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IMPROVED METHODS FOR USE IN STUDIES ON 
Phytophthora cinnamomi Rands AND OTHER
Phytophthora SPECIES

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ABSTRACT

A satisfactory method was devised for obtaining large numbers
of active zoospores of Phytophthora cinnamomi Rands. Aerial mycelium
from cultures grown on a pea agar medium is placed in non-sterile
soil extracts for periods of up to 48 hours. This method has proved
more consistent and provided larger numbers of zoospores than other
methods tried. Seedlings of blue lupin (Lupinus angustifolius L.)
have proved to be a very convenient host plant for infection studies
with P. cinnamomi. Blue lupin seedlings used as a selective bait were
more efficient than the apple technique in soil isolation tests for
P. cinnamomi and were also found suitable for isolation of several other
species of Phytophthora.

INTRODUCTION

The soil-borne pathogen Phytophthora cinnamomi Rands was first
described by Rands, who isolated it from stripe cankers of cinnamon
trees in Sumatra (Rands 1922). P. cinnamomi is the species most
commonly found in association with the serious root rot disease of
Pinus radiata D. Don and other hosts in New Zealand (Newhook 1959a).
This fungus is also associated with a disease of Pinus echinata Mill.
in south-eastern U.S.A., of pineapples in Hawaii, and of cinchona in
Malaya, and is also a major pathogen on over 125 host species. Like
many Phytophthora species, it is almost cosmopolitan.

Little is yet known about the conditions which govern infection
by zoospores of P. cinnamomi or about the behaviour of the fungus
in soil. To be able to investigate these aspects, a method for obtaining

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zoospores in quantity must be available, as well as a suitable test host and a good system of detecting the fungus in soil.

Several workers have described techniques for obtaining zoospores of \textit{P. cinnamomi}, but in this laboratory none of those tested in the past has been found consistently reliable. Hence a systematic testing of previously published techniques with various modifications was carried out, including those found useful for other \textit{Phytophthora} species.

1. METHODS OF OBTAINING ZOOSPORES

A commonly employed method is to grow the fungus on potato dextrose agar (PDA) or V–8 agar from which discs are cut and incubated in non-sterile soil extracts for two to three days. Using this method, Zentmyer (1959) obtained an average of 17.5 sporangia/sq. mm of mycelial disc at a temperature of 24°C, which he had found optimum. In this laboratory, however, it has been found that sporangia are produced relatively sparsely and inconsistently on PDA (both Difco and fresh), while V–8 juice is not available in New Zealand.

Before \textit{P. cinnamomi} was recorded, Wilson (1914) reported that pea, bean, and oat media were efficient and satisfactory for sporangial production by \textit{Phytophthora} species. Waterhouse (1931) grew \textit{Phytophthora} species on bean, oat, potato, and corn agar for sporangial production and found that best results were obtained with bean juice agar. A medium made from chick peas (\textit{Cicer arietinum} L.) was used by Keay (1953) to produce sporangia of \textit{Phytophthora infestans} (Mont.) de Bary. Zak (1954) used inoculated wheat grains in non-sterile soil water for sporangia production by \textit{P. cinnamomi}. Torgeson (1954) used pea broth medium to grow mycelium of \textit{P. cinnamomi} for inclusion in infection studies in soil.

In this laboratory tests for sporangial production were carried out with cultures on PDA containing various organic amendments and with agar media made up from seeds of several species of legumes and cereals and from seeds of other plants. In addition, sporangial production was attempted using inoculated seeds of legumes, cereals, and hemp in non-sterile soil extract. Chick pea agar, and garden pea (\textit{Pisum sativum} L.) agar gave far better results than other substrates tested. A solid medium was found to be most suitable, and garden pea agar was slightly better than bean agar.

The method of preparation of pea medium was basically the same as that used by Keay (1953) for \textit{P. infestans}, but was slightly modified to improve yields of zoospores of \textit{P. cinnamomi}. The medium currently being used is prepared from 200 g dried garden pea seeds, 20 g sucrose, 10 g agar, and 1 litre distilled (or deionised) water. The seeds are washed in tap water for an hour and then soaked in distilled water overnight. Most of the water is drained off and the seeds are homogenised in a Waring blender, after which the homogenate is made up to 1 litre with distilled water. This is simmered for half an hour with continuous stirring, then strained through fine muslin. The sucrose and agar, dissolved by heating with 50 ml of water, are added, and the whole is again made up to 1 litre with hot, distilled water. The medium is sterilised in Erlenmyer flasks for 15 minutes at 12 lb pressure.
Methods for use with *P. cinnamomi*

For zoospore production small amounts (4 mm sq.) of aerial mycelium are stripped from the surface of one-week-old cultures grown on the pea medium described above. These mycelial mats are placed in non-sterile soil extract for 48 hours at 26°C, then rinsed in distilled water and placed in fresh distilled water in a petri dish at 8°C for 15 minutes. They are then returned to room temperature until zoospores are released.

Suitably clear soil extracts are made up by adding approximately 1 g of soil to 100 ml water and allowing to settle for two days before filtering the supernatant through Whatman No. 1 filter paper. An occasional soil extract has been found in which sporangial production by mycelial mats was less than usual. In those cases addition of 1 g of “Bacto-Peptone” in water to 100 g of soil (air-dry basis) has considerably improved yield of sporangia. The addition of “Bacto-Peptone” was suggested by concurrent results from an investigation of the relationship of non-sterility of soil extracts to sporangial production, a full account of which will be published later.

The advantages of pea medium are: 1. Vegetative growth is rapid, providing ample material for mycelial mats, which are preferred to discs of cultures. 2. With a few exceptions sporangia are abundant on the mycelial mats after 24 hours in the soil extract at 24–28°C, with further increases after 48 hours. The average number of sporangia per square millimetre of mycelial mat after 48 hours' incubation is regularly found to be 60 or more, compared with nil or seldom more than 15/sq. mm on PDA.

Although the above technique was developed for production of zoospores of *P. cinnamomi*, it has been found equally satisfactory in this laboratory for sporulation of *Phytophthora nicotianae* var. *parasitica* (Dastur) Waterhouse, *Phytophthora cactorum* (Leb. and Cohn) Schroet, *Phytophthora cryptogea* Pethybr. and Laff., *Phytophthora syringae* Kleb., and *Phytophthora megasperma* Drechsler.

2. BLUE LUPIN AS A TEST HOST FOR *P. cinnamomi*

Much of the research of *P. cinnamomi* in this laboratory has been concerned with the infection of newly germinated seedlings of *Pinus radiata* (Newhook 1959a, 1961). The thick white susceptible radical is a satisfactory medium for infection studies, although one of the disadvantages of pine seedlings is the unevenness, and relatively slow rate of germination and growth. Blue lupin (*Lupinus angustifolius* L.) commonly used as a cover crop and reported as a host of *Phytophthora cinnamomi* in New Zealand by Newhook (1959b), has proved to be more satisfactory for studying host parasite relationships. It has the following suitable features: 1. Seedlings are ready for inoculation 60 hours after sowing. Germination of fresh seed is usually 100 per cent and very uniform. 2. Seedlings have robust white radicals which are easy to use and which show lesions as sharply defined brown or orange-coloured areas a few millimetres behind the root tip (Fig. 1). 3. Lesions develop within 24 hours even with low inoculum densities.
In this laboratory, infection experiments are carried out as follows. Lupin seeds are surface-sterilised by immersing in 50 per cent alcohol and then soaked in distilled water overnight. The seeds are then planted in AGYO perlite (Perlite Products Ltd.) in a wooden tray, the initial water content of the perlite being standardised by applying 1 ml of distilled water/2 c.c. of perlite. No further watering is required. The tray is placed in a glass-house at about 22–26°C. Seedlings with
uniform radicals about 1½ in. long are selected for the experiments. Perlite particles adhering to radicals are removed by washing in a muslin-covered beaker under running water. Seedlings are then placed radicals down, on a plastic-coated rack (5–6 mm mesh) in a plastic container, 3½ in. sq. and 4½ in. deep, filled with distilled water to the top of the rack (Fig. 2). A zoospores suspension is added to the water in the container.

Fig. 2.—Plastic container and rack used for infection studies.

No additional aeration has been found necessary, since lesions almost invariably develop within 24 hours.
Using this method, successful inoculations with *P. cinnamomi* have been obtained with a final inoculum density in the jar of one zoospore/ml.

This method is being used for investigation of strain variation within *P. cinnamomi* in relation to environmental factors. It should also prove useful in other studies on the fungus.

3. BLUE LUPIN AS A BAIT FOR ISOLATING *Phytophthora* FROM SOIL

Tucker (1931) used apples in a technique for the differential isolation of certain species of *Phytophthora* from infected plant tissue. A similar apple technique was also used by Campbell (1949) and Newhook (1959a) for isolations of *Phytophthora* from soil. The technique has given good results, although there are certain disadvantages; for example, saprophytic fungi, such as *Mucor*, *Rhizopus*, and *Penicillium*, if present, will produce rapid soft rots which obscure the presence of *Phytophthora*. *Trichoderma viride* Pers. ex Fr. is frequently present in soil and causes a hard rot of apple, and while both *T. viride* and *Phytophthora* may cause separate isolatable rots in a single apple, it is likely that *T. viride* often suppresses *Phytophthora* growth. Anderson (1951) in Hawaii uses white bases of pineapple leaves suspended in water over soil as a selective bait for *P. cinnamomi*. Unfortunately, this method is restricted to pineapple-growing areas.

In New Zealand we have found newly germinated blue lupin to be a suitable bait for detecting *P. cinnamomi* and some other species of *Phytophthora* in soil. Since this host should be readily available in many parts of the world, the technique may be found useful elsewhere.

A $\frac{3}{4}$ in. layer of the soil to be tested is placed in a plastic container of the type shown in Fig. 2, or another suitable receptacle, and tap water is added to the top of a support rack. Seedlings with 2 in. radicals are placed on the partially submerged rack so that radical tips are almost in contact with the soil. If soil samples are dry at the time of collecting they are allowed to soak for 28 hours before use. The containers are left at room temperature for 48 hours, within which period *Phytophthora*-infected lupins develop brown lesions of varying intensity near the radical tips. No aeration is necessary, owing to the short duration of the test. The identity of the fungus producing the lesions is checked by examination of cultures produced by plating in soil agar.

In 10 comparative tests the method produced results more rapidly than the apple technique. Probably because of its non-sensitivity to saprophytic rot fungi, it gave higher counts of *P. cinnamomi* than the apple technique in all except one test (Table 1).

Higher counts were also obtained from other species of *Phytophthora* present, viz, *P. syringae*, *P. megasperma*, *P. nicotianae* var. *parasitica*, *P. boehmeriae* Sawada. Zoospores from pure cultures of *P. cactorum* and *P. cryptogea* failed to infect lupin radicals. Thus, while the lupin technique is more efficient in isolating certain *Phytophthora* spp. than the apple technique, the latter has the advantage of detecting a wider range of species. Currently both methods are being used concurrently in this laboratory.
<table>
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REFERENCES


