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ACACIA.

CHEMICAL CONSTITUENTS OF ACACIA PULCHELLA AND THEIR EFFECTS UPON PHYTOPHTHORA CINNAMOMI.

FINAL REPORT

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PROJECT TITLE:

Investigation of the effect of chemical constituents of *Acacia* species on *Phytophthora cinnamomi*.

1. Introduction

We commenced work on the jarrah dieback problem in mid-77 in a joint programme between the W.A. Forests Department and the WAIT Chemistry Department supported by funds from ALCOA and from WAIT. Subsequently, in 1978, Dr. Syd Shea of W.A.F.D. and the WAIT Group received support for two years from the Rural Credits Fund of the Reserve Bank. More recently, in 1980, the work has been continued as a joint WAIT-CSIRO programme with two years support from the Dieback Research Fund.

Broadly speaking, the programme is aimed at providing scientific underpinning for proposed alterations of forest management practices which are directed at controlling the jarrah dieback effects of the fungus *Phytophthora cinnamomi (P.c.)* in the jarrah forests of south-west Western Australia. Briefly, it has been established by workers from the W.A.F.D. and CSIRO that certain forest legumes, in particular *Acacia pulchella*, are highly resistant to attack by *P.c.* Further, these legumes appear to be able to confer some similar resistance to other species such as jarrah grown in close proximity in pots. Washings obtained from roots of *Acacia pulchella* have also been shown to be antagonistic to *P.c.* in microbiological cultures. Another fact which makes the present programme highly significant in the total effort directed towards controlling the disease is that by altering the burning regime to provide hotter burns, the forest understorey can be modified to include a greater proportion of antagonistic leguminous species and less of more susceptible species such as Banksia. This represents a significant change in forest management practice and involves increased operational costs, so it is

highly desirable that a major factor in the decision to move to a new regime be thoroughly investigated.

Our main approach has been to extract roots of *Acacia pulchella* and to separate the extracts into fractions which are then bioassayed for activity against *P.c.* The long turnaround time and the variability of results from the bioassay has been a major problem which has plagued us from the outset of the programme. We now appear to have largely overcome this difficulty with the use of an axenic (sterile) bioassay developed by the CSIRO group. This new technique is used in parallel with the original bioassay which uses a non-sterile soil extract (with highly variable properties) to stimulate sporulation. Results from these experiments have established that *Acacia pulchella* roots do in fact contain chemicals which are antagonistic to *P.c.* Using the non-sterile bioassay, a crude aqueous root extract showed strong inhibition of sporangial formation at 250ppm and moderate activity at 125ppm. A phenolic fraction from this extract, probably mainly tannins and related compounds, was active at about 500ppm, suppressing the formation of sporangia. A crude saponin fraction from which glycosides of two principal triterpenes have been isolated has been shown to be active at similar levels.

In the axenic bioassay, antifungal activity takes the form of total inhibition of zoospore release from apparently normal sporangia which are formed. A crude saponin extract shows this activity at concentrations down to 1ppm. Tannins obtained from Karri bark show activity at 400-500ppm.

The low activity and the variability of the results in the non-sterile bioassay is a source of concern because intuitively one would expect that this system is a better model for the situation in the soil than the axenic system. We are presently conducting experiments which have established that this is due to degradation of the active saponin component by microorganisms from the

non-sterile soil extract during the bioassay procedure.

With this success behind us we are now conducting experiments and developing techniques to enable us to extend our work into the field situation. The major project is a substantial pot trial to provide information on the prospects for control of *P.c.* in soils by material generated in roots of *Acacia pulchella*. We have also developed techniques for analysis of the saponins at low levels in soils by high performance liquid chromatography and by gas chromatography - mass spectrometry. Several subsidiary chemical investigations with the objective of providing useful information to other workers in the field are being carried out in periods when the main thrust of the work does not fully occupy us.

2. Report on Progress and Current Activities

2.1 Bioassays

Throughout this study, the WAIT group has collaborated with microbiologists, firstly at the Dwellingup Research Station of the W.A.F.D. and, more recently, at the CSIRO Division of Forests Research, Kelmscott. A detailed outline of the bioassay procedures, prepared by Mr. David Darling, CSIRO, Kelmscott, is included as Appendix I of this report. Until recently, only the bioassay which required the use of a non-sterile soil extract to stimulate the formation of sporangia was used, and it is this bioassay which has been a major factor limiting the rate of progress in the overall study. This is because each bioassay takes at least two weeks to conduct, results can be variable requiring large numbers of replicates, and sometimes sporangial formation would not occur in the controls so that the trial would have to be abandoned. Further, we have been unable to find reproducible pronounced reduction of sporangial numbers in fractions from acacia root extracts at levels less than about 100 ppm, which is a

substantially higher concentration than one might reasonably expect to be required to produce the effects observed in pot trials.

The difficulties outlined above provided the stimulus for the CSIRO microbiologists to develop the axenic bioassay described in Appendix 1. As this assay is carried out in sterile conditions one is not confronted with the variability introduced by the use of soil extract containing a vast range of microorganisms. Further, we now believe that a major source of the variability in our earlier results, and the reason that activity occurs only at unexpectedly high levels, is because the principal active component, the saponins, is progressively degraded in the bioassay medium. In the axenic bioassay we have consistently observed activity of a crude saponin fraction at concentrations of 1 ppm which is an increase of about two orders of magnitude in the activity found in the non-sterile assay. The activity observed however, was quite different from the principal effect by which we had been monitoring activity in the non-sterile bioassay. In the axenic bioassay, sporangia numbers are already low, so attention was focussed on the process of zoospore release. A chemical which is active causes abortion of the release process. The zoospores spill out as an amorphous mass or remain in the sporangia in an apparently unviable form. A typical set of axenic bioassay results is shown in Table 1. In the

Table 1: Typical set of results for axenic bioassay

Concentration of saponin	Numbers of sporangia	Percentage abortion
0	Control	10%
1	Unchanged	20%
10	Unchanged	40%
100	Decreased	60%
500	Decreased	90%

non-sterile system, because sporangial numbers are substantial, activity of fractions has been monitored by observation of a decrease in sporangial numbers. In the light of the results found in the axenic bioassay, we have reassessed the results obtained in the non-sterile bioassay. In trials where there was a substantial decrease in sporangial numbers at a concentration of 500ppm, there was also in fact pronounced inhibition of zoospore release at a concentration of 100ppm. Further, the microbiologists in the team are presently extending the bioassay to observe the effects of test chemicals upon such operations as the germination of zoospore cysts and upon chlamydospore germination. Another response of *P.c.* to chemicals is for mycelial lysis to occur. Sometime ago, our co-workers at Dwellingup reported that some lysis of mycelium was observed with fractions from acacia extracts. Our CSIRO co-workers have not observed this effect, in fact, when preliminary results from the pot trial are considered, it now appears that the material in acacia roots is ineffective against mycelia, both in living plants and as extracts.

Preliminary attempts have been made to bioassay volatile material. In this, the volatile material is placed in a petri dish and the culture, in a second inverted petri dish, is suspended above it. On one occasion, significant inhibition of mycelial growth was observed, but in five subsequent replicate trials no effect was observed.

2.2 Isolation of material from roots of *Acacia pulchella*

Extraction of material from acacia roots and fractionation of the extracts presents a number of problems. Aqueous extracts froth very badly when evaporation is attempted due to the presence of the saponins. In an aqueous medium, there is also the possibility of microbiological or enzymatic alterations to extracted material. Further, manipulations using heat in the presence of oxygen might conceivably cause degradation of a

variety of chemical types. The series of experiments outlined in the summary: Extraction of Acacia Roots has been carried out and the isolates have been bioassayed. It appears from this work that heating and exposure to air do not have an appreciable affect on the activity of the extracts. The results also indicated, however, that the active factor in the roots is probably degraded by microorganisms or by enzymes during storage or extraction using aqueous media. Accordingly, the extraction procedure we reported previously (Alexander, et al., Aust. J. Chem., 1978, 31, 2741-2744) for isolation of material from roots has been modified to obtain large quantities of material from roots for use in the pot trial. We now pre-extract with ethanol to deactivate enzymes and microorganisms, then an aqueous extract is obtained which is quickly spray dried. This extract is presently being assayed to determine its content of saponin.

In order to determine whether other highly active material occurs in acacia roots, an ethanolic extract of the roots was chromatographed on silica gel and the fractions were bioassayed in the usual way. As described in the summary, the activity of these fractions against *P.c.* was found to be quite low. Also, the phenolic fraction of the root extract was further fractionated into a weak acid, strong acid, and neutral fraction. Bioassay of these fractions revealed only a moderate antagonistic effect to *P.c.*

Summary: Extraction of Acacia Roots

a. Roots which had been shredded and air-dried were stored for three months in the laboratory. Extracts obtained from these roots by extraction with either ethanol or water failed to show any activity against *P.c.* at concentrations of 200 ppm.

Conclusion: Although the test was carried out at the rather low concentration (in the light of more recent experience) of 200 ppm, it would seem to indicate that degradation of the active principle occurs during storage of shredded roots.

b. Fresh roots which had been cut into small pieces were immediately placed in ethanol and soaked for one week. The ethanol washings were filtered and the ethanol evaporated, then a water-soluble fraction of this material was obtained by trituration. This component showed strong activity at 500 ppm and weak activity at 250 ppm and the activity was not significantly affected by boiling the aqueous solution. The root pieces from this procedure were air-dried, ground to a fine powder, and extracted with water. This extract showed strong activity both at 500 ppm and 250 ppm and moderate activity at 125 ppm.

Conclusion: Treatment of freshly cut root pieces with ethanol appears to preserve the activity of the active principle. The active material is extractible with ethanol, but after ethanol extraction, strongly active material can be extracted with water. Taken with experiment 1 above, the results of this trial strongly suggest that unless micro-organisms and enzymes are deactivated with ethanol, the material which is active against *P.c.* is degraded by enzymes or microorganisms after roots are macerated.

c. A sample obtained by thoroughly extracting finely ground *Acacia pulchella* roots with ethanol was column chromatographed on silica gel. The fractions were combined on the basis of thin layer chromatography and subjected to bioassay. Most of these fractions were totally inactive against *P.c.* at concentrations of 200ppm, although one fraction showed low to moderate activity.

Conclusion: If there was some highly active principle in the extract, apart from the saponins and the tannins already identified, then it should have been discovered in this experiment.

2.3 Pot Trials

A substantial pot trial as summarised in Table 2 is underway. The broad objective of this trial is to provide information on the prospects for field control of *P.c.* by material generated in the roots of *Acacia pulchella*. In particular, a major aim is to evaluate the ability of material derived either from extraction of roots or directly from plants growing in the pots to control infectivity in jarrah and banksia seedlings. More specifically, the aims of the trial are as follows:

(a) To confirm that *Acacia pulchella* seedlings which are growing in pots with jarrah seedlings are able to confer significant resistance to infection by *P.c.* upon the jarrah seedlings. This work is to be extended to assess whether roots left in the soil after the upper parts of the plants are removed are effective in controlling *P.c.* Also, jarrah seedlings were watered with water obtained by leaching pots containing uninfected acacias in order to assess whether the effective agents are present when infection has not occurred, and are transported in water. Further, the trial in which jarrah is replaced with banksia is aimed at determining whether the protection provided by the acacia is potent enough to protect even more susceptible forest species.

(b) To evaluate various extracts and fractions of *Acacia pulchella* roots for effectiveness in controlling infectivity of jarrah and banksia seedlings by *P.c.* Also, karri bark tannin is being tested similarly to evaluate the effectiveness of tannins in controlling infectivity.

(c) To discover whether *Acacia pulchella* seedlings grown near to jarrah seedlings, but in separate pots, are capable of protecting jarrah from infection. If this is in fact the case it would suggest that volatile material from the acacia leaves may be a factor in the protective capacity of the acacia.

(d) In order to establish whether any of the protective factors from *Acacia pulchella* are macromolecules or microorganisms associated with the acacia roots, acacias were grown inside dialysis membranes in pots containing jarrah seedlings. Unfortunately the dialysis membranes failed prematurely so this study has had to be aborted. We propose to use the pots which have been therefore made available to carry out a further study. It is possible that the *P.c.* antagonist is generated in response to the presence of *P.c.* We will therefore establish a smaller trial to evaluate the capacity to protect jarrah seedlings of washings obtained from leaching infected pots compared with those obtained from uninfected pots. In designing these trials, care has been taken to avoid the technical problem of droughting of pots caused by the powerful water-pumping action of the acacias, thereby leading to anomolous results.

TABLE 2: SCHEME FOR POT TRIALS

Abbreviations: *A* = *Acacia pulchella*, *J* = *Jarrah*, *B* = *Banksia grandis*, *P.c.* = *Phytophthora cinnamomi*

Concentrations quoted are as w/w of the entire contents in the pots.

Plants in Pot	No. of Pots	Inoc. with P.c.	Description and Comments
A & J	13	YES	Both sides of membrane* inoculated with *P.c.*
A & J	21	NO	Control.
A & J	13	YES	*A* side inoculated. Jarrah side inoculated 2 weeks later.*
A & J	13	YES	*J* side inoculated only.
			Acacia (*A*) and jarrah (*J*) seedlings were grown separated by a semipermeable membrane to see if there was any protection afforded by separated root systems. i.e. (1) Are anti-fungal agents capable of leaching through a membrane to provide protection? (2) Are anti-fungal chemicals only produced in response to *P.c.* infection? (3) Are the protecting agents (either chemical, bacterial or other) too large to penetrate the membrane? Note: Towards the end of the trial it was observed that the dialysis tubing membrane had failed so the original objectives are no longer achievable. It is however possible that a postmortem will show that the root systems are sufficiently separated to allow some useful information to be obtained about the capacity of acacia to protect when root systems are more separate. *Not proceeded with as membranes were shown to be degraded.
A & J	26	YES	To check the original observations of the protective capacity of acacia and to act as controls for various other parts of the trial.
A & J	21	NO	
A & J	13	NO	Control) Grown together until infection with *P.c.* then Acacia seedlings cut
A & J	24	YES	Test) off at ground level to see if the roots are able to confer protection after ablation of the upper parts.
J	13	YES	Pots watered with washings obtained by leaching uninfected acacia pots. If protection observed this would implicate water soluble chemicals or microorganisms in the soil of the acacia pot which are present even if the plant is not under attack from *P.c.*
J	13	NO	
J	39	NO	Negative control.
J	13	YES	Control not near any pots containing acacia seedlings.
J	13	YES	Control surrounded by acacia seedlings. The aim of this trial was to check if protection is associated with nearness of acacia plants, thereby implication volatile material from the leaves of acacia seedlings.
J	26	NO	)
J	26	YES	) Karri bark tannin at 500 ppm added to pots
J	13	YES	Crude water-soluble acacia root extract added to soil once a week (50 ppm in soil)
J	13	YES	)
J	13	NO	) As above except acacia root extract was added to make the soil concentration 500 ppm.
J	13	NO	)
J	26	YES	) Saponin fraction was added (50 ppm) once a week
			The aim of this trial was to see if any of the above fractions affords protection to the jarrah seedlings.
A	26	NO	These pots act both as controls and to assess the immunity of the acacias to *P.c.* infections.
A	52	YES	
B & A	39	YES	The aim of this trial is to check if acacia (*A*) can protect banksia seedlings (*B*) in the same way that jarrah seedlings are reported to be protected.
B & A	13	NO	
B	6	NO	)
B	7	YES	) Controls for various infection studies.
B	6	NO	)
B	7	YES	) Karri bark tannin at 500 ppm (added once a week).
B	6	NO	)
B	7	YES	) Acacia extract at 500 ppm
			The aim of this trial was to assess the capacity of the various extracts to affect infection of banksia seedlings.

2.4 Development of Methods for Assaying Levels of Saponins in Soils

A number of investigations have been commenced as a preliminary to carrying out assays of saponins in soil in field situations. Methods have been developed for rapid analysis of saponins by high performance liquid chromatography (HPLC) and by gas chromatography-mass spectrometry GC-MS-DS) of silylated saponin material. These techniques are being used to monitor the persistence of saponins in both the non-sterile soil extract used in the bioassays, and in soils. It appears that the saponin is certainly labile in both these media, that is, it is biodegraded. We believe that this observation explains the earlier variability of bioassay results in non-sterile systems and indicates that experiments are required to evaluate levels and persistence of this *P.c.* antagonist in soil in the field situation. Our initial observations suggest that the degradation does not simply involve hydrolytic removal of the sugars of the glycosides to give the aglycone, but rather that it results in some alteration of the triterpene aglycone.

2.5 Associated Studies

Several subsidiary chemical investigations aimed at providing information useful to other workers in the field are being carried out in periods when the main programme of work outlined above does not fully occupy us.

2.5.1 Taxonomic classification of *Phytophthora* species using gas chromatography

At present a number of different isolates of *P.c.* are known, each of which has a different morphology, yet all are classified as *P.c.* As this variability makes classification so much harder, it is suggested that chemotaxonomic identification might be simpler and more rapid. As different families of fungi have different quantitative and qualitative lipid profiles, we intend to use this variability to see if we can build a chemical "fingerprint" for each of the different species and strains of *Phytophthora* so that they can can be readily classified.

The lipid fraction obtained from mycelium of a range of *Phytophthora* species, and different strains of the same species, have been examined by gas chromatography (GLC) of the fatty acid methyl esters. Our initial results have been disappointing in so far as the differences between samples appear to be too subtle to enable a simple examination of the GLC trace to provide a direct identification tool. An American group recently reported a similar situation with species of *Candida* grown in cultures containing glucose, maltose, or sucrose. When the medium contained only lactose, however, a distinctive fatty acid fingerprint was obtained from the GLC. We now propose to test whether a similar effect is observed with *Phytophthora*.

We have recently upgraded our GLC facilities with the acquisition of integrator recorders, fused silica capillary columns, and a new injector system which we hope will greatly simplify the mechanics of this study.

2.5.2 Lipid Compositions of Forest Species

It has been reported that in culture conditions *Phytophthora* require certain lipids such as sterols and linoleic acid. We are therefore investigating the lipid compositions of jarrah, banksia, and acacia to establish whether there is any correlation between composition and susceptibility to infection by *P.c.* We also propose to identify any changes in lipid composition which occur after infection of a host plant by *P.c.*, that is, whether the vulnerability of a species such as banksia is related to an increased production of lipids required by the pathogen.

2.5.3 Cyanogenic glycosides - their role in the susceptibility of various plant species

As part of her master's thesis (U.W.A.) Ms. Ros Hart observed that *P.c.* metabolises cyanogenic glycosides with the liberation of

hydrogen cyanide, and further that *P.c.* is capable of existence in the resulting cyanide-rich environment. She then proposed that *P.c.* might be utilising such liberated HCN to kill cells in advance of invasion. This type of advance toxicity is known for other host pathogen systems and HCN is implicated in at least two cases.

It is possible that *P.c.* utilises cyanogenic glycosides present in the host root tissue and uses the liberated HCN against the plant. We propose to test the root wood and root bark of both resistant and susceptible plants from the jarrah forest to see if there is any correlation between the concentration of cyanogenic glycosides and *P.c.* susceptibility.

BIOASSAYS OF ROOT EXTRACTS AND LEACHATES WITH *Phytophthora cinnamomi* USING POLYCARBONATE MICRO-FILTRATION MEMBRANES AS MYCELIAL SUPPORT.

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INTRODUCTION:

Bioassays of the effects of organic compounds and chemical extracts on the behaviour of the water mould *Phytophthora cinnamomi*, require the fungus to be presented to the test substances in a viable, axenic, non-sporing condition, and to be recovered intact, as free as possible from extraneous material, for microscopic examination.

Squares of various woven materials[4,5] have been used as a support to grow mycelium of the fungus in liquid culture. The mycelial cover is often uneven with luxuriant growth occurring outside the perimeter of the mat. The thickness of the material makes microscopic examination very laborious and time consuming due to the large depth of field to be studied. The niches formed by the weave encourage dense mycelial growth and trap extraneous material making microscopic examination difficult, if not impossible. The woven material absorbs the liquid nutrient solution with the mycelium and however well rinsed, some solution must carry over into the test culture and may influence the fungus or the test substance. Squares of cellophate[2] are also used as support for the mycelium but are unsatisfactory because the mycelium often slips off the support and the cellophane itself could be a food source for micro-organisms.

"Nuclepore " membranes (Membrane Filtration Industries, 12 Deason Ave., Richmond, S.A. 5033), made from polycarbonate, in various pore sizes and diameters, are chemically inert, robust and pliable to handle, and are transparent, eliminating the necessity for a clearing technique which might produce artifacts. The membranes do not take up any stain used to enhance observations of the fungus and provide a regular surface with a small depth of field.

DESCRIPTION OF THE TECHNIQUE:

1. Preparation of the mycelial mats

1.1 Green pea agar[1] is prepared by intermittently macerating 200g frozen green peas in 500 ml deionised water in a blender, at

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high speed for a total of five minutes. This fine suspension is placed in a water bath set at 50°C. In a separate vessel 15g agar is added to 300 ml deionised water and melted in a water bath at 90°C. It is then placed in the 50°C bath to cool. The two suspensions are mixed with at the same temperature and made up to one litre with warm deionised water. The agar is dispensed in 20 ml aliquots into pourers, which are then sealed and sterilised at 103.4 kPa for 15 minutes.

1.2 "Nuclepore" membranes, with a pore size of 8 microns and a diameter of 13mm, are placed in batches of twelve in vials containing deionised water, sealed and sterilised.

1.3 A template is prepared to establish the centre and twelve points equidistant from the centre (36mm) and equally spaced around the circumference (16mm) of a 9cm petri dish.

1.4 Molten green pea agar is poured into 9cm petri dishes. When the agar has set each dish is placed on the template and inoculated in the centre with a 4mm plug cut from the growing edge of a cornmeal agar culture of the fungus. The sterilised membranes are poured into a sterile petri dish and placed one to each of the twelve spots on the circumference of the dish. During transfer excess water is drained from the membranes on the edge of the petri dish. The plates are incubated for 5 days at 26°C in darkness.

2. Description of Bioassays

2.1 The non-sterile bioassay

2.1.1 A soil leachate is prepared by mixing 1 L of steralised deionised water with 1Kg of a soil known to be conducive to sporangial production of *P. cinnamomi*. This mixture is allowed to stand for 72 hours before filtration through a Whatman No. 1 filter paper and a "Nuclepore" 3 micron membrane. The filtered leachate is dispensed in ten millilitre aliquots into 6.5cm sterile plastic petri dishes to which one ml of the chemical to be tested is added, swirled gently to mix and incubated at 26°C in darkness to equilibriate. Three replicate dishes are prepared for each chemical with an equal number of distilled water controls.

2.1.2 The following day the dishes are transferred to a bio-clean work station where the membranes are peeled from the surface of the 5 day old cultures and placed in the dishes, one at a time, until each test dish contains four membranes. The surface of each membrane is covered with an even layer of mycelium, free of any spore propagules, and anchored through the 8 micron pores. The dishes are placed on sheets of plate glass, surrounded by a frame of polystyrene foam, and covered with a second plate glass sheet, and then incubated for 5 days at 26°C under continuous fluorescent light (4,300°A).

2.2 Axenic Bioassay

The infested membranes obtained from 1.4 are washed with five changes of a mineral salt solution every thirty minutes to induce sporangial production. The membranes are then transferred to the chemical extracts diluted in the same mineral salt solution at 1 part chemical extract to 10 part mineral salt solution.

The incubation procedure is the same as that described for the non-sterile method.

3. Rating the Bioassay

3.1 After 5 days incubation the dishes are removed from the incubator in batches of not more than nine to obviate any changes caused by variation in the laboratory environment before the membranes are fixed on microscope slides. Two membranes from each dish are removed, placed on slides, drained briefly with filter paper, stained with 0.1% aqueous trypan blue and warmed to intensity the stain. A self-sealing mounting medium[3] is dispensed onto the membranes which are then covered with a cover slip and the slide labelled. The remaining pair of membranes in the dish are placed in a cold room at 4°C for 30 minutes, and then returned to the incubator at 26°C for two hours before staining and mounting as described above.

3.2 The membranes are examined on a Reichert "Visopan" projection microscope at X 125 and X 500 magnification. Sporangial production is rated on a scale, 0 = Nil; Very Low = the occasional sporangium here and there; Low = 1-5; Moderate = 5-25; High = 25-50; and

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Very High = >50 sporangia per sq.mm of membrane.

The chilled membranes are further rated for sporangial release of zoospores, abortion of zoospores, direct hyphal germination and no apparent change. Notes are also made on the condition of the hypha and the presence of other spore forms such as chlamydospores and oospores.

BENEFITS OF THE TECHNIQUE:

A method for the rapid assessment of the effects of chemical extracts or other organisms on the biology of *Phytophthora cinnamomi* has been developed. Polycarbonate membranes by "Nucleopore" are autoclavable, pliable, chemically inert and transparent, and provide an ideal support for mycelium of *P. cinnamomi*. An even growth of hyphae over and through the pores of the membrane enable observation of the fungus over an area of 132.7 sw.mm with a very short depth of field. When the membranes bearing the mycelium are peeled off the agar there is no carry over of nutrient solution, the membrane is flat and regular, does not stain with trypan blue and remains fully transparent without clearing, which may cause artifacts in the specimen.

The Reichert "Visopan" projection microscope allows for systematic counting of 0.5mm squares, both vertically and horizontally, by an indexing device. The projection screen provides a fully focused image which is less tiring to the eyes of the operator, therefore increasing the rate at which slides can be assessed.

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