

Stimulation of Sexual Reproduction in the A2 Mating Type of *Phytophthora cinnamomi* by Oleic Acid and Lipids from Avocado Roots

A. I. Zaki, G. A. Zentmyer, J. J. Sims, and N. T. Keen

Department of Plant Pathology, University of California, Riverside 92521.
Accepted for publication 28 July 1982.

ABSTRACT

Zaki, A. I., Zentmyer, G. A., Sims, J. J., and Keen, N. T. 1983. Stimulation of sexual reproduction in the A2 mating type of *Phytophthora cinnamomi* by oleic acid and lipids from avocado roots. *Phytopathology* 73:199-203.

Lipids extracted from avocado roots stimulated the formation of oogonia and oospores in four A2 mating type isolates of *Phytophthora cinnamomi*. Fractionation of the extracts showed that biological activity was associated with fractions that yielded spectral data similar to those of unsaturated fatty acids and their triglycerides. Authentic oleic acid, triolein, and, to a lesser extent, palmitoleic and linoleic acids, also stimulated the formation of oogonia and oospores in these isolates. Neither the natural lipids from

avocado roots nor the authentic compounds stimulated sexual reproduction in the A1 mating type. Stimulation of sexual reproduction in the A2 mating type with oleic acid or triolein occurred when the fungal isolates were grown in V-8 broth for 48–72 hr, washed momentarily in sterilized distilled water, and placed between two sheets of cheesecloth impregnated with 0.5–10 mg of the test substances. Oogonia and oospores did not form when these conditions were varied.

Sexuality in *Phytophthora cinnamomi* Rands is controlled by two compatibility factors (16). Crosses between A1 and A2 mating type isolates normally result in the formation of oogonia and oospores (4–6). The nature of sexual compatibility in *P. cinnamomi* and in other heterothallic *Phytophthora* species is not known. It is difficult to determine because a successful mating between the two opposite mating types requires intimate contact and conjugation of their hyphae and is dependent on the nutritional and environmental conditions under which they are grown (21). Attempts by different workers to isolate from *Phytophthora* species hormones or hormonelike substances such as those found in certain aquatic fungi (1) have, so far, been without success. Oospores, however, are formed when isolates of opposite mating types are placed in close contact with each other but are physically separated by a polycarbonate membrane (9). In the absence of one of the two mating types, the other (usually an A2 mating type isolate) may produce oospores under the influence of certain biological (2, 12, 17), chemical (13, 18, 19), or physical (14) stimuli. Production of oospores in aged cultures of single mating type isolates (17) or under the influence of chloroneb (12) has recently been shown to be due to the appearance of the opposite mating type (10). Zentmyer (20) observed the formation of oospores by A2 mating type isolates of *P. cinnamomi* in aqueous extracts of avocado roots. In this report, we describe the influence of certain components of the lipid fraction from avocado roots and of various authentic lipids on the formation of oogonia and oospores in A2 mating type isolates of *P. cinnamomi*.

MATERIALS AND METHODS

Isolate Pc40 of *P. cinnamomi* (A2 mating type isolate from avocado in California) was used throughout this work. In certain tests, isolates Pc94 and Pc190 (A2 mating type isolates from avocado in California), isolate Pc38 (A2 mating type isolate from avocado in Honduras), isolate Pc138 (A1 mating type isolate from avocado in California), and isolate Pc97 (A1 mating type isolated from camellia in California) were also used. The fungus was grown for 1–3 wk on cleared V-8 agar that was prepared by mixing 608 ml of Campbell's V-8 juice with 10 g of CaCO₃. The mixture was centrifuged at 4,000 rpm for 20 min. Two hundred milliliters of the supernatant was mixed with 800 ml of distilled, deionized water

and 15 g of agar and autoclaved at 15 psi for 20 min. Plugs of these cultures (0.5 cm in diameter) were transferred to 5-cm petri plates containing cleared V-8 broth and incubated at 21 C for 1, 2, or 3 days. The mycelial mats (approximately 0.8 cm in diameter) that formed under these conditions were washed momentarily in sterilized, distilled water and tested for production of oogonia and oospores in the presence of avocado root extracts or various authentic lipid substances. The test method involved impregnation of sterilized cheesecloth sheets (1 cm²) with a known amount of the test substance in 10–200 μ l of hexane (normally 0.5–10.0 mg of the test substance for two sheets). The solvent was allowed to evaporate, and a mycelial mat was placed between two impregnated sheets that were placed in a 5-cm petri plate containing 7 ml of sterile, distilled water (Fig. 1). The plate was

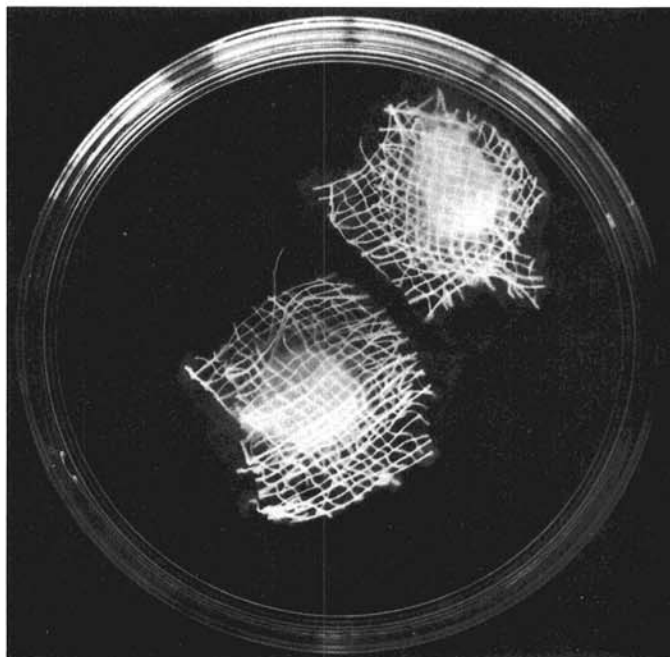


Fig. 1. The "cheesecloth" method of bioassay. Mycelial mats of isolate Pc40 of *Phytophthora cinnamomi* were placed between two layers of sterilized cheesecloth impregnated with oleic acid or other lipids. Sterilized distilled water was added and plates were incubated in the dark for 4 days at 21–24 C and examined for production of oogonia.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

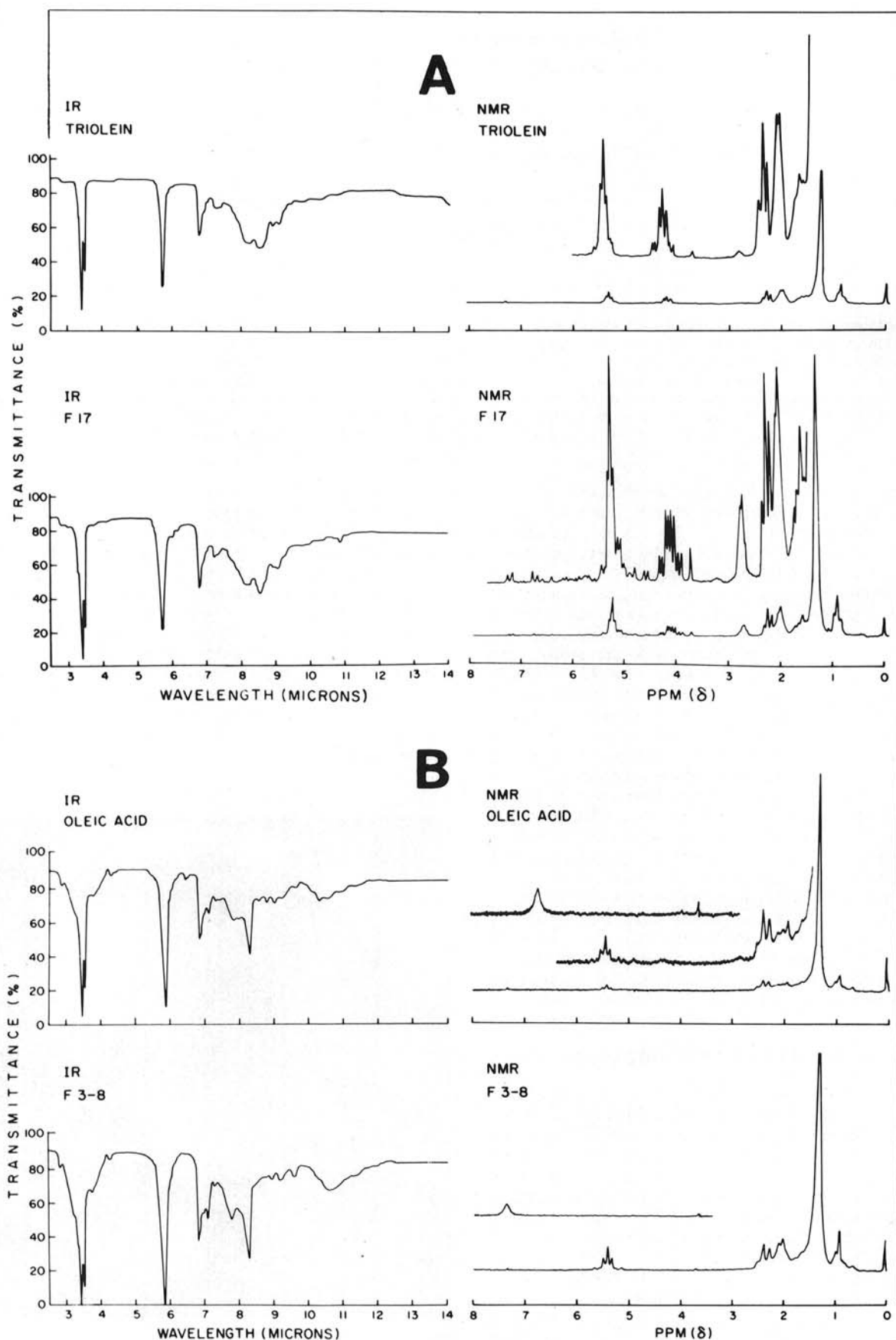


Fig. 2. Nuclear magnetic resonance (NMR) and infrared (IR) spectra of lipids that stimulated oospore formation in A2 mating type isolates of *Phytophthora cinnamomi*. **A**, Spectra of triolein and of fraction F17 obtained by fractionation of hexane extracts of avocado roots on a 45-cm silica gel column with 3% ethyl acetate in hexane. **B**, Spectra of oleic acid and of fraction F3-8 obtained by fractionation of hexane extracts of avocado roots using high pressure liquid chromatography and a Biosil silica gel column. The column was eluted with 10% ethyl acetate in hexane.

placed in an incubator in the dark at 21 or 24 C for 4–14 days and examined periodically under a bright-field microscope at a magnification of 160 or 400.

Avocado feeder roots were collected from trees in the field or from Mexican seedlings grown in a nutrient solution in the greenhouse (21). Air-dried roots were extracted with 95% ethanol (25 ml of ethanol per gram of root tissue). Ethanol was evaporated in vacuo, and the residue was partitioned in equal volumes of water and chloroform. The chloroform solution was dried with anhydrous MgSO₄ and evaporated to dryness. The residue was then partitioned in equal volumes of hexane and 90% methanol in water. The hexane fraction was concentrated and fractionated on a 45 × 2.5-cm silica gel column (M/L D-37 Davison grade H, 60–200 mesh silica gel), using 250-ml portions of hexane and hexane-ethyl acetate mixtures. The various fractions were concentrated and tested for their stimulatory effect on the formation of sexual structures in *P. cinnamomi*. The active fractions were further purified by high pressure liquid chromatography (HPLC; Biosil column eluted with 10% ethyl acetate in hexane) and bioassayed. Infrared (IR) and nuclear magnetic resonance (NMR) spectra were obtained of those fractions that induced the formation of oogonia and oospores, and these were compared with the spectra of authentic lipid compounds. These included oleic acid (Sigma grade, 99% pure, Sigma Chemical Co., St. Louis, MO), triolein (glyceryl trioleate, synthetic, 99% pure, U.S. Chemical Co., Cleveland, OH), *cis*-linoleic acid (Sigma grade III, 99% pure), palmitoleic acid (*cis*-9-hexadecanoic acid, Sigma grade, 99% pure), and β -sitosterol (Sigma grade II, from soybean). Triolein was further purified by thin-layer chromatography (silica gel 254, hexane/diethyl ether/acetic acid, 90:10:1, v/v, as the developing solvent). The other lipid compounds were tested without further purification. Solutions of each compound were prepared by dissolving 50 mg in 1.0 ml of hexane or diethyl ether.

RESULTS

Effect of lipids from avocado roots on the formation of oogonia and oospores in isolate Pc40 of *P. cinnamomi*. Substances in the ethanol extracts from avocado feeder roots induced the sexual reproduction in isolate Pc40 of *P. cinnamomi*. One to 40 oospores formed in each 0.8-cm mycelial mat. When the residue of the ethanol extracts was partitioned between water and chloroform, the oospore-inducing activity was found only in the chloroform fraction. The chloroform fraction, like the ethanol fraction, induced the formation of variable numbers (0–150) of oogonia and oospores per mycelial mat. When the chloroform solution was partitioned between 90% methanol and hexane, the activity moved to the hexane fraction, which consistently induced the formation of a large number of oogonia (>300 per mycelial mat). The majority of these were slightly pigmented and formed morphologically mature oospores (spores with thick walls and amphigynous antheridia). After the hexane fraction was concentrated and fractionated on a silica gel column, the oospore-inducing activity was associated with substances that eluted with 1, 2, or 3% ethyl acetate in hexane. The IR and NMR spectra of the substances that were eluted with 3% ethyl acetate in hexane (fraction F17) resembled but were not identical to those of triolein (Fig. 2A). No further purification or characterization was made of these substances.

When the crude hexane fraction from avocado roots was chromatographed using HPLC, a number of fractions were collected that induced the formation of oogonia and oospores in isolate Pc40. None of these fractions appeared to contain a single compound, but the IR and NMR spectra of fraction F3-8 (Fig. 2B) suggested the occurrence of one or more unsaturated fatty acids similar to oleic acid. Attention was accordingly directed to the possible biological activity of authentic fatty acids and their triglycerides.

Influence of authentic lipids on the formation of oogonia and oospores in isolate Pc40 of *P. cinnamomi*. Preliminary experiments revealed the formation of oogonia and oospores by isolate Pc40 of *P. cinnamomi* under the influence of various lipids, notably oleic

acid and triolein, but the formation of these sexual structures was variable and inconsistent. High production occurred on certain mycelial mats, whereas similarly treated mats produced few oogonia in localized areas or did not form any sexual structures. Such variability was not observed when the hexane fraction from avocado roots was used. It was then found that the type of medium in which the mycelial mats were grown before treatment with the test substance and the duration of growth in this medium strongly affected the formation of oogonia and oospores in the presence of oleic acid and triolein. Oogonia and oospores were not formed in mycelial mats that had grown for only 24 hr in V-8 broth or those grown for 24, 48, or 72 hr in media other than V-8 broth (potato-dextrose broth or liquid synthetic medium [15]). Oogonia and oospores were consistently formed, however, when mycelial mats were treated with oleic acid or triolein following growth for 48–72 hr in V-8 broth.

Mycelial mats that were grown for 48 hr in V-8 broth and then placed between two sheets of cheesecloth impregnated with 0.5 mg of oleic acid or triolein produced low numbers of oogonia (six oogonia per mycelial mat for oleic acid and 28 oogonia per mycelial mat for triolein) 4 days after treatment. Oogonia were generally produced in localized areas of the mycelial mats, perhaps because of the uneven distribution of the test substance on the impregnated cheesecloth sheets. As the amount of oleic acid or triolein was increased (up to 2.5 mg of oleic acid or 10 mg of triolein), the number of oogonia produced per mycelial mat also increased, although the difference was not significant statistically at the 5% level (Table 1). Distribution of the oogonia on the mycelial mats was generally uniform under these conditions. Five and 10 mg of oleic acid produced lower numbers of oogonia than did 2.5 mg. The total number of oogonia did not appear to increase as the time of incubation increased from 4 to 14 days after treatment with oleic acid or triolein. The majority (>50%) of the oogonia produced 4 days after treatment subsequently formed morphologically mature oospores (Fig. 3). A considerable proportion (<50%) appeared to remain as oogonia with thin walls that failed to form oospores. The aborted oogonia were distinctly different in appearance from chlamydospores, which lacked the amphigynous antheridia. No attempt was made to test the viability of these sexual structures. Palmitoleic acid (2 mg per mycelial mat) stimulated the formation of 20 oogonia per mycelial mat. Linoleic acid (0.5 and 2.5 mg per mycelial mat) induced the formation of three and 20 oogonia, respectively, per mycelial mat. Higher amounts of linoleic acid (5 or 10 mg per mycelial mat) appeared to be toxic to the fungus and did not induce the formation of oogonia. The addition of β -sitosterol to oleic acid or triolein did not lead to an increase or decrease of the total number of oogonia and oospores produced as compared to the level produced with oleic acid or triolein alone. Mycelial mats placed between cheesecloth impregnated with the solvents hexane

TABLE 1. Production of oogonia by mycelial mats of isolate Pc40 of *Phytophthora cinnamomi* grown in V-8 juice broth for 48 hr and subsequently placed between two sheets of cheesecloth impregnated with different amounts of oleic acid or triolein

Oleic acid or triolein used to impregnate two sheets of cheesecloth (mg)	Mean ^a number of oogonia per mycelial mat (8 mm in diameter) observed 4 days after treatment with	
	Oleic acid	Triolein
0	0 e	0 e
0.5	6 e	28 de
1.0	39 cde	67 bcd
2.0	62 bcd	64 bcd
2.5	139 a	73 bcd
5.0	117 ab	87 abd
10.0	96 ab	92 abc

^a Each value represents the mean of the number of oogonia observed on six mycelial mats. Values followed by a common letter are not significantly different from each other at the 1% level. The compound mean of all oleic acid treatments is not significantly different from the compound mean of all triolein treatments at the 5% level.

or diethyl ether did not form oogonia.

The amounts of oleic acid or triolein that stimulated high production of oogonia and oospores in isolate Pc40 of *P. cinnamomi* also induced the formation of high numbers of oogonia and oospores in isolates Pc94, Pc190, and Pc38 (A2 mating type isolates of *P. cinnamomi*). Oogonia and oospore production was not observed under any conditions by isolates Pc97 or Pc138 (A1 mating type isolates of *P. cinnamomi*).

DISCUSSION

Among the controversial aspects of sexuality in *Phytophthora* is

the induction of sexual reproduction in unpaired normally heterothallic cultures by several types of stimuli. Thus, *P. cinnamomi* as well as other species of *Phytophthora* can function as homothallic fungi when the proper chemical stimulus is applied, particularly to the A2 mating type. Hormonal control (9) and nutritional regulation (18,21) of sexual reproduction in *Phytophthora* have been proposed.

Data presented in this report indicate that formation of oospores by the A2 mating type isolates of *P. cinnamomi* can be stimulated in the absence of any external nutrients by supplying the fungus with oleic acid or other derivatives of this fatty acid, notably triolein.

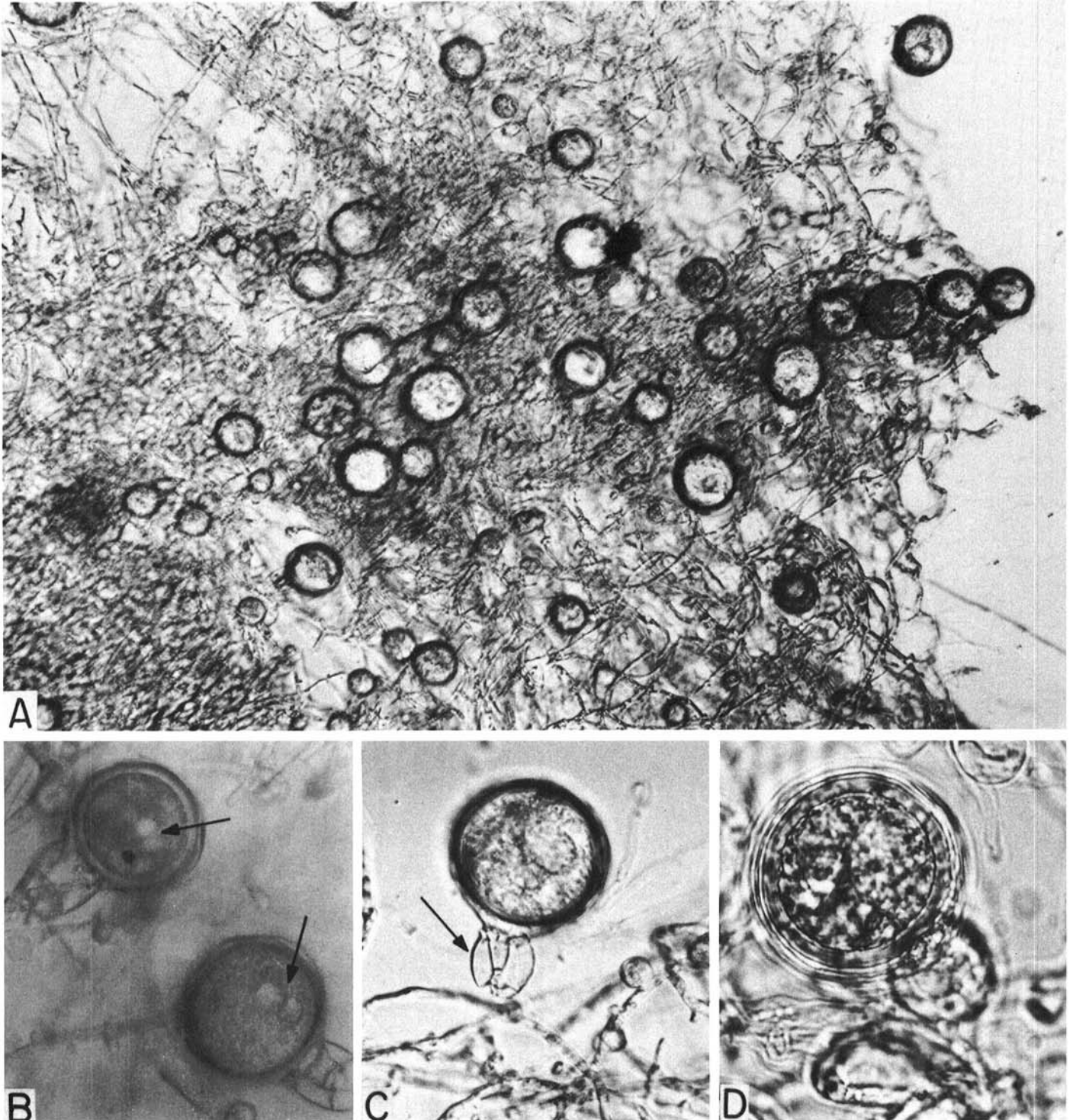


Fig. 3. Oogonia and oospores of different stages of maturity produced by isolate Pc40 of *Phytophthora cinnamomi* in the presence of oleic acid. **A**, Oogonia and oospores ($\times 160$) observed under the light microscope; **B**, partially developed oospores ($\times 400$), showing pellucid bodies (arrows); **C**, an oospore, showing well-developed amphigynous antheridium (arrow) typical of *P. cinnamomi*; and **D**, a mature oospore ($\times 400$), showing various layers of the spore wall.

This was accomplished by the cheesecloth method of bioassay. Nevertheless, this stimulation was only apparent under a narrow range of specific growth and cultural conditions that preceded the treatment of the fungus with these lipid compounds. The required growth period before exposure of the fungus to oleic acid could indicate that the fungus had to reach a certain stage in its vegetative development (or a certain stage of physiological maturity) in V-8 broth before it could respond to the presence of chemical stimuli in the absence of other nutrients by producing oogonia and oospores.

Oleic acid might function by triggering certain metabolic events leading to the initiation of sexual reproduction by normally self-sterile A2 isolates of *P. cinnamomi*. Alternatively, oleic acid or a metabolic product might function as a sexual hormone or stimulate the action of a naturally occurring, but yet unidentified sexual hormone. An alternative hypothesis is that oleic acid may act as a carrier of another unidentified factor normally present in the growth medium, such as a sterol. Various workers have implicated sterols in the stimulation of oospore formation in homothallic (3,7,8) or heterothallic (15) *Phytophthora* spp. Elliott et al (3) found that the addition of oleic acid to a medium with 10 mg of cholesterol per liter enhanced the production of oospores by the homothallic fungus *P. cactorum*. Harnish (7) reported that triolein alone was not active in inducing sexual reproduction in *P. cactorum*, but in combination with a low concentration (>0.5 mg/l) of β -sitosterol, it enhanced oospore production as much as sixfold. In our experiments, β -sitosterol alone did not stimulate the formation of oospores in isolate Pc40 of *P. cinnamomi*, and addition of β -sitosterol to oleic acid or triolein did not further increase the formation of oogonia and oospores.

The absence of oospore formation under the influence of oleic acid or triolein in the A1 mating type isolates of *P. cinnamomi* was consistent with findings by other workers (14,20). Oogonial formation by the A2 mating type isolates of *P. cinnamomi* occurred 3 or 4 days after treatment with oleic acid or triolein. The length of this period is the same as that required for the homothallic formation of oogonia under the influence of various stimuli and is also the same as the period required for the formation of oogonia in normal crosses between the A1 and the A2 mating types of this fungus.

The lipids extracted from avocado roots stimulated the formation of greater numbers of oogonia and oospores than did oleic acid or triolein. In addition, active fractions from the silica gel and HPLC columns yielded spectral data that were not identical to authentic samples of these compounds. Apparently, therefore, more than one natural substance may be responsible for the biologic activity of avocado root extracts. Further purification and characterization of these substances remain to be done. Oleic acid and its ester derivatives are commonly found in plants. Other derivatives of this fatty acid are structural components of certain chemical constituents of suberin, which in turn is a major component of plant roots (11). Possibly, these naturally occurring chemical derivatives of oleic acid stimulate sexual reproduction in the A2 mating type isolates of *P. cinnamomi* more efficiently than the free acid does. Avocado root extracts and, to a lesser extent, root extracts of other *Persea* sp. and unrelated plant species

(Zentmyer, *unpublished*) were stimulatory to oospore formation in the A2 mating type isolates of *P. cinnamomi*. Further purification and characterization of the factors responsible may prove them to be structurally related to oleic acid.

LITERATURE CITED

1. Barksdale, A. 1963. The role of hormone A during sexual conjugation in *Achlya ambisexualis*. *Mycologia* 55:627-632.
2. Brasier, C. M. 1971. Induction of sexual reproduction in single A² isolates of *Phytophthora* species by *Trichoderma viridae*. *Nature* (Lond.) New Biol. 231:283.
3. Elliott, C. G., Hendrei, M. R., and Knights, B. A. 1966. The sterol requirement of *Phytophthora cactorum*. *J. Gen. Microbiol.* 42:425-435.
4. Galindo A., J., and Gallegly, M. E. 1960. The nature of sexuality in *Phytophthora infestans*. *Phytopathology* 50:123-128.
5. Galindo A., J., and Zentmyer, G. A. 1964. Mating types in *Phytophthora cinnamomi*. *Phytopathology* 54:238-239.
6. Gallegly, M. E., and Galindo, J. 1958. Mating types and oospores of *Phytophthora infestans* in Mexico. *Phytopathology* 48:274-277.
7. Harnish, W. N. 1967. Effects of sterols on sexual reproduction of *Phytophthora cactorum*. *Proc. W.V. Acad. Sci.* 40:99-104.
8. Hendrix, J. W. 1965. Influence of sterols on growth and reproduction of *Pythium* and *Phytophthora* spp. *Phytopathology* 55:790-797.
9. Ko, W. 1978. Heterothallic *Phytophthora*: Evidence for hormonal regulation of sexual reproduction. *J. Gen. Microbiol.* 107:15-18.
10. Ko, W. H. 1981. Reversible change of mating type in *Phytophthora parasitica*. *J. Gen. Microbiol.* 125:451-454.
11. Kolattukudy, P. E. 1980. Biopolyester membranes of plants: Cutin and suberin. *Science* 208:990-1000.
12. Mukerjee, N., and Roy, A. B. 1962. Microbial influence on the formation of oospores in culture by *Phytophthora parasitica* var. *sabdariffae*. *Phytopathology* 52:583-584.
13. Noon, J. P., and Hickman, C. J. 1974. Oospore production by a single isolate of *Phytophthora capsici* in the presence of chloroneb. *Can. J. Bot.* 52:1591-1592.
14. Reeves, R., and Jackson, R. M. 1974. Stimulation of sexual reproduction in *Phytophthora* by damage. *J. Gen. Microbiol.* 84:303-310.
15. Ribeiro, O. K., Erwin, D. C., and Zentmyer, G. A. 1975. An improved synthetic medium for oospore production and germination of several *Phytophthora* species. *Mycologia* 67:1012-1019.
16. Savage, E. J., Clayton, C. W., Hunter, J. H., and Gallegly, M. E. 1968. Homothallism, heterothallism, and interspecific hybridization in the genus *Phytophthora*. *Phytopathology* 58:1004-1012.
17. Tsao, P. H., Ugale, R., Hobbs, S., and Farih, A. 1980. Control of homothallic oospores formation in *Phytophthora parasitica* by culture manipulations. *Trans. Br. Mycol. Soc.* 75:153-156.
18. Waterhouse, G. M. 1950. Identification of *Phytophthora* species by means of oospores produced in dual cultures. *Proc. Int. Bot. Cong.* 7th. Stockholm. 1950:413-414.
19. Zentmyer, G. A. 1952. A substance stimulating sexual reproduction in *Phytophthora cinnamomi*. (Abstr.) *Phytopathology* 42:24.
20. Zentmyer, G. A. 1979. Stimulation of sexual reproduction in the A² mating type of *Phytophthora cinnamomi* by a substance in avocado roots. *Phytopathology* 69:1129-1131.
21. Zentmyer, G. A., Klure, L. J., and Pond, E. C. 1979. The influence of temperature and nutrition on the formation of sexual structures by *Phytophthora cinnamomi*. *Mycologia* 71:55-67.