# Stimulation of Sexual Reproduction in the $A^2$ Mating Type of *Phytophthora cinnamomi* by a Substance in Avocado Roots

G. A. Zentmyer

Professor and plant pathologist, Department of Plant Pathology, University of California, Riverside 92521.
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## ABSTRACT

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Oospore formation was induced in unmated isolates of the $A^2$ mating type of *Phytophthora cinnamomi* by a substance in an extract or diffusate of avocado (*Persea americana*) roots. Thus, this heterothallic fungus functioned as a homothallic organism when provided with a specific organic (hormone-like) stimulus. The sexual stage also was initiated by the root extract in $A^2$ mating types of *P. capsici* and *P. drechsleri*, but not in $A^2$ types of several other *Phytophthora* spp. $A^1$ isolates of *P. cinnamomi* did not produce oospores in the avocado root extract. Similar stimulation was not found in extracts from roots of several other hosts of the pathogen. The stimulatory substance appears to be organic in nature, is nonvolatile, heat-stable, water-soluble, and can be extracted by several organic solvents.

Sexual reproduction in fungi is a complex and intricate process, with varied mechanisms of induction and stimulation. Little is known about the specific mode of action of substances inducing sexual reproduction, except for substances known as sex hormones or pheromones (10), eg, antheridiol (1,12,14), sirenin (11–13,19), and the trisporic acids (4,28). There are many reports of the induction or stimulation of sexual reproduction in fungi by other microorganisms (17,20–22,26), but no reports of such activity from extracts of higher plants. The substance described in this paper does not meet the criterion for a sexual hormone: ". . . a diffusible substance playing a specific role in the sexual reproduction of the organism by which it is produced (28)," but does meet Machlis'(12) designation of an "erogen," a substance controlling the induction and differentiation of sexual structures.

The genus *Phytophthora* includes homothallic species and heterothallic species with two mating types designated as $A^1$ and $A^2$ (7). *Phytophthora cinnamomi* Rands generally is regarded as a heterothallic species (6,27). The $A^2$ type is the common form with world-wide distribution, but the $A^1$ type is very limited geographically and in the number of hosts (30).

In an earlier note (29), I reported that *P. cinnamomi* could act as a homothallic fungus, forming the sexual stage when a culture derived from a single zoospore was placed in an aqueous extract of avocado (*Persea americana* Mill.) roots. Thus the species was shown to be basically bisexual, or monoecious according to the terminology of Esser and Kuenen (5). The original isolate tested was from avocado and later was found to be of the $A^2$ mating type. $A^1$ isolates were not identified at that time. Several other isolates of *P. cinnamomi* from chestnut, pine, and heath also produced oospores in the avocado root extract, as did an isolate of *P. drechsleri*; these all later were found to be of the $A^2$ mating type. This paper is an elaboration of the early brief report.

In the past few years, several other reports on the production of the sexual stage by $A^2$ mating type isolates of *P. cinnamomi* and several other species of *Phytophthora* have been published. Brasier (2) reported that *Trichoderma* spp., especially *T. viride*, produce a volatile substance in culture that initiates sexual reproduction in $A^2$ types of *P. botryosa, P. cambivora, P. capsici, P. cinnamomi, P. cryptogea, P. drechsleri, P. infestans, P. nicotianae,* and *P. palmivora*. Mircetich and Zentmyer (15) in 1966 reported production of a few oospores by *P. cinnamomi* ($A^2$ isolate) in mycelial mats buried in nonsterile soil; this could have been a response to *Trichoderma*, although the effect was not known at that time. Reeves and Jackson (23) buried mats of *P. cinnamomi* along with small pieces of roots in several soils, and observed oospore production in association with actively sporulating *Trichoderma*.

Noon and Hickman (18) found that incorporation of the organic fungicide chloroneb (2,5-dichloro-1, 4-dimethoxybenzene) in agar media stimulated oospore production by $A^2$ types of *P. capsici*. Reeves and Jackson (24) reported that wounding $A^2$ cultures of *Phytophthora* with a scalpel or cork borer also induced sexual reproduction.

## MATERIALS AND METHODS

Small feeder roots (approximately 1.0–1.5 mm in diameter) were collected from avocado trees in the field or from Mexican seedlings growing either in nutrient solution or in soil in the greenhouse. Roots were washed thoroughly, cut into pieces approximately 1 cm

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in length, and (in most experiments) were placed in jars and sterilized with propylene oxide. In some tests, roots were used without propylene oxide sterilization. Roots of other *Persea* spp. and of other hosts of *P. cinnamomi* were included in some of the tests.

To test for oospore production, two methods were used: (i) To prepare a root extract, root sections were placed in jars or flasks with sterile demineralized water (SDW) at the rate of 1–2 g of roots per 100 ml of SDW, and autoclaved for 15 min. The clear brown supernatant (root extract) was decanted and kept sterile at 4 C. (ii) To prepare root diffusates, sterile root sections were placed directly in small (6-cm diameter) petri dishes and covered with 7 ml of SDW. The first method was used in most experiments.

Cultures used in these tests were from the *Phytophthora* culture collection at the University of California, Riverside. Cultures were either grown on one of several agar media including V8 and synthetic agar (25) or in one-fifth strength V8 broth. Disks (5 mm in diameter) from agar cultures or 24- to 48-hr-old mats from liquid cultures were placed directly in the root extracts or diffusates. For most of the tests, an $A^2$ isolate of *P. cinnamomi* (Pc 40, ATCC 32992) was used. After placing disks or mats of the fungus in the root extract or diffusate, petri dishes were incubated in the dark at 21 C.

Various tests, including autoclaving, boiling, evaporating to dryness in a rotary evaporator, dialyzing, and extracting with several solvents, were run to provide information on the nature of the oospore-inducing compound.

## RESULTS

In the avocado root extract and in the root diffusate, typical amphigynous antheridia and oogonia were formed by single zoospore cultures of $A^2$ isolates of *P. cinnamomi* in 3–5 days, and thick-walled oospores in 4–7 days at 21 C (Fig. 1). Oospores produced in the root extract or diffusate usually were golden brown. Oospores varied in abundance from 25 or 30 to over 300 on a 5-mm disk. No oospores were formed on the V8 agar disks or the mats that were placed in SDW. Oospores were formed in the autoclaved root extract when it was prepared either from roots previously sterilized with propylene oxide or from roots not treated with propylene oxide.

Sex organs produced in the avocado root extract usually were larger than those produced by pairing $A^1$ and $A^2$ isolates (9). The average size of oogonia and oospores in the root extract was 41.8 and 35.7 $\mu$m, respectively, compared to averages of 35.7 and 31.7 $\mu$m for the same structures in paired cultures. Most of the oospores formed in the extract were typical and well formed, although some were quite abnormal, with poorly developed and misshapen oogonia and oospores.

Oospores were extracted from agar disks or mats and placed on water agar or clear V8 agar for germination. Approximately 5% of the oospores germinated, similar to results with paired cultures. All of the progeny from 20 germinated oospores were $A^2$ mating type and there was little variation in cultural appearance.

Fifty-two isolates of *P. cinnamomi*, of either $A^1$ or $A^2$ type, were tested for oospore production in the avocado root extract. Sexual reproduction was initiated only in the $A^2$ mating type; none of the 10 $A^1$ isolates produced oospores. Four of the 42 $A^2$ isolates did not respond to the root extract. This has been confirmed in many tests during the past several years. Results with 16 isolates are shown in Table 1.

Eight other *Phytophthora* spp. also were tested for oospore production in the avocado root extract. Oospores were produced in $A^2$ isolates of *P. capsici* and *P. drechsleri*, but not in $A^2$ isolates of *P. cambivora*, *P. cryptogea*, *P. infestans*, *P. meadii*, *P. nicotianae* var. *nicotianae*, *P. nicotianae* var. *parasitica*, or *P. palmivora* (Morphological Form 1) (32).

Aqueous extracts from roots of other hosts of *P. cinnamomi*, camellia (*Camellia japonica*), American chestnut (*Castanea dentata*), and heath (*Erica regerminans*), did not induce oospores in either mating type of *P. cinnamomi*, nor was an extract of cacao (*Theobroma cacao*) roots effective. Oospores were formed in extracts of roots of *Persea borbonia*, *P. indica*, and *P. skutchii*, although, in general, production in these other species was considerably less than in extracts from roots of *P. americana*. *Persea borbonia*, and *P. skutchii* are very resistant to *P. cinnamomi*, whereas *P. indica* and most cultivars of *P. americana* are very susceptible (31). Thus, there is no obvious relation between resistance to *P. cinnamomi* and stimulation of oospore production in the few species that have been tested.

**Tests for characterization of oospore-inducing factor.** The substance that stimulated oospore production was heat stable (to autoclaving for 20 min at 1.05 kg/cm$^2$ [15 lb]) at the normal pH of the extract (pH 5.5–6.0). The extract retained full activity after evaporation to dryness in a rotary evaporator, and some activity after boiling and evaporation to dryness. Dilution of the extract with water caused a proportionate reduction in the number of oospores produced. The factor was dialyzable, but only a portion of the activity was recovered by concentrating the dialysate. Activity was removed from aqueous solution by activated charcoal; some of

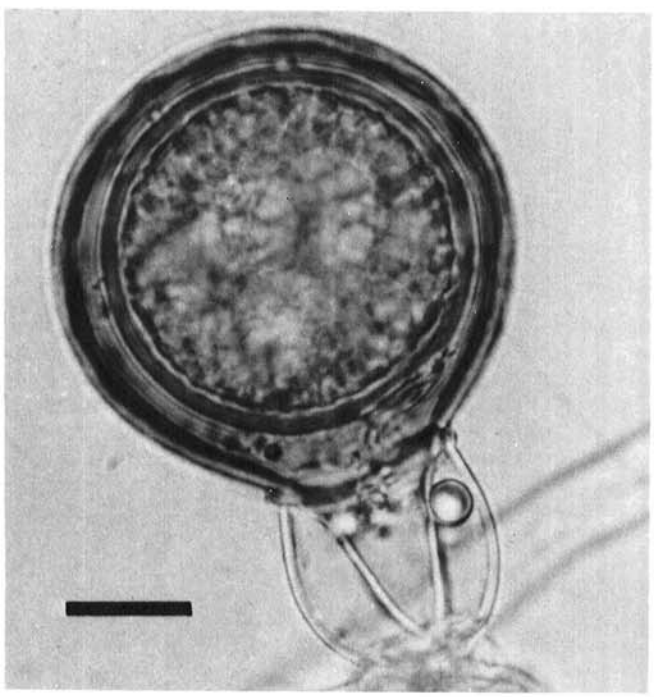

**Fig. 1.** Antheridium, oogonium, and oospore produced by a single-zoospore culture of $A^2$ mating type of *Phytophthora cinnamomi* in avocado root extract (× 2,125). Scale bar represents 10 $\mu$m.

TABLE 1. Production of oospores by $A^1$ and $A^2$ mating types of *Phytophthora cinnamomi* in avocado root extract

| Culture no. | Host | Origin | Mating type | Oospore production |
|---|---|---|---|---|
| Pc 35 | Avocado | Hawaii | $A^2$ | + |
| Pc 40 | Avocado | California | $A^2$ | + |
| Pc 62 | Macadamia | Hawaii | $A^1$ | – |
| Pc 70 | Myrtle | California | $A^2$ | + |
| Pc 73 | Eucalyptus | Australia | $A^2$ | – |
| Pc 97 | Camellia | California | $A^1$ | – |
| Pc 138 | Avocado | California | $A^1$ | – |
| Pc 140 | Apricot | Maryland | $A^2$ | + |
| Pc 163 | Avocado | Cameroon | $A^2$ | + |
| Pc 179 | Macadamia | Australia | $A^2$ | + |
| Pc 184 | Eucalyptus | Australia | $A^1$ | – |
| Pc 200 | Chestnut | USSR | $A^2$ | + |
| Pc 240 | Heath | California | $A^2$ | + |
| Pc 264 | Yucca | Australia | $A^1$ | – |
| Pc 277 | Rhododendron | Oregon | $A^2$ | + |
| Pc 320 | Camellia | Florida | $A^1$ | – |

the activity was recovered from the charcoal by extracting with acetone. Extraction with chloroform or ether removed the factor from aqueous solution indicating its organic nature; it was recovered from the ether fraction. Stimulation of oospore production was most active when the pH of the root extract was 5.8. There was no activity at pH 3, slight at pH 4, moderate at pH 8, and slight at pH 9. Additional tests are underway to attempt to characterize the active principle.

## DISCUSSION

The active substance in the avocado root extract acts similarly to other factors initiating sexual reproduction in single-zoospore cultures of heterothallic species of *Phytophthora* in that it is effective only with the $A^2$ mating type. This was the case with the volatile chemical produced by *Trichoderma* (2) and with chloroneb (18). Wounding of hyphae also stimulated oospore production only with $A^2$ isolates (24). This does not imply that all of these phenomena involve identical chemicals or an identical type of stimulus.

The apparently volatile nature of the stimulant produced by *Trichoderma* differentiates this from the factor in the avocado root extract. Moss et al (16) purified a metabolite from culture extracts of *T. viride* that induced oospores in *P. cinnamomi*. They identified the chemical as 6-(pent-1-enyl)-α-pyrone, but concluded that this chemical was not, on its own, responsible for the stimulation because, as the compound was purified, it became increasingly difficult to demonstrate biological activity.

The finding of Reeves and Jackson (24) that mechanical damage to hyphae of $A^2$ types of *Phytophthora* induces oospore formation introduces a different concept. They suggested that damage could induce oospore formation either through a physical or a chemical stimulus, and postulated that the "*Trichoderma* effect" also may result from damage to *Phytophthora* hyphae.

The selectivity of the stimulus for oospore production to $A^2$ mating types is somewhat analogous to the specificity of the sex hormones antheridiol and sirenin in fungi, which stimulate only one mating type. The trisporic acids, however, induce zygophore formation in both mating types of *Mucor mucedo* (28).

Sterols stimulate sexual as well as asexual reproduction and growth in some species of *Phytophthora* and *Pythium* (8); however, this response has only been reported with homothallic species or with paired $A^1 \times A^2$ isolates.

The seemingly unique presence of the factor in avocado roots is difficult to reconcile with the enormous host range of *P. cinnamomi* (over 900 plant hosts) (31). With additional testing, similar factors may be found in other plants.

Stimulation of $A^2$ types of *Phytophthora* to form oospores may be significant in relation to survival of the pathogen. In the case of *P. cinnamomi,* the infrequent occurrence of the $A^1$ type (3,30) and the wide distribution of the $A^2$ type may be related to the homothallic properties of the latter type. The $A^2$ type apparently has become well adapted to the production of oospores by some mechanisms other than the stimulation by the appropriate mating type. This ability provides the $A^2$ type with an efficient survival mechanism. In a different context, stimulating the pathogen to produce the sexual stage possibly could be considered a host defense mechanism, reducing production of sporangia which have enormous potential for accelerating disease development through the formation of zoospores. Preliminary tests show a significant reduction in sporangium production when the avocado root extract is added to a nonsterile soil extract that normally stimulates production of sporangia.

The substance in the avocado root extract is a unique example of an apparently specific chemical from a higher plant that can provide the stimulus for initiation of the sexual stage in a fungus.

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