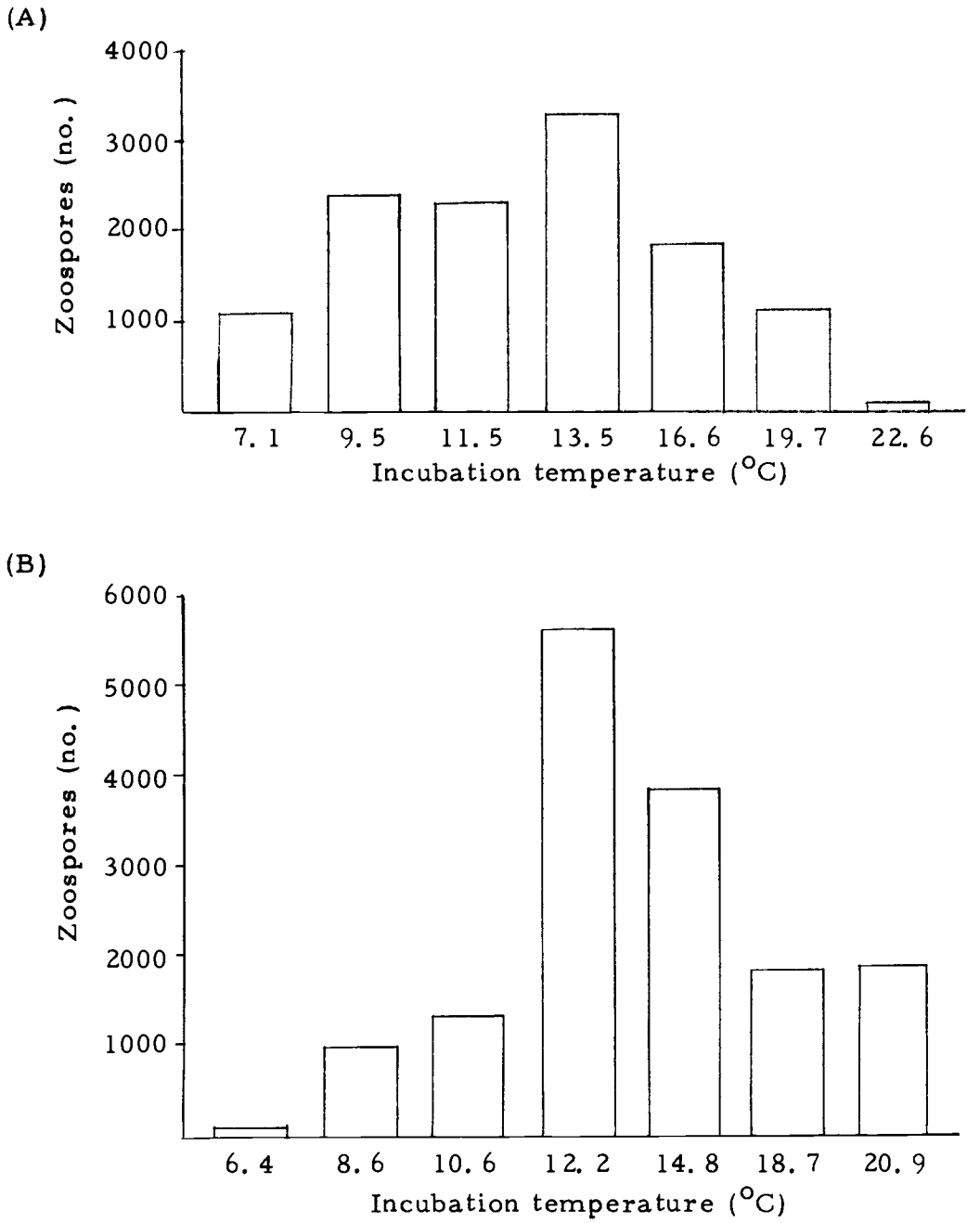

Figure 9. Zoospores released by sporangia of Phytophthora lateralis incubated in deionized water at seven temperatures; (A) under continuous illumination, (B) continuously in the dark.

DISCUSSION

The literature contains a limited amount of information about the physiology of growth and reproduction of Phytophthora lateralis. While the voluminous literature on Phycomycetes contains information useful in the culture of P. lateralis, much of this is not directly applicable. The intention of this study was to define optimum cultural conditions for production of hyphae and spores and to learn the germination requirements of these spores. This knowledge will enable the development of consistently effective laboratory techniques for inoculum production. Hopefully, it will aid our understanding of the relationship of the chemical and physical factors studied to the biology of P. lateralis in the cedar forests.

A beneficial effect on growth of many fungi can be achieved by culture in moving media. Culture agitation may increase growth by replenishing nutrients, by decreasing the concentration of staling products adjacent to the hyphae, by increasing surface area between the medium and the atmosphere (i. e., increasing gaseous exchange), and by physically fragmenting colonies to accomplish formation of new colonies. Experiments show that growth (dry weight) of P. lateralis in liquid media was increased by shaking (Tables 1 and 2). In 21 days of shake culture, about six times as much growth developed in shaken cultures as in those kept stationary. Phytophthora studies by several

investigators showed that although shaking increases growth over stationary controls, greater growth is obtained if shaking is begun after 2 or 3 days of still culture. I found that a rest period prior to shaking benefited growth of P. lateralis (Table 2). Cultures incubated stationary for 3 days prior to shaking produced maximum dry weight.

P. lateralis does not form typical colonies on conventional, rather violent, laboratory shakers. Possibly this results from injuries made critical by the aseptate nature of the hyphae. Where I used a wrist action shaker with speed adjusted to the minimum providing what was judged as good mixing, growth was atypical, consisting of numerous, small, extremely dense colonies. Incubation on a commercial tissue culture Roller-drum provided the increased growth rates associated with moving media, and allowed morphologically normal development.

My observations indicate that colony fragmentation and spore dispersal are not responsible for the increased growth of P. lateralis found in shake culture, at least not in the Roller-drum type of culture, since each culture tube invariably contained a single colony, which retained its morphological integrity throughout incubation. The possibility that sterols may float, and thus be more available in shake culture to the otherwise submerged fungus, was rejected experimentally; I determined that sterols are honogeneously distributed when added as an ethanol solution to aqueous media. Increased availability

of other nutrients due to shake culture was not tested. In still culture, nutrients may become depleted at the hyphal surfaces, since replenishment depends on diffusion from other areas in the medium (a relatively slow process, compared to homogeneous distribution in shake culture). The removal of excess volatile inhibitors such as carbon dioxide and ammonia as a cause of increased growth in shake culture was not tested specifically, although the better growth realized in sealed, shaken culture vessels compared to the growth in aerated vessels (Table 3) tends to discredit this hypothesis. Still, the fact that phytophthoras produce ammonia in vitro (Leal et al., 1970), and that a species of Phytophthora was inhibited by low concentrations of ammonia (Gilpatrick, 1969) indicates that further work be done before ammonia dissipation is disqualified as a factor in increasing growth in shake culture.

High growth rates of P. lateralis in shake culture were consistently associated with an increasingly basic reaction of the medium as growth progressed, typically to greater than pH 7 from an initial pH of about 4.6 (Tables 1 and 2). The comparatively slow growth rates of stationary controls were marked by an increasingly acidic reaction of the medium to about pH 4. The hypothesis was proposed that a basic reaction is more favorable to growth, and that as the pH of the medium is made more basic by fungus metabolism, the growth rate increases, limited only by eventual depletion of the nutrients.

This hypothesis was tested by examining growth of P. lateralis cultures on media with initial acidity levels between pH 4.5 and 7.0. Data in Tables 5 and 6 show that P. lateralis grows best on an acidic medium, with the optimum pH near 4.5 or 5.5. There was essentially no growth at pH 6.5 or 7.0. Cameron and Milbrath (1965) found that P. lateralis grew poorly at pH 4.5, a fact which contrasts with my results; this difference may be due to their use of a synthetic medium, whereas I used a natural medium fortified with sterols. They determined the optimum initial pH for P. lateralis was 5.5, with less growth developing at 6.5 and 7.0. Their data and mine show that a more basic pH is not conducive to good growth. This information refutes the hypothesis that the fungus grows better as it causes the reaction of the culture medium to become more basic. It appears that high pH associated with high growth rates in shake cultures limits, rather than stimulates, further growth. Media buffered in the acid range were used to determine whether higher growth rates could be realized by controlling the pH. The buffer systems which were used, phosphate and citrate-phosphate, proved to be fungistatic even at low concentrations (0.001 M). Further work requires the determination and use of a biologically inert buffer system which is effective at about pH 4.5 to 5.5, or development of a replacement culture technique for P. lateralis.

The influence of the overall concentration of nutrients in culture

media on sporangial formation remains a controversial issue. The Literature Review contains reports supporting and refuting the sporulation requirement of replacing nutrients with water (or salt solution) for different phytophthoras. It is my feeling that some form of nutrient deprivation, with a concomitant reduction in the growth phase, occurs in leached mycelia as well as in mycelia kept in nutritionally depleted media. I found that P. lateralis produces abundant sporangia in culture media, and the fungus was not separated from the culture medium in most of the sporulation experiments reported in this study. However, before sporangia were counted, colonies had developed for 11 to 14 days in petri dishes only 55 mm in diameter. As long as accumulation of staling products is not a factor in inhibiting sporulation, nutrient depletion and nutrient removal should be expected to elicit a similar response, namely sporangial formation.

Concerning calcium requirements in fungi, Steinberg (1948, p. 423) states, "Conclusions concerning indispensability of chemical elements, based on growth levels well within the range of extreme starvation, have been made and even generally accepted." In my tests for a growth requirement for calcium by P. lateralis, synthetic media were used to allow formulations with known amounts of calcium. P. lateralis does not grow well on synthetic media, and the required long incubation periods, coupled with the low dry weights, approach Steinberg's range of starvation (Table 8; Figure 3). Nevertheless, the

data show that calcium concentrations of about 10^{-4} to 10^{-3} M stimulate growth of P. lateralis (Table 8), and that in a synthetic medium with sterols 10^{-1} M calcium inhibited growth (Figure 3A). In synthetic medium with sterol, the optimum calcium concentration was clearly 10^{-3} M, whereas in similar medium without sterol the optimum calcium concentration was not distinct. There was more than 50 mg dry weight growth after 24 days in media with both 10^{-3} M calcium and β-sitosterol. This growth exceeds the sum of growth in medium with sterol but no calcium (Figure 3A) and medium with 10^{-3} M calcium but no sterol (Figure 3B). Evidently there was a synergistic interaction between calcium and β-sitosterol (or the sterol solvent, discussed below). Hendrix and Guttman (1970) report that P. parasitica var. nicotianae was stimulated synergistically by calcium and sterol in glucose-nitrate synthetic medium. Hendrix et al. (1969) reported a calcium inhibition phenomenon similar to that expressed by P. lateralis (Figure 3). Dry weight growth in medium with sterol and 10^{-1} M calcium was less than in the calcium free control. In sterol free medium with 10^{-1} M calcium, growth was less than at the optimum calcium concentration, but more than in calcium free medium.

In my tests, calcium was supplied as calcium chloride, since the high solubility of this salt allowed preparation of a wide range of concentrations. After tests showed that 10^{-3} M calcium provided optimum growth of P. lateralis, I routinely used 10^{-3} M calcium carbonate

in media since this salt is completely soluble at room temperature at this concentration, and is the form of calcium used in most culture media. Evidence cited in the Literature Review showed that anions of calcium salts had no effect on growth of phytophthoras. Although I did not test the effect on growth of different calcium concentrations in V8-sterol medium, a very low inherent calcium concentration was recently shown for this medium (Hylin et al., 1970). Thus, addition of 10^{-3} M calcium would not raise the calcium concentration to 10^{-2} M, which might possibly be inhibitory.

Sterols increase growth and sporulation of some phytophthoras (Hendrix, 1965). I found dry weight growth of P. lateralis was essentially unaffected by concentrations of β-sitosterol between 0 and 200 ppm (Table 7). However, a striking stimulation by the sterol solvent, ethanol, was discovered. Nearly twice as much total growth developed in medium with ethanol than in control medium with no ethanol. This may be due to utilization of ethanol carbon, or to a solvent effect of the ethanol on some otherwise less available nutrient in the medium. Ethanol, at approximately the same concentration as in my test, stimulated growth of Phytophthora hevea (Leal et al., 1968). These workers showed that the fungus metabolized the ethanol.

Chlamydospore production by P. lateralis was stimulated by sterols. The optimum concentration of β-sitosterol was 20 ppm (Table 9). These data show that the sterol solvent, ethanol, only slightly

depressed the number of chlamydospores in each culture, but since growth was stimulated by ethanol, chlamydospore density (spores per mg dry weight) appears greatly reduced in the ethanol control. Hendrix (1965) reported that sterols were required for chlamydospore formation by P. lateralis in a synthetic medium. My data show that, although not an absolute requirement for sporulation in a natural medium, sterol greatly stimulates chlamydospore production by P. lateralis.

Sporangial production by P. lateralis growing in a natural medium was stimulated by sterol, with maximum production of spores in media containing at least 10 ppm β-sitosterol (Table 15). Ethanol inhibited formation of sporangia more than it did chlamydospores, but inhibition was more than compensated for by a sterol concentration as low as 1 ppm.

It is significant that even in V8-calcium media, considered one of the best substrates for growth and sporulation of Phycomycetes (Ho and Hickman, 1967; Kennedy and Erwin, 1961; Tsao, 1969), the addition of sterol greatly increased chlamydospore and sporangial production by P. lateralis. The fact that no spores formed in sterol free synthetic medium, and that some spores formed in V8-calcium medium to which no sterols were added (Tables 9 and 15), suggests that this latter, natural medium contains some sterols. However, the stimulation of chlamydospore production by relatively low amounts of

added sterol (1 and 2 ppm) indicate that the quantity of sterol which is inherent in V8 media is very low (Table 9).

Temperature and light influence sporulation of P. lateralis. In both agar and broth media, sporangia formed in the highest numbers at 14 to 15 C on cultures illuminated 12 hrs each day (Tables 16 and 17). In media containing sterol, an alternating light-dark routine increased sporangial production over that of dark controls (Table 16). In a similar medium, but without β-sitosterol, illuminated cultures produced very few sporangia at any temperature tested.

Obviously, there is an interaction between light and sterol in respect to sporangial formation (Table 16). Further work is needed to clarify whether light affects the sterol before or after it is incorporated into the fungus. If sporulation stimulation is the result of a photochemical reaction between light and sterol, then it would be advantageous to determine the reaction product for use as a sporulation stimulus, thus replacing the light requirement. There is some foundation in the literature for these lines of speculation. Certain sterols are converted to D vitamins when treated with light (Baniecki and Bloss, 1969; Rosenheim and Webster, 1927). Leach (1965) isolated a substance which replaced the light requirement for sporulation of several fungi. This substance, formed in the mycelium (and not the medium) after exposure to light. When this substance was extracted and applied to dark grown, non sporulating cultures, these sporulated.

Data in Table 17 show that colonies which are continuously illuminated produce fewer sporangia than those which are illuminated 12 hrs daily. However, the lowest incubation temperature of illuminated colonies tested (10 C) was not low enough to determine the optimum temperature for sporangial production by continuously illuminated colonies. Thus, before conclusions on the effect of photoperiod may be drawn, further tests must be done with the range of temperatures extended below 10 C. Also, stimulation of sporulation may be a response to a total dosage of light, rather than periodicity of illumination *per se*. Further comparisons of sporulation under continuous and alternating light should include different light intensities as treatments.

Chlamydospores of *P. lateralis* are produced almost exclusively between 20 and 24 C, with most spores developing at about 24 C (Tables 11, 12 and 13). Spore morphology data (Figure 6C) show that chlamydospores which formed at cooler temperatures are smaller and lack the thick walls characteristic of spores formed above 20 C. It is significant that 24 C was both the optimum temperature for chlamydospore production and the maximum temperature for linear and dry weight growth. This indicates that the fungus produces chlamydospores as a survival mechanism in response to the approach of temperatures sufficiently high to cause cessation of growth and possibly death of hyphae. Although it has not yet been tested, chlamydospores most

likely survive exposure to temperatures sufficiently high as to be lethal to hyphae. Chlamydospore formation at high temperatures supports Trione's (1959) hypothesis that chlamydospores are an important means of oversummering for this fungus.

Light consistently inhibited chlamydospore formation in my experiments. This is shown by the data in Figure 6 and Tables 11, 12 and 13. Light intensity as low as 25 foot candles inhibits chlamydospore production by almost two-thirds, as compared to the dark control (Table 14). Light inhibition of chlamydospores may or may not play a role in the life cycle of P. lateralis in its principally subterranean habitat. However, the significance of this previously unreported inhibition phenomenon to culture identification and in vitro physiological studies is apparent.

Studies on the effect of temperature, light and nutrition on germination of chlamydospores of P. lateralis did not reveal any particularly exacting germination requirements. Chlamydospores of P. lateralis germinated best between 14 and 22 C. In deionized water, many spores which germinated within this temperature range formed functional sporangia on the germ tubes. Chlamydospores were capable of germinating over temperatures ranging from below 11 C to above 26 C (Figure 7). Their ability to germinate at temperatures lethal to growth is interesting. (Trione (1957) reported a somewhat similar phenomenon in that P. lateralis zoospores germinated and

infected foliage at a temperature exceeding the upper limit for mycelial growth.)

Illuminated chlamydospores germinated poorly in deionized water (Figure 7B), yet germinated well in a complete (V8-calcium-sterol-asparagine) nutrient medium (Figure 7A). Although any number of factors may have been responsible for this differential in germination ability, future studies should consider the possibility of an interaction between light and sterol, similar to the phenomenon noted for sporangial formation.

Trione (1957) observed chlamydospore germination infrequently, and then only after an obligatory dormant period at low temperature. In my studies chlamydospores germinated within 48 hrs, without any dormancy period. Possibly, chlamydospores in my studies were more viable since they were produced at optimum conditions of high temperature and darkness, and in media fortified with sterols. Possibly, Trione's chlamydospore germination studies were made in situ (i. e., on spores still attached to the mycelium within a colony). If a germination inhibitor is present in colonies, my method of separating spores from the hyphae and washing several times with water would have removed this dormancy factor. If we extend to chlamydospores a light phenomenon reported for oospore germination of other Phytophthora species, another explanation may be offered for lack of chlamydospore dormancy in my study. The Literature Review contains reports

that show oospore formation generally is inhibited by light (this is consistent with the inhibition of P. lateralis chlamydospore formation by light). Germination of dark grown oospores required light (Berg and Gallegly, 1966; Henry and Stelfox, 1968; Leal and Gomez-Miranda, 1965). However, there was no requirement for light during germination as long as light had been applied to the oospores during formation or maturation, such as by short exposure, insufficient to inhibit spore formation (Klisiewicz, 1970). Relating this to my results, although P. lateralis chlamydospores were produced in darkness, the process of separating these spores from the hyphae was done in the light. Thus, a germination requirement for light may have been met at that time. This is especially feasible when one considers the short exposure (10 min) to light which satisfied the germination requirements of P. drechsleri oospores (Klisiewicz, 1970).

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