Effect of Soil Solution and Two Soil Pseudomonads on Sporangium Production by Phytophthora cinnamomi

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ABSTRACT

Agar-mycelial discs and washed mycelial mats of *Phytophthora cinnamomi* produced a few sporangia, within 24 hr, when incubated at 24 C in a sterile mineral salts solution or in autoclaved soil extracts. Nonsterile soil extracts and solutions containing two *Pseudomonas* isolates from soil induced the gradual development of greater numbers of sporangia over a longer period than did sterile solutions. The bacterial isolates synergistically stimulated formation of sporangia. One- and 2-day-old mats, but not 5-day-old mats, formed sporangia under axenic conditions; young mats also formed sporangia at a more rapid rate than did older mats in nonsterile soil extract. Forced aeration enhanced production of sporangia under both axenic and nonsterile conditions. Glucose at 10 to 1,000 ppm or glutamic acid at 100 ppm inhibited production of sporangia under axenic conditions, and delayed the onset of sporangium production in nonsterile solutions. The suppressing effect of nutrients appeared to be overcome by the bacterial isolates and by microorganisms in nonsterile soil extract. Phytopathology 61:1188-1193.

Additional key words: Fungal morphogenesis, sporulation.

Since the original description of *Phytophthora cinnamomi* Rands (11) in 1922, few investigators have observed sporangium production by this organism in pure culture (8, 13). Mehrlich (10) reported enhanced sporangium production by mycelia placed in solutions of nonsterile soil leachate. Few or no sporangia were formed in autoclaved soil leachate (10) or in filter-sterilized soil solution (14). Living soil microorganisms were thus implicated in the production of sporangia by the fungus in these solutions.

Zentmyer (12) isolated strains of *Chromobacterium violaceum* from Australia and California soils that stimulated sporangium production when the fungus and bacteria were added together to sterilized soil extract. *Pseudomonas* spp. have also been reported to induce or stimulate sporangium production (3, 7, 9). Most workers have concluded that specific soil microorganisms produce some metabolite essential for initiating sporangium production by the fungus (3, 7, 9, 12). It has also been suggested that depletion of nutrients by soil microorganisms in nonsterile solutions may play a role in initiating production of sporangia (13). Recently, Chen & Zentmyer (4, 5) described a method for producing sporangia of *P. cinnamomi* under axenic conditions.

In this paper we report some relationships between the axenic production of sporangia by *P. cinnamomi* and that which occurs in nonsterile soil solutions. The stimulation of sporangium production by the synergistic action of two bacterial isolates is also described.

Materials and Methods.—Soil extracts were prepared from a suspension of 10 g fresh soil in 1 liter of deionized water. After standing at room temperature for 3 days, the solutions were filtered through Whatman No. 42 paper. Portions of the extracts were sterilized by autoclaving at 121 C for 20 min.

The mineral salts solution used as the washing and suspending medium in some experiments was modified from that of Chen & Zentmyer (5). It consisted of 0.005 M KNO_3, 0.005 M $Ca(NO_3)_2$, and 0.002 M $MgSO_4$ (pH 6.5). The solution was autoclaved at 121 C for 20 min. Iron chelate solution (ethylenediamine tetraacetic acid 26.1 g; KOH, 15 g; $FeSO_4 \cdot 7H_2O$, 24.9 g; water, 1 liter) was autoclaved separately, and 1 ml added to the cooled medium.

Phytophthora cinnamomi (SB 216-1), an avocado isolate, was cultured on V-8 agar (200 ml V-8 juice/liter; pH adjusted to 6.5 with $CaCO_3$; agar, 15 g), and in a synthetic medium (SM) consisting of the following ingredients in g per liter: sucrose, 10; L-glutamic acid, 2.2; L-tryptophan, 0.01; KH_2PO_4, 0.5: $MgSO_4$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.2; thiamine hydrochloride, 0.001; β-sitosterol, 0.008; and 1 ml of a solution of minor elements to give 1 ppm Zn and 0.02 ppm each of Cu, Mo, and Mn. The pH was adjusted to 6.5 with KOH before sterilization.

Bacteria were isolated from nonsterile soil extract on dilution plates of trypticase soy-soil extract agar (TSS), consisting of dehydrated trypticase soy broth (Baltimore Biological Lab., Baltimore, Md.), 1.5 g; soil extract, 1 liter; and agar, 15 g. Colonies were transferred to nutrient broth; after 48 hr at 24 C, turbid cultures were tested for sporangium-stimulating activity by the addition of one drop of a 1:10 dilution of the culture in sterile water to the sterile test solutions.

The agar-disc test for sporangium production was carried out in 5-cm petri dishes containing 5 ml of test solution and three 5-mm diam discs cut from the margin of 3- or 4-day-old *P. cinnamomi* colonies on V-8 agar. The dishes were incubated in the dark at 24 C. At daily intervals, sporangia over the surface of the discs were counted with a × 50 stereoscopic microscope and expressed as average number of sporangia

per disc. Four replicate dishes were used for each treatment.

Sporangium production by washed mycelial mats, except where otherwise indicated, was carried out as follows: 40 ml of SM in a 250-ml flask was inoculated with several pieces of a V-8 agar culture of *P. cinnamomi,* and incubated at 24 C for 24 hr. The resulting mycelium was minced with 140 ml of sterile SM in a sterile blender for 30 sec. Ten-ml quantities of the inoculated medium were dispensed aseptically to 9-cm petri dishes, and the dishes incubated for 18-20 hr at 24 C. The medium was decanted from each dish and replaced with 20 ml of sterile mineral salts solution at three 1-hr intervals. The mats were finally suspended in fresh salt solution or other treatment, and incubated at 24 C for 18 hr or longer. Sporangia appearing on the surface of the mats were counted in 10 random microscopic fields (3.8 mm^2) at $\times$ 100. The counts from four replicate mats were averaged and expressed as sporangia per 10 fields.

The effect of aeration on sporangium production was tested on mats washed as described above, except that the mats were finally suspended in 80 ml of solution in 250-ml sterile flasks equipped with plastic spargers. Filtered air, at a slow rate, was passed through duplicate flasks of solution and mycelia for 18 hr. Control flasks were not aerated. Each mat was placed into a sterile dish and examined for sporangium production.

RESULTS.—*Effect of sterile and nonsterile solutions on sporangium production.*—A few sporangia developed within 24 hr on V-8 agar-mycelial discs and on washed mycelial mats placed in sterilized soil extract or salt solution, but numbers did not increase upon further incubation (Table 1). In nonsterile soil extract, sporangium formation was comparably low at 1 day, but the numbers gradually increased daily until they reached relatively high numbers after several days of incubation. Sporangia did not develop on discs or mats placed in sterile deionized water.

The ability of the sterile mineral salts solution to support the consistent production of a few sporangia in 24 hr or less under axenic conditions was confirmed in over 30 separate experiments. The average number of sporangia formed per 5 mm agar-mycelial disc varied widely in these experiments, from as few as 0.6 to as many as 30/disc; however, in most cases, these numbers were in the range of one to six sporangia/disc.

TABLE 1. Sporangium production by agar discs and washed mycelial mats of *Phytophthora cinnamomi* in several solutions

Suspending solution	Sporangia produced[a]			
	V-8 Discs		Washed mats	
	1 Day	3 Days	1 Day	6 Days
Deionized water	0	0	0	0
Mineral salts solution	7	6	14	12
Autoclaved soil extract	5	5	12	17
Nonsterile soil extract	6	>150	9	95

[a] Average number of sporangia per disc produced on three 5-mm agar discs suspended in 5 ml of solution, or average number of sporangia per 10 microscopic fields on washed mats suspended in 20 ml of solution. Four replicates were used for each treatment.

In contrast to sporangium production in sterile solutions in which fully mature sporangia were formed within 24 hr, sporangium production by discs and mats in nonsterile soil extract after continued incubation was gradual, and resulted in sporangia of different sizes and stages of development. Motile zoospores were frequently released in the nonsterile solution throughout the incubation period, but spontaneous release of zoospores from sporangia was only occasionally observed in the mycelia incubated in the sterile solutions. Sporangium development in nonsterile soil extract appeared to be a continuous process for ca. 1 week, after which it gradually declined.

During the course of this experiment (Table 1), bacterial dilution-plate counts on TSS agar were made of the nonsterile soil extract in which the mats were incubated. The total numbers of bacteria increased from an initial count of 2.2×10^5 to 2.7×10^6 bacteria/ml after 6 days. Thus, significant multiplication of bacteria had occurred during the period of marked sporangia increase.

Effect of culture age on sporangium production.—In sterile soil extract, 1- and 2-day-old mats formed a few sporangia observable after 1 day, but the numbers did not greatly increase after further incubation (Fig. 1). Five-day-old mats failed to produce any sporangia in the sterile extract, but formed them abundantly after 5 days in nonsterile soil extract. The younger 1- and 2-day-old mats in nonsterile soil extract formed abundant sporangia earlier in the incubation period than did the older mats. The results suggested that increasing culture age decreased the ability of the mycelia to form sporangia under axenic conditions, and delayed the onset of sporangia production in the nonsterile solution.

Bacterial induction of sporangia production.—During a survey of ca. 100 bacterial colonies isolated on dilution plates from an active nonsterile soil extract, a single colony, designated as No. 48, stimulated sporangium production by *P. cinnamomi* in 1 week when a loopful of the bacteria was transferred to sterilized soil extract containing agar discs of the fungus. Other bacterial isolates did not give this effect. Culture 48, streaked for purity, gave rise to two slightly different colony types. Subcultures of these colonies (isolates 48-1 and 48-2) were maintained in nutrient broth, nutrient agar, and in sterilized soil culture.

Bacterium 48-1 stimulated sporangium development by both agar-mycelial discs and washed mycelia of *P. cinnamomi.* Bacterium 48-2, alone, had no appreciable effect on sporangium production. The two isolates supplied together induced sporangium development to a greater degree than isolate 48-1 alone (Fig. 2, 3).

Nonsterile soil extracts prepared from sterilized soil previously inoculated with the bacterial isolates gave similar results. The mixed bacterial-soil extract mod-

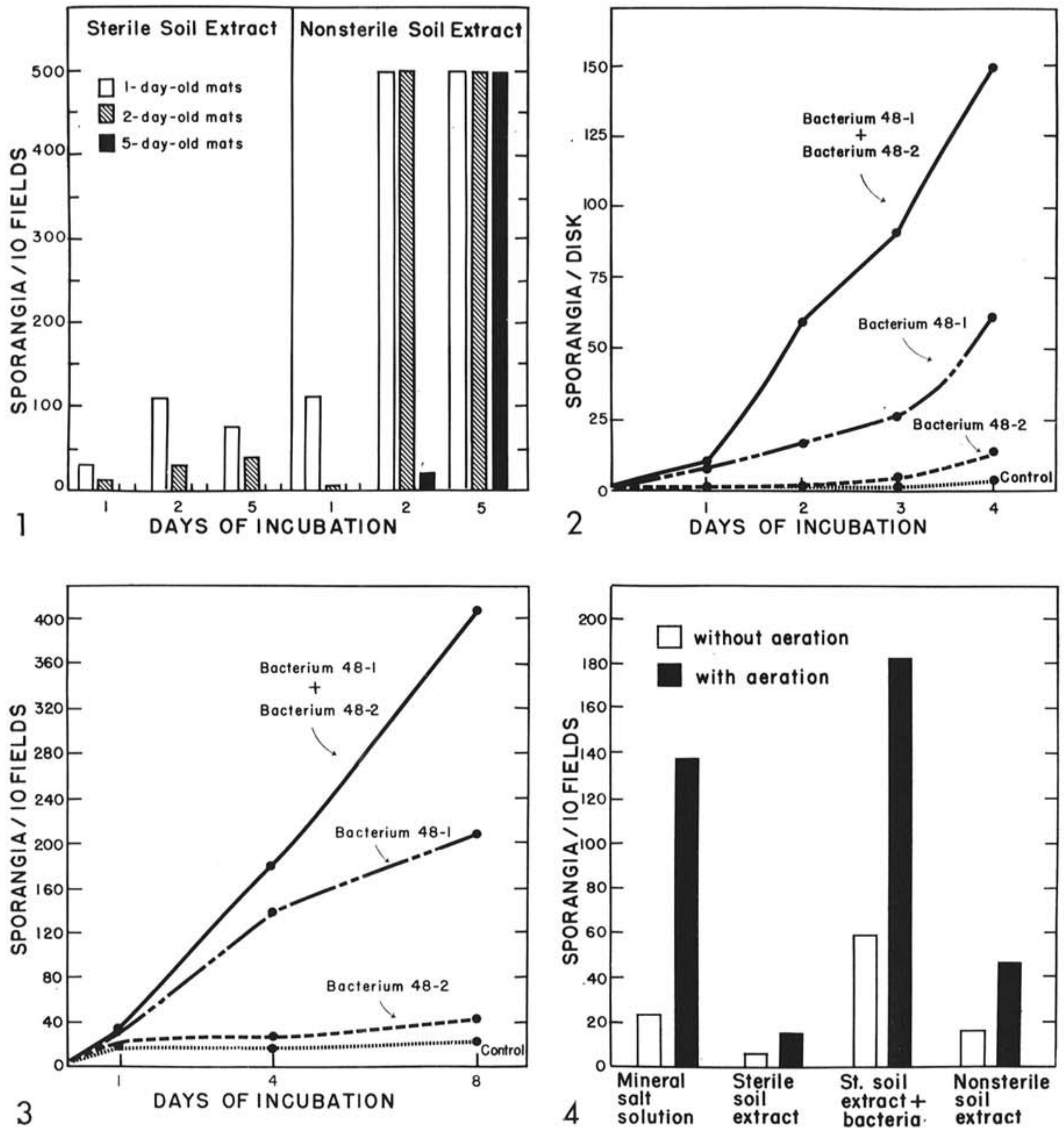


Fig. 1-4. 1) Sporangium production by washed mats of *Phytophthora cinnamomi* of differing culture age. Mats were grown in synthetic medium for the indicated times; each mat was rinsed with 100 ml of sterile deionized water, then suspended in 20 ml of sterile or nonsterile soil extract and incubated at 24 C. **2)** Induction of sporangia on agar-mycelial discs of *Phytophthora cinnamomi* by two bacterial isolates added to sterilized soil extract. **3)** Sporangium production by washed mats of *Phytophthora cinnamomi* in sterilized soil extract seeded with two bacterial isolates. **4)** Effect of aeration on sporangium production by washed mats of *Phytophthora cinnamomi*. Mats suspended in the indicated treatments were aerated by a stream of air passing through the solutions for 18 hr.

erately stimulated sporangium production by agar discs of the fungus within 5 days at 24 C, whereas the extract prepared from the soil culture of isolate 48-2 had no effect. The extract prepared from the soil culture of isolate 48-1 gave only a slight increase in sporangium production over that occurring in sterile controls.

Effect of aeration on sporangium production.—Air, bubbled through solutions containing washed mats of *P. cinnamomi*, markedly stimulated sporangium production within 18 hr (Fig. 4). Sporangia were particularly abundant on mats suspended in aerated solutions of sterile salts solution and in soil extract seeded with the mixed bacterial isolates 48-1 and 48-2. There was

no significant increase in sporulation in the bacteria-free solutions after 48 hr and longer, but numbers of sporangia were greatly increased in the nonsterile soil extract treatment and in the solution seeded with the bacterial isolates.

Effect of nutrients on production of sporangia.—As it has long been known that sporangium production by water molds occurs most readily under low nutrient status (6), the effect of organic nutrients on sporangium development was considered. Agar-mycelial discs cut from the margin of colonies on V-8 agar of increasing thickness to provide increasing amounts of nutrients were incubated in sterile and nonsterile soil extract. The results (Table 2) indicated that greater amounts of nutrients added with the discs from the 10- and 15-ml agar plates reduced the numbers of sporangia formed in sterile soil extract, and appeared to retard the onset of sporangium production in nonsterile soil extract. After further incubation in nonsterile soil extract, sporangia were formed in abundance on discs of all thicknesses.

Glucose at 10 to 1,000 ppm suppressed or retarded sporangium production in varying degrees in both sterile and nonsterile soil extract (Fig. 5). In sterile soil extract, glucose at 1,000 ppm completely suppressed sporangium production throughout the experiment. Numbers of sporangia did not increase in the sterile solution beyond the 2-day period. In nonsterile soil extract, the suppressing effect of 10- and 100-ppm levels of glucose at 1 day appeared to have been overcome after 2 days. After 3 days (not indicated in Fig. 5), sporangia became abundant (> 200 sporangia/10 fields) in the 1,000-ppm glucose treatment as well. Continued incubation beyond 3 days resulted in numbers of sporangia numerically indistinguishable in all of the treatments in nonsterile soil extract.

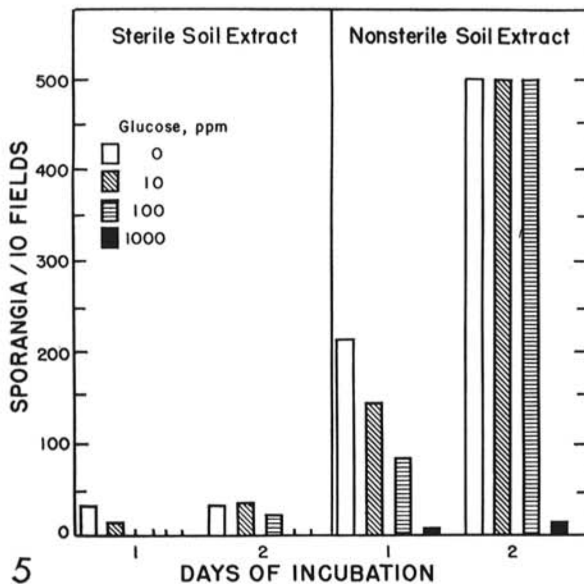
TABLE 2. Effect of agar thickness on sporangium production by discs of *Phytophthora cinnamomi* in soil extracts

Disc thickness[a] (agar vol/plates), ml	Avg no. of sporangia/disc: Autoclaved soil extract, 1 Day	Autoclaved soil extract, 2 Days	Nonsterile soil extract, 1 Day	Nonsterile soil extract, 2 Days
2	21	21	22	>100
5	13	13	5	75
10	5	5	6	6
15	3	3	4	4

[a] 5-mm diam discs cut from the margin of 3-day-old colonies grown on varying volumes of V-8 agar were placed in 5 ml of solution.

The two bacterial isolates supplied together to sterile soil extract stimulated sporangium production by washed mats of the fungus in the presence of either glucose or glutamic acid at 100 ppm (Fig. 6). Glucose at 100 ppm prevented sporangium production in sterilized soil extract, and reduced the number of sporangia formed in the solution with the bacteria. Glutamic acid, also inhibitory to sporangium formation at 100 ppm in sterile solution, apparently permitted greater number of sporangia to develop in the bacterial culture than when it was not present. Glucose supplied along with glutamic acid allowed only slightly fewer sporangia to develop than glutamic acid alone on mats incubated with the bacteria.

The above results suggested that *P. cinnamomi,* which has not been shown to produce sporangia within a medium in which it has grown, might be induced to



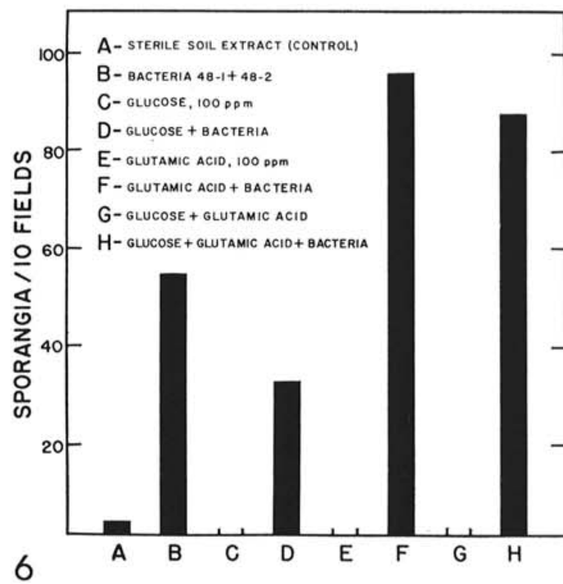


Fig. 5-6. 5) Effect of glucose on sporangium production by washed mats of *Phytophthora cinnamomi.* Mats were suspended in autoclaved or nonsterile soil extract supplemented with several concentrations of glucose. **6)** Effect of glucose and glutamic acid on bacterially induced sporangium production. Washed mats suspended in the treatments were counted after 4 days at 24 C.

do so by inoculation of an appropriately diluted medium with both fungus and bacteria. To test this, sterile V-8 broth diluted 0, 1:5, 1:10, 1:50 with water was seeded with isolates 48-1 and 48-2 and an agar disc of *P. cinnamomi*. After 1 week at 25 C, averages of 12 and 28 sporangia were observed, respectively, on the mats in the medium diluted 1:10 and 1:50, but no sporangia were observed in the 1:5 or 0 dilution. Sporangia were not formed in media without bacteria. Thus, sporangium production by *P. cinnamomi* in its original growth medium was shown to be possible, provided specific bacteria were present and the nutrient level of the medium was low.

Taxonomic relationship of bacterial isolates.—Both of the isolates are gram-negative, motile, polar-flagellated, strictly aerobic bacilli. Accordingly, they belong to the family *Pseudomonadaceae* as defined in *Bergey's Manual* (2). Neither isolate produced fluorescent pigments or reduced nitrate. When studied by standard methods, isolate 48-1 accumulated poly-β-hydroxybutyric acid and utilized D-arabinose, cellobiose and L-threonine, in addition to many other substrates. Based on its cultural and nutritional pattern, this isolate appeared to be related to the nonfluorescent "pseudomallei group" of pseudomonads characterized by Ballard et al. (1). It was not assigned to a species. Isolate 48-2, which differed in several significant respects from isolate 48-1 (in that it did not accumulate poly-β-hydroxybutyric acid, utilize cellobiose, or hydrolyze gelatin), was not identified.

DISCUSSION.—These experiments confirm the observations of Chen & Zentmyer (4, 5) that *P. cinnamomi* can be induced to form sporangia under axenic conditions. Agar-mycelial discs and washed young mycelial mats form at least a few sporangia when inoculated in sterile salt solutions of certain composition and in some sterilized aqueous soil extracts. The age of the mycelia is important: only young, 1- or 2-day-old mycelial mats formed sporangia in these experiments (Fig. 1) and in those of Chen & Zentmyer (5). In addition, the nearly complete exhaustion of external nutrients by thorough washing, or by dilution, and the presence of certain inorganic elements, especially Ca, in the suspending solution, seem to be required (4, 5).

Aeration of solutions in which mycelial mats were incubated greatly stimulated sporangium production. The stimulating effect of increased aeration was apparent after only 18 hr in both sterile and nonsterile solutions. Thus, aeration appeared to affect the early production of sporangia that occurs independent of bacterial stimulation. The greatest numbers of sporangia were produced in aerated solution seeded with the bacterial isolates; however, the effect of aeration seemed additive to the bacterial stimulation.

Except for the isolates of *Chromobacterium violaceum* tested by Zentmyer (12), all of the bacteria that have so far been shown to stimulate sporangia in *P. cinnamomi* have been classed as pseudomonads (3, 7, 9). Isolates 48-1 and 48-2, also, appear to belong to the genus *Pseudomonas*. Loss or attenuation of sporangium-stimulating ability by specific bacterial isolates was noted by previous workers (3, 9, 12). However, the isolates used in these experiments, maintained in nutrient broth and soil culture, have retained their stimulating ability for more than 1 year.

The precise role of bacteria in stimulating sporangium production by *P. cinnamomi* has not been determined. Several investigators have concluded that specific bacteria produce a metabolite that triggers sporangium production by the fungus (3, 7, 9, 12). Marx & Haasis (9) obtained aseptic induction of sporulation in sterilized soil extract separated from nonsterile soil extract by a filter membrane in a special apparatus. Chee & Newhook (3) obtained similar results by continuous passage of filter-sterilized soil extract over a mycelial mat of *P. cinnamomi*. However, in each of these cases, it cannot be ruled out that sporangia were induced by a nutrient-depleting effect of the diluting solution; in effect, the phenomenon of axenic production of sporangia observed by Chen & Zentmyer (4, 5).

Essential to sporangium production is the absence of high levels of organic nutrients. Glucose, glutamic acid, or a complete nutrient culture medium suppressed sporangium production. However, in nonsterile systems this suppression was overcome, suggesting that the bacteria utilize and decrease the exogenous nutrients in these cultures. But since only certain bacteria have been found to induce sporangium production in gnotobiotic culture, nutrient-depletion alone, which could be expected to result from catabolic activities of many organisms, does not seem a completely tenable explanation. Alternatively, the active bacteria might specifically remove a hypothetical inhibitor of sporulation from the fungus or its environment; or, as suggested by Zentmyer (12) and others (3, 7, 8, 9), the bacteria may produce a specific metabolite which induces or stimulates sporangium production.

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