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FUNGICIDAL ACTION OF PHOSPHITE IN AVOCADO ROOT TIPS ON *PHYTOPHTHORA CINNAMOMI*

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ABSTRACT

Avocado seedlings cv. Edranol were injected at a rate of 15 ml / m^2 canopy area with a 10 percent phosphorous acid (H_3PO_3) solution with the pH adjusted to 6.1 with KOH. Gas-liquid-chromatography (glc) analysis showed that a phosphite (PO_3^{3-}) concentration of 9.8 ppm was reached in avocado roots 14 days after injection with H_3PO_3 and gave an 87% control of root colonisation by P cinnamomi *(Pc) as determined by the detached root technique. A maximum phosphite concentration of 53.2 ppm 6 weeks after injection failed to arrest infection by Pc. A maximum phosphite level of 2.45 ppm was reached after an Aliette leaf spray and gave a 47 % control of root colonisation by Pc Scanning electron microscopy (SEM) showed no difference in the ability of the zoospores of Pc to recognize, encyst, germinate and to form appresoria on H_3PO_3 treated roots as opposed to the untreated roots.*

INTRODUCTION

A lot of effort has gone into research of the *in vitro* and the *in vivo* antifungal activity of Fosetyl-Al (aluminium tris-o-ethyl phosphonate, 80% wettable powder formulation) the first commercial fungicide that moves in a basipetal direction after foliar application (Bertrand, Ducret, Debouge and Horriere, 1977) and, or Phosphorous acid against members of the Perenosperales (Coffey & Bower, 1984 ; Fenn & Coffey ,1984 ; Coffey & Joseph ,1985 and Griffith, Smillie & Grant ,1990). Fosetyl-Al is degraded to H_3PO_3 and ethanol in plant tissue (Bompeix, Ravisé, Raynal, Fettouche & Durand, 1980 ; Trique, Ravisé & Bompeix , 1981 ; Luttringer & De Cormis ,1985 and Saindrenan, Darakis & Bompeix ,1985). Evidence for a direct mode of action (Fenn & Coffey ,1985 and Fenn et al, 1989) and for both direct and indirect modes of action (Smillie, Grant & Guest, 1989) exists. The use of chromatography for quantitative analysis of the systemic fungicide Aliette and its breakdown product H_3PO_3 is commonly known (Quimette & Coffey, 1988 ; Schutte, Botha, Bezuidenhout & Kotzé, 1988 and Fenn & Coffey, 1989). The fact that *P. cinnamomi* isolates with resistance to Fosetyl-Al and a cross-resistance to phosphorous acid were detected under field conditions in France (Vegh, Le Roux, Le Berre & Lanen, 1985) places doubt on the long-term effectiveness of these fungicides for root rot control, especially after reports such as the development of metalaxyl resistant strains of *P. cinnamomi* and the lack of control after 2 seasons

use (Darvas et al. 1984). This emphasizes the importance to understand the exact mode of action of these fungicides. The aim of this study was to try to shed more light on the reaction encountered when a zoospore encysts on an avocado root containing phosphite.

MATERIALS AND METHODS

Plant material

6 month old *Persea Americana* cv. Edranol seedlings (susceptible) Snyman, Snyman & Kotzé, (1984) were used throughout all experiments. According to Botha, Wehner & Kotzé *(1990)* the cultivar Edranol is also the least resistant to colonisation by *P. cinnamomi.*

Treatments

Plants were injected with a 10% phosphorous acid solution at a rate of 15 ml/m^{-2} canopy areas. The H_3PO_3 solution was partially neutralized with potassium hydroxide to a pH of 6.1 to reduce the effect of phytotoxicity to the trees (Whiley, Pegg, Saranah & Langdon, 1991)

Fosetyl-Al was applied as a foliar spray until run-off at a rate of 3 g a.i./ litre (80% wettable powder formulation). Paper cloth was wrapped around the base of the trunk and plastic was used to cover the pots to prevent any run-off solution contacting the plant medium. The foliar spray was repeated on two consecutive days.

Pathogen isolate and zoospore production

P. cinnamomi isolated from avocado roots in the Nelspruit area was used. The pathogen was grown on pea-agar (Chen & Zentmyer, 1970) and a method of Gisi et al (1980) was used for production of sporangia. Zoospore release was induced as described by Botha et al (1989). Concentrations of 10^3 to 10^4 $m\ell^{-1}$ motile zoospores were obtained in this manner.

Roots

The method of excised root tips to determine tolerance in avocado rootstocks was described by various researchers (Kellam & Coffey, 1985; Dolan & Coffey 1986). As protection by phosphite against invasion by *P. cinnamomi* was expressed as a reduction in the rate of lesion extention after wound inoculation (Smillie, Grant & Guest, 1989) and reduction in lesion length in tomato leavelets inoculated with *P. capsici,* due to HPO_3^{2-}, fungicidial action in this paper is expressed through reduction in linear colonisation by *P. cinnamomi* after inoculation with motile zoospores on excised avocado root tips as described by Botha et al, 1989.

Twenty excised root tips (ca. 40 mm in length) of each plant (two plants in each treatment and each treatment repeated twice) were placed perpendicularly onto two parallel glass rods in 90 mm diameter petri dishes, containing 15 ml water agar in each as described by Botha et al (1989). Each root tip was inoculated in the region of root elongation with 10 µℓ of zoospore suspension containing 10^3 to 10^4 ml^{-1} motile zoospores. The roots were then incubated in the dark at 25 °C for 48 hours. Linear colonisation was determined by aseptically cutting the root tips in 3 to 4 mm segments after surface disinfecting for 5 s in 70 % ethanol. The root segments were then plated

out sequentially on a selective medium (PARPH) (Tsao & Guy, 1977). After incubation at 25 °C for two days the segments from which *P. cinnamomi* developed were measured to obtain total length of root colonisation. Root colonisation was determined every 7 days after injection until termination of trial.

Preparation for Scanning Electron Microscope (SEM)

Each week after inoculating roots with the 10 µℓ motile zoospores, root tips (ca. 10 mm in length) were excised at 3, 6,24 and 48 hours after inoculation and fixed in a 2.5 % Gluter aldehyde (GTA) solution with a pH of 7.3. The roots were then washed three times in a 0,075M phosphate buffer, followed by washing once for 15 minutes in each of a 20, 50, 70 and 90 % ethanol solution. Samples were critical point dried with CO and mounted for gold coating in a Eiko IB 2 ion coater. The Hitachi S450 scanning electron microscope was used throughout the experiment.

Gas-liquid-chromatography analysis (glc)

After a root sample of the control or untreated plants was homogenised by mortar and pestle in liquid nitrogen, a 1 g of the fine sample was added to 10 ml distilled water and homogenised futher with an ultra turrax. The sample was centrifuged for 5 min at 6000 rpm. Two ml methoxy-ethanol and 0.1 mℓ of the sample was transferred to a vial and 10 µℓ of formic acid was added to the solution. Derivation was done by separately dissolving a 10% KOH and 20% N-methyl-N nitroso-p-toluolsulfonamide in 50 mℓ 2-methoxy-ethanol. Nitrogen gas was bubbled through this mixture of two solutions which turned from clear to slightly yellow, after which another 10 end formic acid was added to clear the solution again.

A series of standard phosphite concentrations 1, 5, 10, 20 and 100 ppm processed in the same way as above were graphically analysed and used as a model to determine the phosphite in the different samples. The conditions of sample analysis are presented in Table A. 0,5 µℓ of both the standard and sample solutions were injected into the glc.

TABLE A : Conditions for determining phosphite concentrations with gas liquid-chromatography

Detector	Type	NPSD (Carlo Erba)
	Temperature	190˚C
Column	Type	Carbowax
	Temperature	140˚C
	Length	2 meters
Injector	Temperature	150˚C
"Carrier Gas"	Type	N_2
	Flow rate	0,80 kg/cm^{-2}
Air	Flow rate	0,80 kg/cm^{-2}
Hydrogen Gas	Flow rate	0,80 kg/cm^{-2}
Registrator	Sensitivity	1mV
	Paper speed	30 cm/h
Sample size		0.5µℓ

RESULTS

The colonisation of root tips by *P. cinnamomi* after inoculation remains high in the control roots but a decrease is noticed 7 days after injection with H_3PO_3. 14 Days after injection the decrease in colonisation is significant and remains significant till 56 days after injection (Figure 1; Table 1). Colonisation of root tips from trees treated with Aliette decreases after 7 days but is only significant after 14 days, and stays significant till 35 days. After 42 days the difference between the control and the Aliette treatment is very slight and insignificant (Table 2).

From Tables 1 and 2 it is clear that there is a variance between the colonisation of the control roots at the weekly intervals. This is due to the fact that the amount of motile zoospores used as inoculum could not be standerdised without encystment of an amount of zoospores. Therefore the colonisations in millimetres in the control roots were relayed to a 100 representing the percentage of colonisation. Seen in the light that the highly susceptable *Persea Americana* cv Edranol were used through out the experiment the roots from the treated trees would also have been colonised to the same extent if not for the treatment. The colonisations of the treated roots were then also calculated as a percentage using the colonisation of the control results from each of the 7 daily intervals. These results are reflected in Table 3 and Figure 3, and show that 7 days after injection with H_3PO_3 root colonisation was reduced to 76 % (24 % control in root colonisation) due to the action of the phosphite. 14 Days after injection root colonisation was reduced to 13 % (87 % control in root colonisation). A mean of more or less 85 % control in colonisation was found from week 2 up to week 7 and then it decreased to a 68 % control in week 8 after injection.

Root colonisation 7 days after the Aliette leaf spray was reduced to 83 % (17 % control of root colonisation). Fourteen and 21 days after the leaf spray the root colonisation was reduced to 53 % (47 % control of root colonisation) from where it steadily increased to 89 % (11 % control of root colonisation) after 7 weeks.

The distribution of phosphite in avocado roots after injection with H_3PO_3 or an Aliette leaf spray can be seen in Figure 2 or Table 4 as monitored on a weekly basis. 7 Days after injection with H_3PO_3 there is 2.7 ppm phosphite in the roots. 14 days after injection there is 9.5 ppm phosphite present in the root tips. The concentration of phosphite then slowly rises to 18.9 ppm 5 weeks after injection and then suddenly increases to 53 ppm in week 6 and then decreases to 41 ppm in week 7. The amount of phosphite 1 week after the Aliette leaf spray was 1.05 ppm followed by 1.6 ppm in week 2 with a maximum of 2.45 in week 3. The concentration of phosphite stayed above 2 ppm till week 6 and dropped to 0.5 ppm in week 7. The natural presence of phosphite in the plants ranged between 0.25 and 0.5 ppm over the period tested.

Figures 4 and 5 show the germ tubes and appresoria very clearly on the untreated and treated roots respectivly at a 400 magnification 3 h after inoculation. Figures 6 and 7 are a 2000 x magnification of the same roots also at 3 h. Figures 8 and 9 show the cysts, germ tubes, appresoria and mycelium very clearly on both treated and untreated roots 6 h after inoculation with no significant differences. Even 48 h after inoculation there are no major differences visible between treated and untreated roots as seen in Figures 10 and 11.

TABLE 1
Linear colonisation of root tips from Edranol seedlings injected with a 10% phosphorous acid solution and then inoculated with *P. cinnamomi* at 7 day intervals after treatment.

Days after treatment	Control	H_3PO_3
0	25.3 a	25.3 a
7	25.3 a	19.28 a
14	35.95 a	6.18 b
21	36.88 a	5.39 b
28	35.79 a	6.11 b
35	37.81 a	5.52 b
42	36.7 a	7.92 b
49	36.58 a	5.42 b
56	36.7 a	12.08 b

Each value is the mean derived from 80 roots from two consecutive experiments. Values not followed by the same letter differ significantly according to Duncan's multiple range test (P = 0.05)

TABLE 2
Linear colonisation of Edranol root tips treated with an Aliette leaf spray and then inoculated with *P. cinnamomi* at 7 day intervals after treatment.

Days after treatment	Linear colonisation of feeder roots (mm)	
	Control	Aliette
0	36.02 a	36.02 a
7	32.61 a	28.54 a
14	39.6 a	21.06 b
21	38.33 a	20.39 b
28	31.9 a	21.19 b
35	40.0 a	22.75 b
42	33.7 a	30.22 a

Each value is the mean derived from 80 roots from two consecutive experiments. Values not followed by the same letter differ significantly according to Duncan's multiple range test (P = 0.05)

TABLE 3
Root colonisation of Aliette and H_3PO_3 treated trees expressed as a percentage using the root colonisation of the control trees as norm

Days after treatment	Control	Aliette	H_3PO_3
0	100	100	100
7	100	82.6	76.2
14	100	53.2	14.5
21	100	53.2	16.8
28	100	56.9	17.0
35	100	66.4	14.6
42	100	89.5	18.7
49	100	-	14.8
56	100	-	33.1

TABLE 4
The phosphite concentrations in the roots of the Control, Aliette and H_3PO_3 treated trees as determined by means of gas liquid chromatography

Days after treatment	Phosphite concentrations (ppm) in roots		
	Control	Aliette	H_3PO_3
0	0.4	0.4	0.4
7	0.5	1.05	2.7
14	0.4	1.6	9.48
21	0.25	2.45	14.03
28	0.4	2.3	14.4
35	0.25	2.05	18.85
42	0.3	2.4	53.16
49	0.3	0.5	41.15
56	0.4		28.5

DISCUSSION

The colonisation of root tips by *P. cinnamomi* after inoculation remains high in the control roots due to the absence of phosphite in the roots. The decrease in root colonisation is evident 7 days after injection with H_3PO_3 with 2.7 ppm of phosphite present in the root tips (24 % control of root colonisation). An 87 % control of root colonisation was accomplished with a 9.8 ppm presence of phosphite in the root tips 14 days after injection with H_3PO_3. Although the concentration of phosphite in the roots increased to 53.2 ppm 6 weeks after injection and then decreased to 41.2 ppm in week 7 after injection the ability to control root colonisation did not increase but stayed at a mean of 85 % from week 2 till week 7 after injection. Initially there is a close relationship between the concentration of phosphite present at the site of invasion (in this study up to 9.5 ppm) and the extent to which protection was expressed, this phenomena was also found by Smillie, Grant and Guest, (1989). The relationship between the extent of protection and the concentration of phosphite at the site of invasion did not apply for

phosphite concentrations higher than 9.5 ppm and up to 53.2 ppm and these phosphite concentrations were never fungitoxic, for root colonisation by the pathogen could never completely be arrested in roots containing these high concentrations of phosphite and the fungus could be isolated from these roots after inoculation. This phenomena was also reported by Smillie, Grant and Guest (1989) who found that lesion extension in tobacco was arrested in plants treated with phosphite, though the pathogen was not killed but was isolated from apparently healthy tissue ahead of the lesion both in phosphite treated and control plants. Smillie et al, (1989) found that phosphite did not provide full control in lupin, suggesting that a well developed, dynamic defence system in the plant is essential if the phosphite is to halt the fungal attack completely, and therefore the mode of action might best be described as mixed rather than direct or indirect.

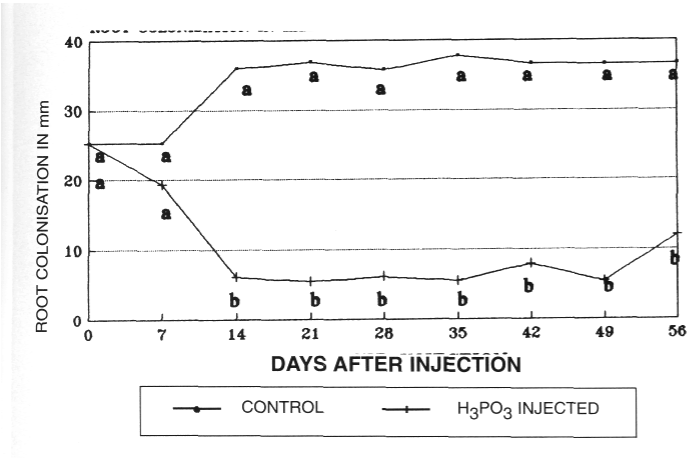

FIG 1.

Linear colonisation of root tips from Edranol seedlings injected with a 10% phosphorous acid solution and then inoculated with *P. cinnamomi* at 7 day intervals after treatment. Positions (values) not followed by the same letter differ significantly according to Duncan's multiple range test (P = 0.05

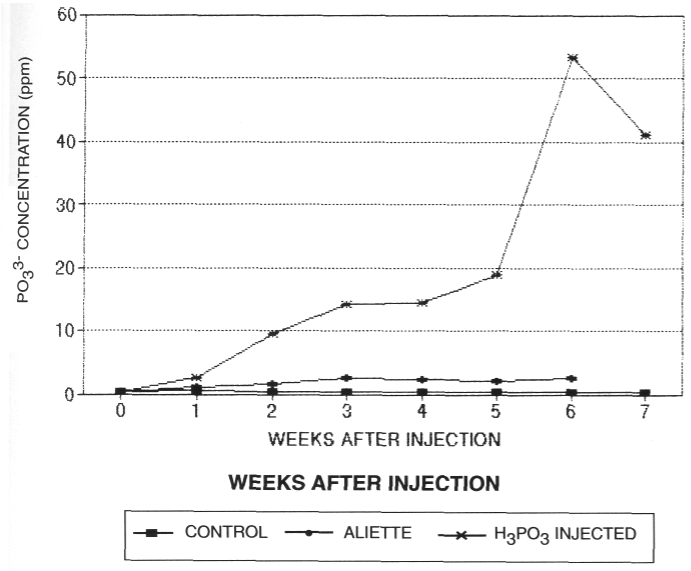

FIG 2

Distribution of phosphite in avocado roots over a period of 7 weeks after treatment with either Aliette or H_3PO_3

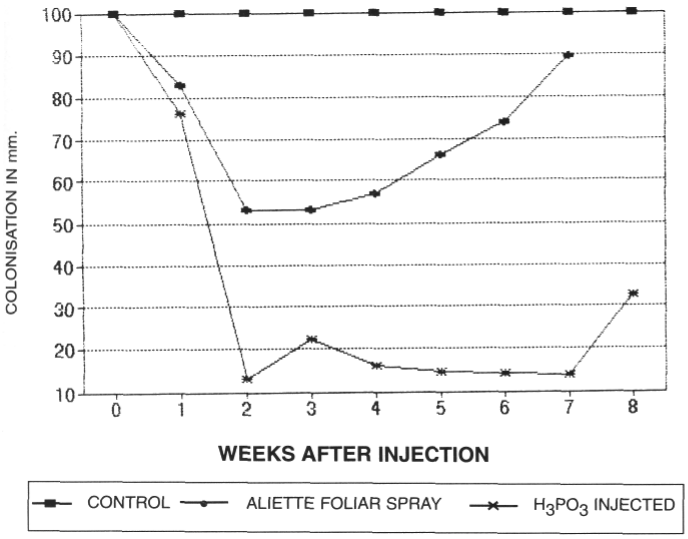

FIG 3

Fungicidial action of a 10% Phosphorous acid injection and an Aliette leaf spray expressed by the inability of *P cinnamomi* to colonise Edranol root tips.

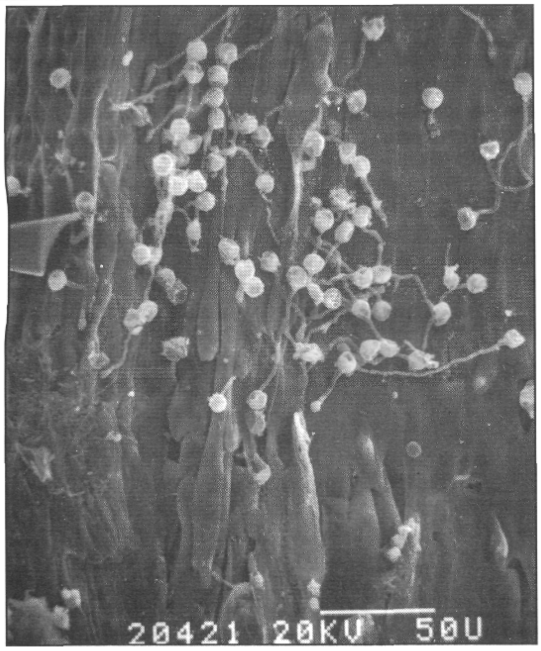

FIG 4
Encysted zoospores of *P. cinnamomi* germinating on the surface of an untreated Edranol root 3 hours after inoculation. Germ tubes and appresoria clearly visible. (Mag: 400 X)

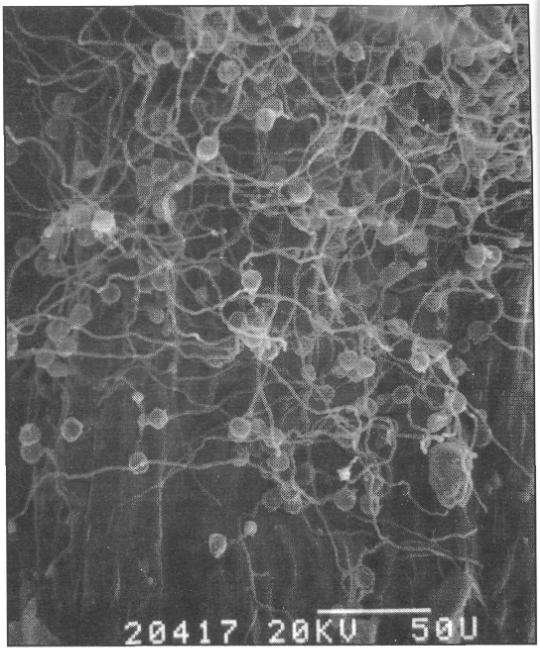

FIG 5
Encysted zoospores of *P. cinnamomi* germinating on the surface of an H_3PO_3 treated Edranol root 3 hours after inoculation. Germ tubes, appresoria and mycelium clearly visible (Mag: 400 X)

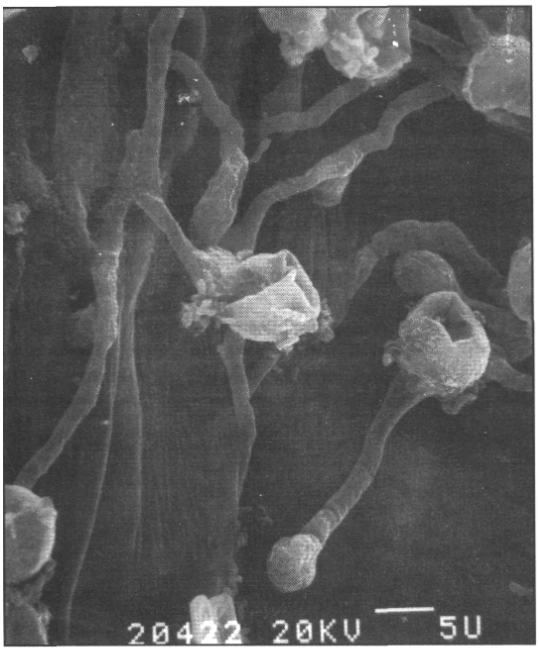

FIG 6
Encysted zoospores of *P. cinnamomi* germinating on the surface of an untreated Edranol root 3 hours after inoculation. Germ tubes and appresoria clearly visible. (Mag: 2 000 X)

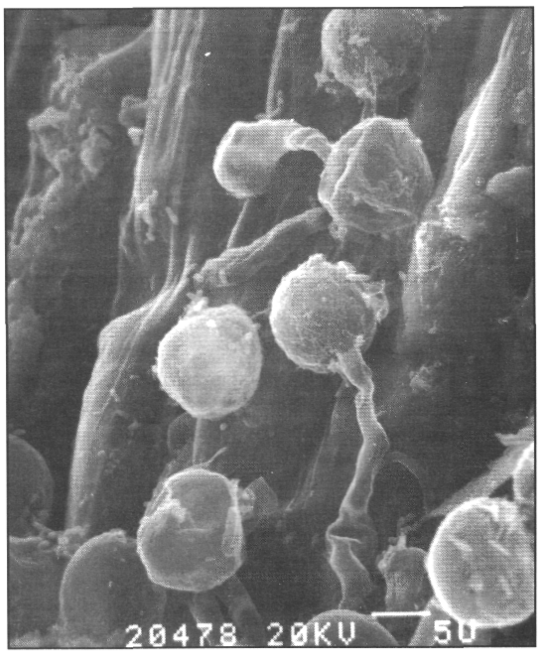

FIG 7
Encysted zoospores of *P. cinnamomi* germinating on the surface of an H_3PO_3 treated Edranol root 3 hours after inoculation. Germ tubes, appresoria and mycelium clearly visible (Mag: 2 000 X)

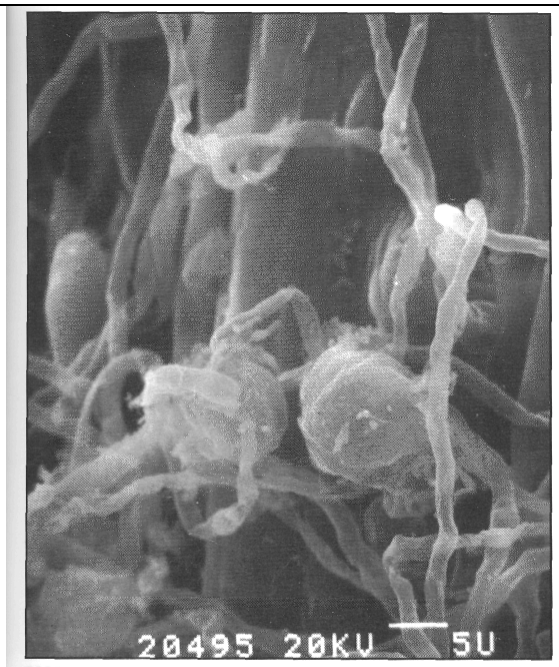

FIG 8
Encysted zoospores of *P. cinnamomi* after germination on the surface of an untreated Edranol root 6 hours after inoculation. Cyst and mycelium clearly visible. (Mag: 2 000 X)

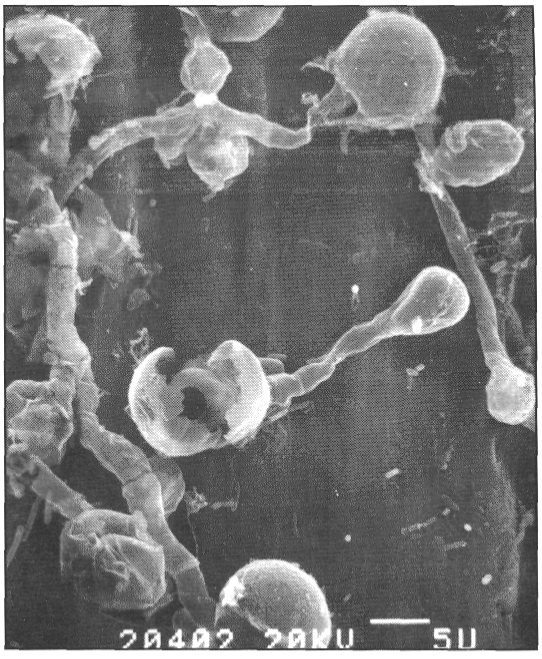

FIG 9
Encysted zoospores of *P. cinnamomi* after germination on the surface of an H_3PO_3 treated Edranol root 6 hours after inoculation. Germ tubes, appresoria and mycelium clearly visible (Mag: 2 000 X)

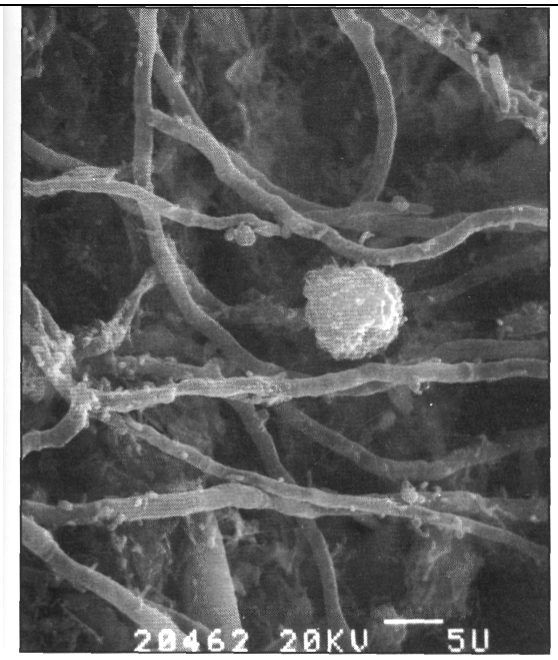

FIG 10
Encysted zoospores of *P. cinnamomi* after germination on the surface of an untreated Edranol root 48 hours after inoculation. Cyst and mycelium clearly visible. (Mag: 2 000 X)

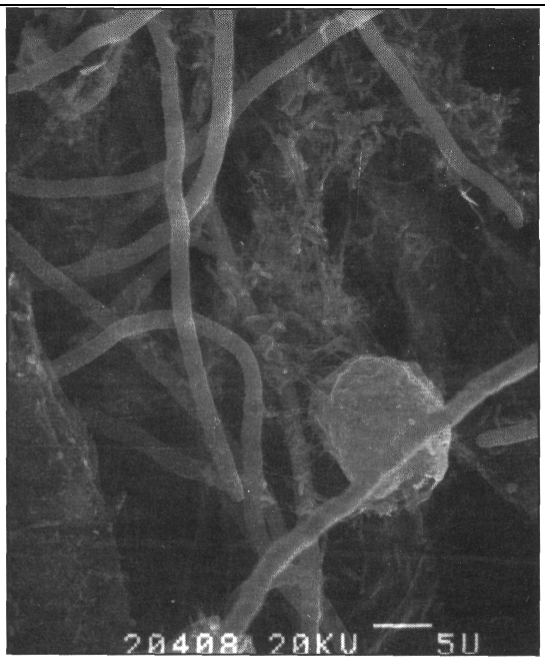

FIG 11
Encysted zoospores of *P. cinnamomi* after germination on the surface of an H_3PO_3 treated Edranol root 48 hours after inoculation. Germ tubes, appresoria and mycelium clearly visible (Mag: 2 000 X)

In this study the same tendency in the distribution of the phosphite concentration in an avocado root was found as by Schutte, Botha, Bezuidenhout and Kotzë (1988). Both studies showed a moderate increase in phosphite up till 35 days after injection followed by a steep increase in phosphite at 42 days after injection from where the phosphite then decreased in the days to follow.

Although it is difficult to determine exactly how much of the product was taken up by the leaves when the Aliette was applied twice on two consecutive days as a foliar spray the concentration of phosphite that reached the roots was never more than 2.45 ppm which gave a 47 % control of root colonisation. This observation supports the report by Darvas (1982) that Fosetyl-Al applied as a foliar spray at 0.3 % a.i. on a monthly basis failed to control the disease on replanted trees (insufficient leaves for uptake of product on small trees) but the same treatment was found to be satisfactory on large trees (sufficient leaves for uptake of product).

By means of the SEM it is clear that the zoospores of *P. cinnamomi* still recognized, encysted, germinated and formed appresoria on roots of H_3PO_3 treated trees. This indicates that if phosphite is exuded from the roots at all, it is not in quantities high enough for inhibition of cyst formation, cyst germination and appresorium formation. With the SEM the effect of the phosphite on the cysts, zoospores and mycelium of *P. cinnamomi* or the root surface can however not be distinguished from *P. cinnamomi on* roots not containing phosphite.

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