Nutritional Factors Affecting Responses of Sporangia of *Pythium ultimum* to Germination Stimulants

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ABSTRACT

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Our study was designed to examine nutritional factors regulating germination of *Pythium ultimum* sporangia in response to glucose and seed-exudate stimulants. As mycelial cultures aged on media containing soybean lecithin, glucose-responsive germination of sporangia produced from these mycelia increased from less than 5% at 4 days to nearly 100% at 14 days. Responses of sporangia to cotton and cucumber seed exudate also increased during the 14-day period. Mycelial cultures growing on living plant tissue gave rise to sporangia unresponsive to glucose or cotton seed exudate after 14 days. By 18 days, these sporangia were sensitive only to seed exudate. Growth of mycelial cultures on media containing decreasing levels of various nutrients gave rise to sporangia with increasing sensitivity to seed exudate. Germination of sporangia in response to glucose remained low regardless of the concentration of most nutrients in the medium. Decreasing levels of lecithin in the medium, however, increased the response of sporangia to glucose. As sporangia aged from 4 to 15 days, germination responses to glucose, as well as to both cucumber and cotton seed exudates, increased. Results suggest that nutrient deprivation can qualitatively and quantitatively affect responses of sporangia to germination stimuli.

Additional keywords: fatty acids, lipids, soilborne pathogens, spermosphere.

Germination of oospores and sporangia of soilborne *Pythium* species represents a key step in the interaction of these pathogens with their host plants. In nearly all cases, sporangia and oospores of seed- and root-infecting *Pythium* species germinate only after being stimulated by plant-derived molecules present in seed and root exudates (32). Although the exact nature of the stimulatory molecules has never been identified, research conducted during the 1960s and 1970s indicates that sugars and amino acids are the likely stimulatory molecules responsible for propagule germination in spermosphere and rhizosphere habitats (1–4,10,15,27, 28,32,39,42,43).

We demonstrated previously that the sensitivity of *Pythium ultimum* Trow sporangium germination to different stimulants was heavily dependant on the composition of the culture medium on which the sporangium-bearing mycelial cultures were grown (33). Sporangia derived from cultures grown on common synthetic culture media germinated readily in response to various individual and combined sugars and amino acids commonly found in seed exudates. In contrast, sporangia produced on metabolically active plant tissue (diseased seeds and radicles) or on a mineral salts medium supplemented with soybean lecithin failed to respond to these same molecules. Sporangia produced on all media remained fully germinant in response to unfractionated seed exudates, however.

Such results have raised questions about the identity of molecules that initiate *Pythium*-seed interactions in soil and about the physiological and biochemical factors affecting responses of sporangia to various stimulants. The previous results suggested an important role for nutritional regulation in the germination of *P. ultimum* sporangia and, in part, the virulence of *P. ultimum* populations. A number of studies have shown that nutritional factors can affect the virulence of soilborne fungal propagules (30,36,46,47), but the mechanisms by which nutrition affects pathogen responses to plants are not understood. The present study was initiated to understand more clearly how cultural and nutritional factors regulate qualitative and quantitative responses of sporangia of *P. ultimum* to germination stimulants.

MATERIALS AND METHODS

Culture of *P. ultimum* and preparation of sporangia. *P. ultimum* isolate P4 was used throughout the study and has been described previously (33). This particular isolate was chosen because of its prolific production of sporangia; oogonia were only rarely formed under the culture conditions of this study. Cultures were maintained at 18–21 C on a wheat leaf-water medium (40). Prior to use in experiments, cultures were removed from the wheat leaf-water medium and plated onto 2% water agar (WA) containing penicillin G and rifampicin at 50 μg/ml each (medium designated as WARP) to reduce potential problems with bacterial contaminants. After 2–4 days of growth at 27 C on WARP, cultures were transferred to a sporangium-production media. In some experiments, cultures were transferred to WA, cornmeal agar (CMA), or potato-dextrose agar (PDA) before transfer to a sporangium-production medium.

A defined mineral salts medium (SM+L) (33) was used in most experiments to produce sporangia of *P. ultimum*. The medium contained the following ingredients per liter of distilled water: 180 mg of D-glucose, 1.3 mg of L-asparagine, 1 g of soy lecithin (as α-phosphatidyl choline; Sigma Chemical Co., St. Louis, Cat P-5638), 5.3 mg of $(NH_4)_2SO_4$, 2.4 mg of $MgSO_4 \cdot 7H_2O$, 1.1 mg of $CaCl_2$, 3.1 mg of K_2HPO_4, 1.6 mg of KH_2PO_4, 1.7 μg of thiamine HCl, and 30 g of agar. In some experiments, this medium also was used without the addition of soybean lecithin (medium designated as SM). Sporangia also were produced on 3% WA amended with surface-disinfested seeds of cotton (*Gossypium hirsutum* L. 'Acala SJ-2') (designated as WCS medium). Prior to transfer to the molten agar, seeds were surface-disinfested by soaking for 10 min in a 0.05% solution of sodium hypochlorite containing one to two drops of Tween 20 as a wetting agent.

Mycelial cultures of *P. ultimum* were grown at 27 C for between 4 and 18 days. With the aid of a cork borer, disks (5 mm diameter) were cut from the colony of *P. ultimum* growing on all synthetic media and from the colony area around the periphery of rotted

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seeds in the WCS medium. Sporangia were produced from mycelial cultures as described previously (33). Briefly, colonized agar disks were placed in sterile petri plates and leached for two consecutive 10-min periods in approximately 20 ml of a leaching buffer (pH 5.8) containing 0.01 M $Ca(NO_3)_2 \cdot 4H_2O$, 0.004 M $MgSO_4 7H_2O$, and 0.005 M KNO_3 (11). Buffer was replaced with fresh buffer after each 10-min leaching period. Finally, disks were leached for 3 h, after which the buffer was removed and disks were rinsed with sterile distilled water and incubated overnight at 24 C. During the leaching process, plates containing disks were wrapped in aluminum foil to exclude light. Sporangia used in all experiments were formed at 24 C. This leaching procedure resulted in a rapid, synchronous formation of sporangia, and, with the exception of sporangium aging experiments, all sporangia evaluated were of the same age. In experiments designed to test the relationships between sporangium age and responses to stimulants, sporangial disks were left sealed in petri plates for 1–18 days after leaching solution was removed.

Collection and preparation of seed exudates. Seeds of cotton (*G. hirsutum* cv. Acala SJ-2) and cucumber (*Cucumis sativus* L. 'Marketmore 76') harvested during 1991 and 1992 were sorted to remove damaged and deformed seeds, and seeds of good quality were surface-disinfested as previously described. A total of 48 g of seeds (~400 cotton seeds or 1,825 cucumber seeds) was added to flasks containing 400 ml of sterile distilled water and placed on a rotary shaker at 27 C. After 4 h, flask contents were filtered through Whatman no. 1 filter paper to remove seeds and large particulates, and the exudate was passed sequentially through 0.8- and 0.2-μm polysulfone membrane filters. Prior to the filtration of the cucumber seed exudates, samples were centrifuged (10,000 *g* for 10 min) to remove a viscous colloidal material that formed during the collection process. All filtrates were evaporated under vacuum at 40 C to a volume of ~3 ml and then were transferred to small preweighed test tubes and freeze-dried. Exudate residues were weighed and reconstituted at various concentrations ranging from 0.5 to 20 mg/ml in a 10 mM ammonium acetate buffer (pH 5.5) and passed through a 0.2-μm filter before use in germination assays. During the course of the study, several batches of seed exudate were prepared. Each batch, therefore, was tested to determine its specific activity in stimulating 1-day-old sporangia produced from 4-day-old SM+L mycelial cultures of *P. ultimum* prior to use in experiments. The comparative specific activities of batches of cotton and cucumber seed exudate are presented in Figure 1.

Germination assays. In addition to responses of sporangia to seed exudates, 1 mM D-glucose was used in some experiments to assess germination responses. Glucose also was routinely included in some germination experiments to ensure that sporangia produced from cultures growing on a lecithin- or cotton seed-containing medium were not sensitive to this stimulant.

For germination assays, leached disks containing sporangia were placed on sterile glass slides (three disks per slide). Aliquots (10 μl) of the appropriate concentration of exudate or glucose solution were then added to each disk, and cultures were sealed and incubated at 24 C. Within 30 min, solutions were absorbed into the sporangial disks. After 3–5 h, sporangium germination was assessed by staining disks with 0.03% acid fuchsin in 85% lactic acid, examining microscopically (×400), and counting the number of germinated and nongerminated sporangia. All sporangia within a random 6.5-mm^2 area of the disk were assessed for germination on each replicate disk, for a total of 30–700 sporangia counted per treatment. Sporangia were considered germinated if a developing germ tube was visible.

Experimental design and data analysis. Experiments were established as a randomized complete block design with each experimental run as a block. All experimental runs contained at least three replicate blocks, and all experiments were performed at least three times. Percent data were transformed (arcsine of square roots) prior to analysis of variance. Means were separated using the least significant difference test. EC_{50} values (effective concentration [micrograms of exudate per sporangial disk] to induce 50% germinated sporangia) were used to express the sensitivity of sporangia grown under various culture conditions to seed-exudate stimulants. EC_{50} values were calculated from log-probit plots of exudate concentration (20–200 μg per disk) versus percent germinated sporangia from different batches of exudate and from sporangia produced from mycelia of different ages produced in different nutritional environments. Chi-square values were used to estimate goodness-of-fit for the EC_{50} estimates. All tabular and graphic data represent results from one representative experiment.

RESULTS

General responses of sporangia to germination stimulants. Regardless of the culture conditions, sporangia of *P. ultimum* isolate P4 were generally highly sensitive to both cotton and cucumber seed exudates. The absolute level of germination response varied, however, not only among the different batches of seed and seed exudates (Fig. 1), but also among experiments. EC_{50} values for different batches of seed exudate (based on responses of sporangia derived from 4-day-old SM+L mycelial cultures) varied widely: ranging from 15 to 200 and from 135 to 750 μg per disk for cotton and cucumber seed exudates, respec-

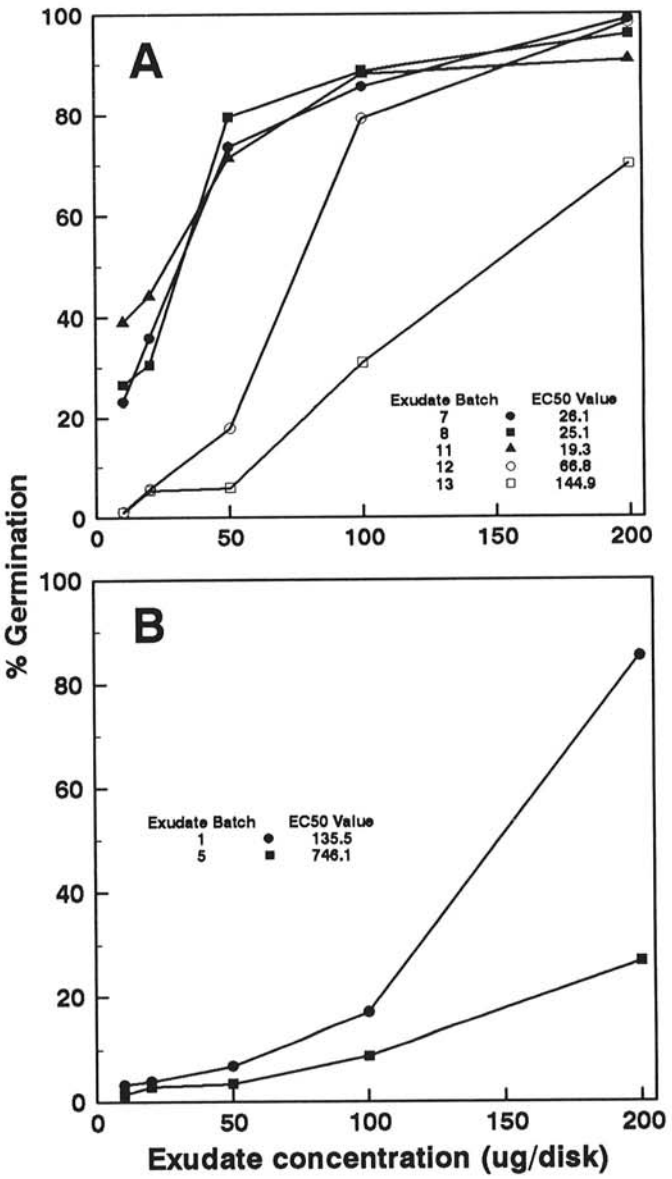

Fig. 1. Relationships between exudate dosage and germination response of 4-day-old mineral salts medium plus lecithin-produced sporangia of *Pythium ultimum* for **A,** batches of cotton seed exudate and **B,** batches of cucumber seed exudate. Specific activity of exudates expressed as EC_{50} values.

tively. Despite variations in absolute levels of germination among different batches of exudate, the general pattern of sporangial responses to stimulants under specific culture conditions or aging treatments (detailed below) was remarkably consistent.

Responses of sporangia to D-glucose were quite consistent from experiment to experiment. Sporangia derived from 4- or 5-day-old SM+L mycelial cultures were never responsive to D-glucose at concentrations of 2 mM or less. However, the age of the culture from which sporangia were derived and the age of sporangia greatly affected these responses, as well as other more specific culture conditions (described below).

The fact that the numbers of sporangia produced on the SM+L medium were considerably greater (>10% more in many experiments) than for those produced on the SM medium or on other culture media is important to note.

Effect of culture age on responses of sporangia to germination stimulants. Responses of *P. ultimum* sporangia to both cotton and cucumber seed exudates were markedly affected by the age of the mycelial culture from which sporangia were derived. As mycelial cultures on SM+L aged from 4 to 14 days, germination of resulting sporangia became much more sensitive to seed-exudate stimulants. EC_{50} values decreased from 141.3 to 4.0 μg per disk for cucumber seed exudate (>35×) and from 66.2 to <1.0 μg per disk for cotton seed exudate (>66×) (Table 1). Regression coefficients for linear regressions of EC_{50} values over culture age for each of the exudate sources were −13.1 ($R^2 = 0.81$) for cucumber and −5.5 ($R^2 = 0.70$) for cotton seed exudate.

TABLE 1. Response of sporangia of *Pythium ultimum* (isolate P4) to cotton and cucumber seed exudates as affected by the age of the culture from which they were derived[y]

Culture age (days)	Exudate			
	Cucumber[z]		Cotton[z]	
	EC_{50}	χ^2	EC_{50}	χ^2
4	141.3	97.2	66.2	85.1
6	59.6	15.7	18.0	21.5
8	84.5	6.4	19.7	17.7
10	14.6	25.4	2.1	14.7
12	6.0	5.5	3.6	2.7
14	4.0	14.1	<1.0	141.8

[y]Sporangia derived from each culture were ≤24 h old at the time of the assay. Experiments were performed with cotton seed exudate batch 12 and cucumber seed exudate batch 1.

[z]EC_{50} value = the effective concentration of exudate (micrograms per disk) required to induce 50% germination of sporangia. Values were calculated from log-probit plots of exudate concentration versus percent germinated sporangia from each culture age. Chi-square values were estimated at an α level of 0.05.

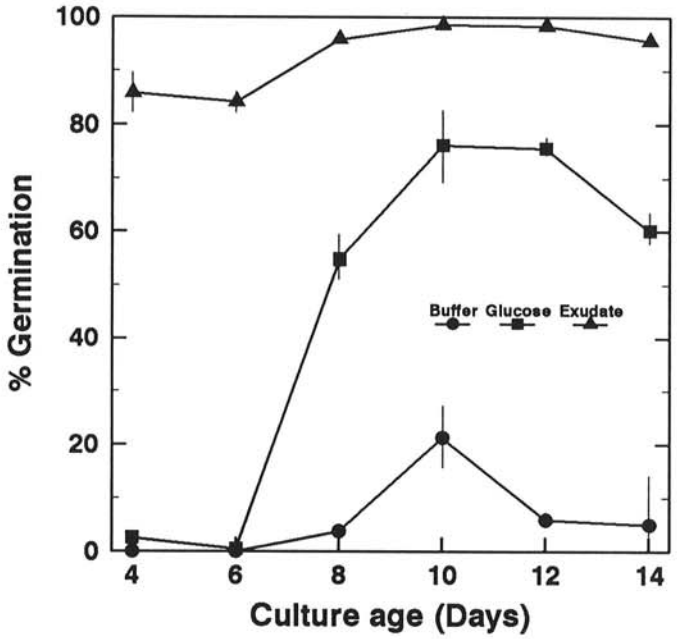

Fig. 2. Responses of sporangia derived from cultures of different ages to ammonium acetate buffer, 1 mM D-glucose, and cotton seed exudate (100 μg per disk). Vertical lines through data points represent standard errors.

Although 4-day-old mycelial cultures grown on SM+L gave rise to sporangia that did not germinate in response to 1 mM D-glucose, older cultures gave rise to sporangia that were increasingly sensitive to glucose (Fig. 2). More than 60% of the sporangia derived from 14-day-old SM+L cultures germinated in response to 1 mM D-glucose as compared with less than 10% from 4-day-old SM+L cultures. Sporangia derived from fresh 4-day-old SM+L cultures, after transfer from 14-day-old cultures to a fresh medium, once again did not respond to 1 mM D-glucose. Responses to ammonium acetate buffer remained between 0 and 20% throughout the 14-day period.

Effect of sporangium age on responses to germination stimulants. If sporangia derived from either 4- or 5-day-old SM+L cultures of *P. ultimum* were themselves allowed to age, they became more sensitive to cotton and cucumber seed exudates (Table 2). EC_{50} values for 1-day-old sporangia produced from a 4-day-old mycelial culture were 500.6 and 211.0 μg per disk for cucumber and cotton seed exudates, respectively. In contrast, EC_{50} values for 3-day-old sporangia produced from a 4-day-old mycelial culture were 48.4 and 27.4 μg per disk for cucumber and cotton seed exudates, respectively. Germination rates of 8- and 15-day-old sporangia were between 90 and 100% in response to 100 μg of exudate per disk (Fig. 3). As observed previously, overall responses of culture-derived sporangia to both glucose and seed exudates became consistently greater as mycelial cultures aged. However, aging of sporangia from 1 to 15 days did not affect responses to ammonium acetate buffer in the absence of exudates or glucose.

Effects of culture history on responses of SM+L-produced sporangia to germination stimulants. Sporangia varied in their sensitivity to cotton seed exudate depending on the medium on which mycelial cultures of *P. ultimum* were grown prior to transfer to the sporangium-production medium (SM+L). Prior growth (4 days) of cultures on nutrient-rich media, such as PDA, SM+L, or CMA, resulted in sporangia that were less responsive to cotton seed exudate than were sporangia derived from SM+L cultures previously grown on nutrient-deficient WA (Table 3). EC_{50} values ranged from 17.2 μg per disk for sporangia derived from SM+L cultures previously grown on WA to 43.6 μg per disk for those derived from cultures previously grown on PDA.

As the SM+L mycelial cultures aged from 4 to 12 days, sporangia derived from mycelia previously grown on PDA, SM+L, and CMA became increasingly sensitive to cotton seed exudate. For example, if previously grown on WA, the sensitivity of

TABLE 2. Comparison of the stimulatory activities of cotton and cucumber seed exudates in relation to culture and sporangium ages[y]

Age		Exudate			
Culture (days)	Sporangium (days)	Cucumber[z]		Cotton[z]	
		EC_{50}	χ^2	EC_{50}	χ^2
4	1	500.6	10.7	211.0	61.2
	2	79.0	25.3	43.2	14.3
	3	48.4	18.6	27.4	0.1
5	1	115.5	23.6	77.1	29.8
	2	14.0	12.0	14.1	9.4
	4	20.3	12.6	35.8	43.0

[y]Experiments were performed with cotton seed exudate batch 13 and cucumber seed exudate batch 5.

[z]EC_{50} value = the effective concentration of exudate (micrograms per disk) required to induce 50% germination of sporangia. Values were calculated from log-probit plots of exudate concentration versus percent germinated sporangia from each culture age-sporangium age combination. Chi-square values were estimated at an α level of 0.05.

sporangia derived from 4- and 12-day-old SM+L cultures did not change (EC_{50} = 17 and 13 μg, respectively). Conversely, the sensitivity of sporangia arising from cultures previously grown on richer media increased nearly 10 times as sporangium-producing cultures aged from 4 to 12 days. Regressing EC_{50} values for sporangia previously cultured on each culture medium over culture age gave rise to regression coefficients for PDA, SM+L, CMA, and WA of −4.2 (R_2 = 0.67), −4.1 (R_2 = 0.94), −2.7 (R_2 = 0.56), and −0.5 (R_2 = 0.37), respectively.

Effects of medium composition on responses of sporangia to germination stimulants. Sensitivity of sporangia produced from mycelia grown on synthetic media differed from that of sporangia produced on living cotton seeds (Table 4). Sporangia produced on SM medium (without lecithin) were highly sensitive to both 1 mM D-glucose and cotton seed exudate at all culture ages tested. Response of SM-produced sporangia to the ammonium acetate buffer also increased as the age of the culture increased. Sporangia derived from cultures growing on the same SM medium, but amended with 0.1% lecithin (SM+L), were highly sensitive to cotton seed exudate but became sensitive to D-glucose only when produced from cultures between 8 and 14 days old. Slight increases in response to ammonium acetate buffer were observed over the 18-day period.

Sporangia derived from mycelial cultures growing on germinating cotton seeds were not sensitive to any of the stimulants during the first 14 days of culture aging. Little or no germination in response to D-glucose or buffer was observed among sporangia produced from 8-, 14- or 18-day-old cultures. Sporangia produced from 18-day-old mycelial cultures germinated only in response to cotton seed exudate.

Altering the levels of various nutrients in the SM+L medium produced different responses of sporangia to D-glucose and cotton seed exudate. In general, as nutrient levels of the culture medium were increased, the sensitivity of sporangia decreased (Fig. 4). For example, adjusting glucose concentrations in the SM+L culture medium from 0 to 50 mM reduced the response of sporangia to cotton seed exudate from near 100 to 18.2% (Fig. 4A). In contrast, responses of sporangia to 1mM D-glucose were not evident at any glucose concentration tested. Similar trends were observed for increasing concentrations of asparagine (0–5 mM) and ammonium sulfate (0–8 mM) in the culture medium (Fig. 4B and C). As lecithin concentrations in the culture medium were increased, responses of sporangia to cotton seed exudate dramatically decreased (Fig. 4D). In the absence of lecithin in the culture medium, nearly 100% of the sporangia produced from 4-day-old mycelial cultures germinated in response to cotton seed exudate. Furthermore, under these conditions, sensitivity of sporangia to glucose also increased (Table 4; Fig. 4D). At lecithin concentrations of 0.1% or more, sporangia were not sensitive to 1 mM D-glucose. At concentrations of 1% or more, sporangia were not sensitive to cotton seed exudate. Even after aging cultures for 14 days, sporangia produced from aged cultures that had grown on media containing 1% lecithin did not respond to D-glucose or seed exudate (data not shown).

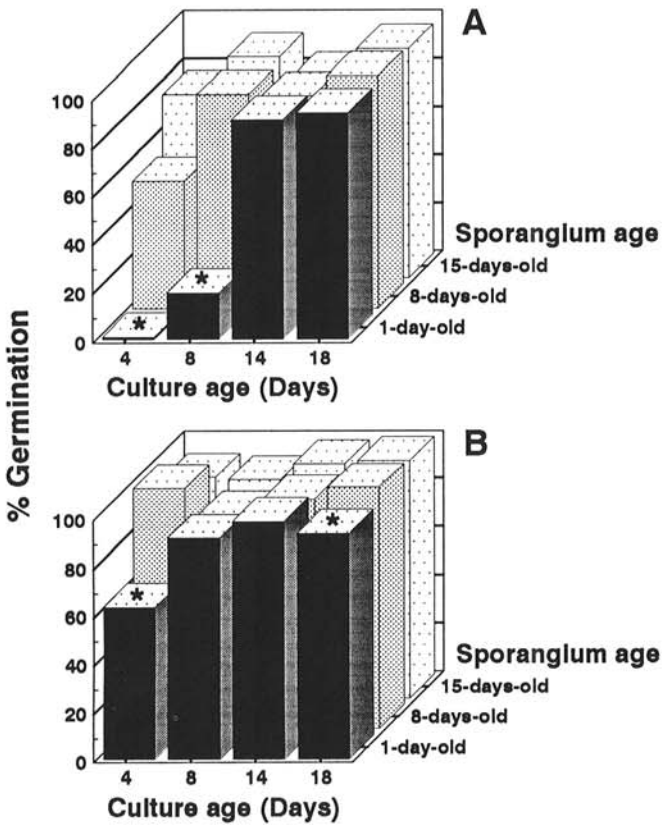

Fig. 3. Responses of sporangia of different ages (derived from mineral salts medium plus lecithin cultures of different ages) to germination stimulants. **A,** Responses to 1 mM D-glucose and **B,** responses to cotton seed exudate (100 μg per disk). Means with an asterisk (*) are significantly less (P = 0.05) than the means within the same culture age group according to the LSD test.

TABLE 3. Response of sporangia of *Pythium ultimum* (isolate P4) to cotton seed exudate as affected by growth of fungal culture on different culture media prior to growth on SM+L medium[y]

SM+L culture age (days)	Previous culture medium[z]							
	PDA		SM+L		CMA		WA	
	EC_{50}	χ^2	EC_{50}	χ^2	EC_{50}	χ^2	EC_{50}	χ^2
4	43.6	17.6	41.4	33.6	33.9	46.3	17.2	11.0
5	16.2	4.7	31.6	21.6	10.4	8.7	18.5	24.2
6	17.0	6.4	24.6	44.0	21.0	6.5	13.0	47.2
12	1.0	24.4	5.2	15.7	4.3	20.1	13.7	8.2

[y] Sporangia derived from each culture were ≤24 h old at the time of the assay. Prior to transfer to a mineral salts medium containing 0.1% lecithin (SM+L), mycelial cultures were grown for 4 days on potato-dextrose agar (PDA), SM+L, cornmeal agar (CMA), and 2% water agar (WA). Experiments were performed with cotton seed exudate batches 8 and 11.

[z] EC_{50} value = the effective concentration of exudate (micrograms per disk) required to induce 50% sporangium germination. Values were calculated from log-probit plots of exudate concentration versus percent germinated sporangia from each culture age-culture medium combination. Chi-square values were estimated at an α level of 0.05.

TABLE 4. Effects of culture medium and culture age on percent germination of sporangia of *Pythium ultimum* to germination stimulants

Germination stimulant[w]	Culture medium[x]	Culture age[y] (% germination)			
		4 days	8 days	14 days	18 days
Buffer	SM+L	1.4 b[z]	1.1 b	6.9 b	23.4 b
	SM	5.6 a	31.4 a	73.9 a	75.0 a
	WCS	0.0 b	0.0 b	0.0 c	0.0 c
D-glucose	SM+L	1.1 b	19.2 c	90.8 a	93.7 a
	SM	87.3 a	79.3 a	98.2 a	98.7 a
	WCS	0.0 b	31.8 b	14.5 b	0.0 b
Exudate	SM+L	62.7 b	91.2 b	98.3 a	93.1 a
	SM	90.0 a	100.0 a	98.7 a	100.0 a
	WCS	0.0 c	25.7 c	6.1 b	100.0 a

[w] Buffer consists of 10 mM ammonium acetate (pH 5.5); D-glucose was used at concentrations of 1 mM, and cotton seed exudate (batch 12) was added at the rate of 100 μg per sporangial disk. To each sporangial disk, 10 μl of solution was added.

[x] SM+L = a mineral salts medium containing 0.1% lecithin, SM = mineral salts medium without the lecithin, and WCS = water agar in which surface-disinfested cotton seeds germinated.

[y] Sporangia produced from mycelial cultures growing on SM+L medium. All sporangia were ≤24 h old.

[z] Numbers in each column, grouped with each stimulant, and followed by the same letter are not significantly (P = 0.05) different according to the LSD test.

DISCUSSION

This study expands previous research (33) in which we found that the germination sensitivity of sporangia to various sugars and amino acids, but not to seed exudates, was affected by the substrate on which their mycelial progenitors were grown. In the present study, we hypothesized that the nutrient status of the culture medium, as well as that of the *P. ultimum* sporangium, can regulate responses of sporangia to stimulants. Part of the rationale for this hypothesis came from our understanding of sporangium formation in *P. ultimum*, in which relationships between substrate nutrient levels and endogenous levels in sporangia are closely coupled (35,42).

Under nutrient-limiting or stress conditions, hyphal protoplasm is mobilized into primary sporangia or into newly formed secondary sporangia (35,42). During sporangium formation, constituents of the culture medium that have been incorporated into hyphal protoplasm, as well as metabolites synthesized during mycelial growth, become packaged within the sporangium. The nutritional status of the mycelium, therefore, greatly impacts the nutritional status of the sporangium.

The main conclusion from this study is that both quantitative and qualitative increases in sensitivity of sporangia to germination stimulants are due to the depletion of endogenous nutrient reserves available to or within sporangia of *P. ultimum*. The principal endogenous storage materials found within mycelia and propagules of *P. ultimum* consist largely of lipids, including triacyl glycerols and fatty acids (16,29,45). In some instances, *Pythium* species have been sought for their high content of long-chain unsaturated fatty acids, which may be exploited for the potential industrial production of fatty acids (14).

Our conclusion of increased germination sensitivity of sporangia accompanying nutrient depletion is based on several lines of evidence. The most direct evidence comes from experiments in which germination responses of sporangia derived from mycelia grown on nutrient-rich media were compared with those derived from mycelia grown on nutrient-deficient media. Inverse relationships were observed between concentrations of either glucose or lecithin in the culture medium and sensitivity of sporangia to cotton seed exudate or to 1 mM D-glucose.

The mechanisms that regulate responses of sporangia to glucose are unknown but may involve changes in sporangial membrane lipid composition. This can be affected by either the fatty acid and glyceride components of the lecithin formulation or the sterol contaminants commonly found in many phosphatidyl choline preparations (23–25,34). Despite the fact that *Pythium* species contain abundant phospholipids, including significant amounts of phosphatidyl choline and phosphatidyl ethanolamine (16), it is unlikely that the phospholipid portions of these molecules are directly responsible for regulating germination responses. Phosphatidyl choline-supplemented cultures of *P. ultimum* do not take up appreciable amounts of the phospholipid headgroup, although endogenous levels of unsaturated fatty acids increase in mycelia (23), presumably in part, through the action of extracellular triacylglycerol lipase enzymes that have particular affinities for glycerides containing predominantly *cis*-isomer unsaturated fatty acids (31). Presently, the role of fluctuating levels of endogenous fatty acids in regulating sporangium germination responses are

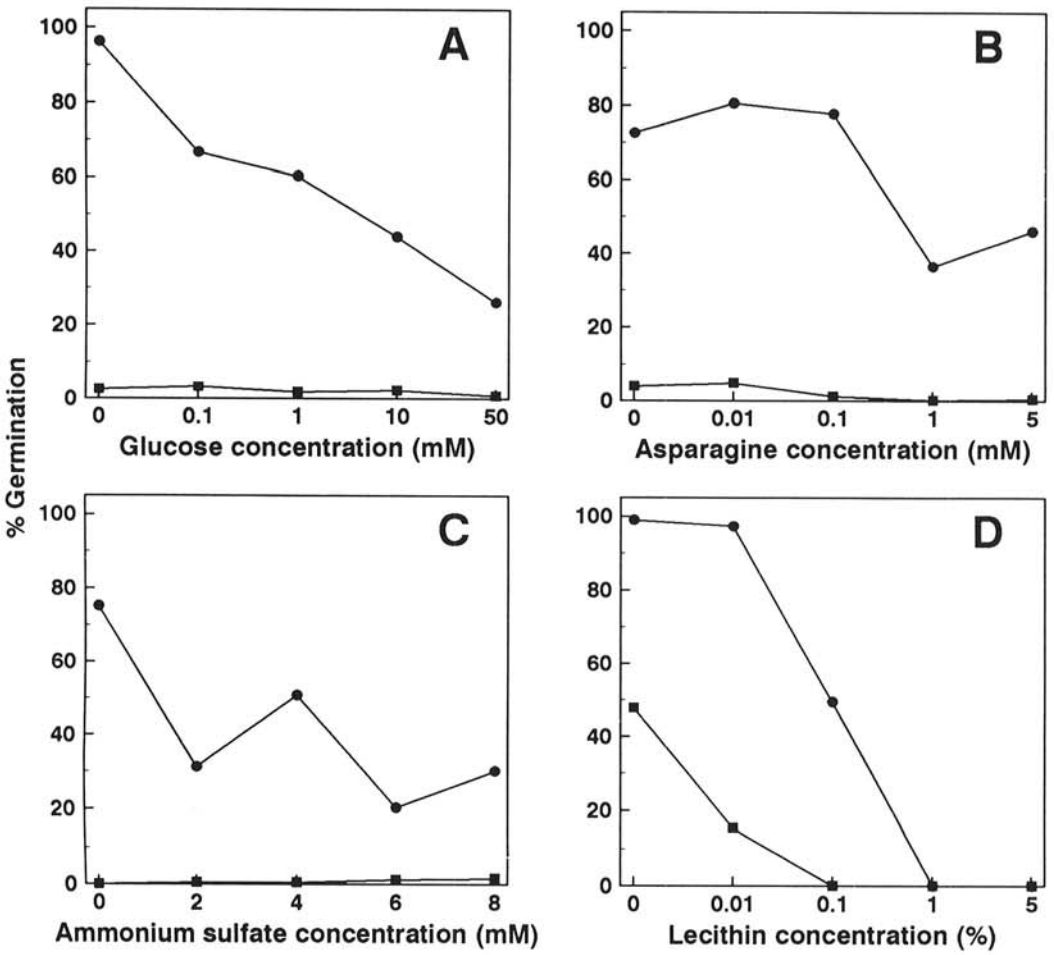

Fig. 4. Responses of *Pythium ultimum* sporangia to 1 mM D-glucose (■) and 100 μg of cotton seed exudate (●) per disk as affected by the levels of specific nutrients in culture medium. **A,** glucose, **B,** asparagine, **C,** ammonium sulfate, and **D,** lecithin. Regression coefficients for responses of sporangia to exudate and glucose, respectively, regressed over nutrient concentration were **A,** glucose: −1.1 ($R^2 = 0.52$) and −0.04 ($R^2 = 0.71$); **B,** asparagine: −7.1 ($R^2 = 0.75$) and −0.7 ($R^2 = 0.76$); **C,** ammonium sulfate: −5.0 ($R^2 = 0.54$) and 0.2 ($R^2 = 0.94$); and **D,** lecithin: −16.6 ($R^2 = 0.94$) and −2.3 ($R^2 = 0.65$). All sporangia were produced from 4-day-old mycelial cultures.

unknown.

Several other lines of evidence support the main conclusion that sensitivity of *P. ultimum* sporangia to germination stimulants is related to depletions of endogenous cellular components. First, culture history can dramatically affect responses of *P. ultimum* sporangia to germination stimulants. Nutrient levels in media from which mycelial cultures were transferred affected the responses of sporangia derived from subsequently cultured mycelia. Sporangia were most sensitive if SM+L mycelial cultures had initially grown on WA. Two possible explanations for this observation are that: 1) elevated levels of endogenous nutrients can be carried-over during culture transfers or 2) the previous culture conditions in some way alter the mycelial physiology for a period of time thereafter. Although *P. ultimum* cultures grown on a lipid-supplemented medium have been shown to carry-over factors stimulatory to oospore formation when transferred to lipid-free basal media (23), specific evidence for the latter hypothesis is lacking. Other studies have shown that the endogenous reserves in propagules and the subsequent germination of those propagules can be greatly affected by the substrate. For example, conidia derived from mycelia of *Colletotrichum truncatum* grown on a low-nutrient medium were much more germinant compared to those derived from mycelia grown on a high-nutrient medium (38).

Second, as mycelial cultures age, subsequently produced sporangia become increasingly sensitive to seed exudate and glucose. As mycelia of *Pythium* grow on solid or liquid substrates, they deplete the medium of nutrients incorporating them into new cell constituents. When these nutrients are limiting for growth, *Pythium* must rely on endogenous energy reserves for continued growth, thus, consuming the pool of cytoplasmic nutrients available to the forming sporangia. Even if mycelial cultures initiate growth on a rich medium, depletion of the medium nutrients and endogenous mycelial nutrients would lead to the same effects as seen with cultures grown on nutrient-deficient media.

Third, as sporangia themselves age, they become increasingly sensitive to exudate stimulants and increasingly responsive to glucose. Numerous studies have demonstrated that propagules of many fungi lose endogenous nutrients due to exudation or through respiration (8,9,12,13,17–19,26,41,44). With some soilborne fungal pathogens, respiratory losses of carbon may account for as much as 55–75% of the total carbon losses from propagules (18). Based on previous studies, we reasoned that as sporangia of *P. ultimum* aged from 1 to 15 days their respiration would contribute to cumulative losses of endogenous nutrient reserves, allowing us to examine relationships between endogenous reserves and germination responses.

Our observations confirmed that sporangia became increasingly responsive to glucose and seed exudate stimulants with increasing aging periods. This effect of aging on germination responses of *Pythium* propagules has been described, particularly for oospores of *P. ultimum* and other species of *Pythium* and *Phytophthora* (5–7,20–22,37). It is likely that as critical endogenous nutrients (such as endogenous fatty acids that accumulate in mycelia grown on lecithin-amended media [23]) are lost through respiration sporangia become increasingly responsive to glucose as well as to stimulants present in cotton and cucumber seed exudates.

In a previous study, *P. ultimum* sporangia produced from mycelia grown on a medium containing 0.1% lecithin had sensitivities to cotton seed exudate similar to those produced by mycelia colonizing germinating seeds and other living plant parts (33). Our present study corroborates that observation. However, differential responses of sporangia produced in this manner were observed as culture conditions changed. Responses to D-glucose remained low throughout the 18-day experimental period and only after 18 days did sporangia become sensitive to seed exudate. In experiments with different concentrations of lecithin in the culture medium, high concentrations (>0.1%) eliminated responses of sporangia to D-glucose and seed exudate, even when cultures were aged up to 14 days. In light of Kerwin and Duddles findings (23), this observation may be the result of correlative increases in endogenous levels of unsaturated fatty acids or other lipids. It is possible that during the growth of *P. ultimum* on germinating seeds, the level of fatty acids or other lipids acquired by the mycelium satisfies qualitative or quantitative endogenous requirements more thoroughly than those acquired from the 1% lecithin amendment in our culture media. It is possible, therefore, that more fatty acids must be exhausted before germination responses are observed. Aging of mycelia or sporangia beyond 18 days may further increase germination responses to seed exudate as well as to D-glucose.

It is not technically feasible at this time to extract naturally produced sporangia of *Pythium* species free of soil and plant tissue so that responses of such propagules to specific stimulants can be assessed. It seems reasonable, however, that responses of sporangia produced on germinating seeds would more closely mimic the responses of sporangia produced in the field. We have recently shown that the principal stimulatory molecules present in cotton seed exudate consists of a mixture of unsaturated fatty acids and triglycerides of these fatty acids (T. R. Ruttledge and E. B. Nelson, *unpublished data*). Sporangia produced on synthetic media under a wide range of cultural conditions are highly responsive to these unsaturated fatty acids, but those produced on germinating seeds are initially not sensitive to these fatty acids. We can hypothesize, therefore, that sporangia in soil are likely to undergo a period immediately after production during which they are not highly responsive to fatty acid stimulants present in seed exudates, presumably because their endogenous levels of unsaturated fatty acids meet or exceed a critical level for germination. Clearly, *P. ultimum* has the potential to maintain high endogenous levels of some unsaturated fatty acids under certain culture conditions, particularly when mycelia are grown in the presence of high glucose concentrations (14). This is consistent with our observation of reduced sensitivity to cotton seed exudate from sporangia produced on media containing high glucose concentrations. As sporangia age, they first become responsive to the fatty acid stimulants present in seed exudates; the fatty acids satisfy the endogenous levels lost through respiration and the levels of fatty acids that Oomycetes require during germination processes (29). Only later, as further endogenous reserves are depleted do sporangia become responsive to other stimulants such as glucose.

Our results are in contrast to general concepts of propagule behavior under fungistasis. Based on many years of research, Lockwood and coworkers (17,26) observed that propagules of a large number of soilborne fungal plant pathogens display nutrient-independent or -dependent behavior. For a number of soilborne pathogens, the greater the endogenous spore nutrition, the more germinant those spores are in soil (i.e., the less the requirement for an exogenous germination stimulant). As endogenous reserves are lost through respiration, propagules become increasingly dependent on an exogenous stimulant for germination. Further losses of carbon result in decreasing germination rates and propagule debilitation (8,12,18). We observed that sporangia become increasingly germinant, particularly in the absence of any stimulant, with increasing aging periods. We interpret these results as indicating that sporangia of *P. ultimum* become less dependent on a specific germination stimulant as propagules respire, suggesting a clearly different germination behavior than that described by Lockwood and colleagues (17,26) for propagules of many other soilborne fungal pathogens.

These germination responses, and the mechanisms underlying such responses, have important implications for the ecology, epidemiology, and control of Pythium seed rots. Our results imply a more highly specialized mechanism of germination response regulation than previously recognized. Further elucidation of these processes is currently under investigation and will be important in furthering our understanding of the behavior and control of *P. ultimum* and other Oomycetes in the spermosphere and rhizosphere.

LITERATURE CITED

1. Agnihotri, V. P., and Vaartaja, O. 1967. Effects of amendments, soil moisture contents, and temperatures on germination of *Pythium*

sporangia under the influence of soil mycostasis. Phytopathology 57:1116-1120.
2. Agnihotri, V. P., and Vaartaja, O. 1967. Root exudates from red pine seedlings and their effects on *Pythium ultimum*. Can. J. Bot. 45:1031-1040.
3. Agnihotri, V. P., and Vaartaja, O. 1968. Seed exudates of *Pinus resinosa* and their effects on growth and zoospore germination of *Pythium afertile*. Can. J. Bot. 46:1135-1141.
4. Agnihotri, V. P., and Vaartaja, O. 1970. Effect of seed exudates of *Pinus resinosa* on the germination of sporangia and on the population of *Pythium irregulare* in soil. Plant Soil 32:246-249.
5. Ann, P. J., and Ko, W. H. 1988. Induction of oospore germination of *Phytophthora parasitica*. Phytopathology 78:335-338.
6. Ayers, W. A., and Lumsden, R. D. 1975. Factors affecting production and germination of oospores of three *Pythium* species. Phytopathology 65:1094-1100.
7. Banihashemi, Z., and Mitchell, J. E. 1976. Factors affecting oospore germination in *Phytophthora cactorum*, the incitant of apple collar rot. Phytopathology 66:443-448.
8. Bristow, P. R., and Lockwood, J. L. 1975. Soil fungistasis: Role of spore exudates in the inhibition of nutrient-independent propagules. J. Gen. Microbiol. 90:140-146.
9. Bristow, P. R., and Lockwood, J. L. 1975. Soil fungistasis: Role of the microbial nutrient sink and of fungistatic substances in two soils. J. Gen. Microbiol. 90:147-156.
10. Chang-Ho, Y. 1970. The effect of pea root exudate on the germination of *Pythium aphanidermatum* zoospore cysts. Can. J. Bot. 48:1501-1514.
11. Chen, D. W., and Zentmyer, G. A. 1970. Production of sporangia by *Phytophthora cinnamomi* in axenic culture. Mycologia 62:397-402.
12. Filonow, A. B., and Lockwood, J. L. 1983. Mycostasis in relation to the microbial nutrient sinks of five soils. Soil Biol. Biochem. 15:557-565.
13. Filonow, A. B., and Lockwood, J. L. 1983. Loss of nutrient-independence for germination by fungal propagules incubated on soils or on a model system imposing diffusive stress. Soil Biol. Biochem. 15:567-573.
14. Gandhi, S. R., and Weete, J. D. 1991. Production of the poly-unsaturated fatty acids arachidonic acid and eicosapentaenoic acid by the fungus *Pythium ultimum*. J. Gen. Microbiol. 137:1825-1830.
15. Gindrat, D. 1976. Components in unbleached commercial chitin stimulate *Pythium ultimum* in sugarbeet spermosphere. Phytopathology 66:312-316.
16. Hendrix, J. W. 1974. Physiology and biochemistry of growth and reproduction in *Pythium*. Proc. Am. Phytopathol. Soc. 1:207-210.
17. Hsu, S. C., and Lockwood, J. L. 1973. Soil fungistasis: Behavior of nutrient-independent spores and sclerotia in a model system. Phytopathology 63:334-337.
18. Hyakumachi, M., and Lockwood, J. L. 1989. Relation of carbon loss from sclerotia of *Sclerotium rolfsii* during incubation in soil to decreased germinability and pathogenic aggressiveness. Phytopathology 79:1059-1063.
19. Jasalavich, C. A., Hyakumachi, M., and Lockwood, J. L. 1990. Loss of endogenous carbon by conidia of *Cochliobolus sativus* exposed to soil and its effect on conidial germination and pathogenic aggressiveness. Soil Biol. Biochem. 22:761-767.
20. Johnson, L. F. 1988. Effects of atmospheric gases and light on changes in thickness of oospore walls and on germinability of oospores of *Pythium ultimum*. Phytopathology 78:435-439.
21. Johnson, L. F., and Arroyo, T. 1983. Germination of oospores of *Pythium ultimum* in the cotton rhizosphere. Phytopathology 73:1620-1624.
22. Johnson, L. F., Qian, P., and Ferriss, R. S. 1990. Soil matric potential effects on changes in wall morphology, germination, and lysis of oospores of *Pythium ultimum*. Phytopathology 80:1357-1361.
23. Kerwin, J. L., and Duddles, N. D. 1989. Reassessment of the role of phospholipids in sexual reproduction by sterol-auxotrophic fungi. J. Bacteriol. 171:3831-3839.
24. Ko, W. H. 1985. Stimulation of sexual reproduction of *Phytophthora cactorum* by phospholipids. J. Gen. Microbiol. 131:2591-2594.
25. Ko, W. H. 1986. Sexual reproduction of *Pythium aphanidermatum*: Stimulation by phospholipids. Phytopathology 76:1159-1160.
26. Ko, W.-H., and Lockwood, J. L. 1967. Soil fungistasis: Relation to fungal spore nutrition. Phytopathology 57:894-901.
27. Kraft, J. M., and Erwin, D. C. 1967. Stimulation of *Pythium aphanidermatum* by exudates from mung bean seeds. Phytopathology 57:866-868.
28. Lifshitz, R., Windham, M. T., and Baker, R. 1986. Mechanism of biological control of preemergence damping-off of pea by seed treatment with *Trichoderma* spp. Phytopathology 76:720-725.
29. Lösel, D. M. 1989. Functions of lipids: Specialized roles in fungi and algae. Pages 367-438 in: Microbial Lipids. C. Ratledge and S. G. Wilkinson, eds. Academic Press, Inc., San Diego, CA.
30. McCoy, R. J., and Kraft, J. M. 1984. Comparison of techniques and inoculum sources in evaluating peas (*Pisum sativum*) for resistance to stem rot caused by *Rhizoctonia solani*. Plant Dis. 68:53-55.
31. Mozaffar, Z., and Weete, J. D. 1993. Purification and properties of an extracellular lipase from *Pythium ultimum*. Lipids 28:377-382.
32. Nelson, E. B. 1990. Exudate molecules initiating fungal responses to seeds and roots. Plant Soil 129:61-73.
33. Nelson, E. B., and Craft, C. M. 1989. Comparative germination of culture-produced and plant-produced sporangia of *Pythium ultimum* in response to soluble seed exudates and exudate components. Phytopathology 79:1009-1013.
34. Nes, W. D. 1987. Biosynthesis and requirement for sterols in the growth and reproduction of oomycetes. Pages 304-328 in: Ecology and Metabolism of Plant Lipids. G. Fuller and W. D. Nes, eds. American Chemical Society, Washington, DC.
35. Paulitz, T. C., and Baker, R. 1988. The formation of secondary sporangia by *Pythium ultimum*: The influence of organic amendments and *Pythium nunn*. Soil Biol. Biochem. 20:151-156.
36. Phillips, D. J. 1965. Ecology of plant pathogens in soil. IV. Pathogenicity of macroconidia of *Fusarium roseum* f. sp. *cerealis* produced on media of high or low nutrient content. Phytopathology 55:328-329.
37. Ruben, D. M., Frank, Z. R., and Chet, I. 1980. Factors affecting behavior and developmental synchrony of germinating oospores of *Pythium aphanidermatum*. Phytopathology 70:54-59.
38. Schisler, D. A., Jackson, M. A., and Bothast, R. J. 1991. Influence of nutrition during conidiation of *Colletotrichum truncatum* on conidial germination and efficacy in inciting disease in *Sesbania exaltata*. Phytopathology 81:587-590.
39. Schlub, R. L., and Schmitthenner, A. F. 1978. Effects of soybean seed coat cracks on seed exudation and seedling quality in soil infested with *Pythium ultimum*. Phytopathology 68:1186-1191.
40. Singleton, L. L. 1986. Storage of *Pythium* species on a wheat leaf:water medium. (Abstr.) Phytopathology 76:1143.
41. Sneh, B., and Lockwood, J. L. 1976. Quantitative evaluation of the microbial nutrient sink in soil in relation to a model system for soil fungistasis. Soil Biol. Biochem. 8:65-69.
42. Stanghellini, M. E., and Hancock, J. G. 1971. The sporangium of *Pythium ultimum* as a survival structure in soil. Phytopathology 61:157-164.
43. Stanghellini, M. E., and Hancock, J. G. 1971. Radial extent of the bean spermosphere and its relation to the behavior of *Pythium ultimum*. Phytopathology 61:165-168.
44. Steiner, G. W., and Lockwood, J. L. 1969. Soil fungistasis: Sensitivity of spores in relation to germination time and size. Phytopathology 59:1084-1092.
45. Weete, J. D. 1980. Lipid Biochemistry of Fungi and Other Organisms. Plenum Press, New York. 388 pp.
46. Weinhold, A. R., Bowman, T., and Dodman, R. L. 1969. Virulence of *Rhizoctonia solani* as affected by nutrition of the pathogen. Phytopathology 59:1601-1605.
47. Weinhold, A. R., Dodman, R. L., and Bowman, T. 1971. Influence of exogenous nutrition on virulence of *Rhizoctonia solani*. Phytopathology 62:278-281.