Growth and Zoospore Germination of *Phytophthora* spp. on $P_{10}VP$ Agar with Hymexazol

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ABSTRACT

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Mycelial growth of 49 isolates of eight *Phytophthora* spp., including 25 isolates of *P. cinnamomi*, on $P_{10}VP$ + hymexazol agar in a half-strength potato-dextrose agar (PDA) base ($P_{10}VPH$) was compared with growth on half-strength PDA (½PDA). Hyphal extension rates of isolates from groups I–III and V were suppressed on the $P_{10}VPH$, whereas isolates from group VI showed enhanced growth. The effect of the $P_{10}VPH$ medium on mycelial growth varied among isolates of the same species. The germination of zoospores and the germ tube growth of *P. nicotianae* and *P. palmivora* were suppressed on $P_{10}VPH$; motile zoospores were more sensitive than encysted ones. The age of the medium (1 or 4 days) had no effect on the percentage germination or germ tube length, and the sterility of the zoospore suspension had much less effect than the type of medium. Zoospore germination of 20 Western Australian isolates of *P. cinnamomi* was also suppressed on $P_{10}VPH$. The mean germination of motile zoospores on $P_{10}VPH$ was 72% of that on ½PDA, whereas the mean germination of encysted zoospores was 85% of the control value. The mean germ tube length after 5 hr on $P_{10}VPH$ was 81% of that on ½PDA. Therefore, when $P_{10}VPH$ agar is used for the quantitative detection of *P. cinnamomi* zoospores in soil, viable propagules are underestimated due to the antibiotics in the agar.

Additional key words: *P. cinnamomi*, selective medium.

Following the report by Masago et al (6) of the selective inhibition of *Pythium* spp. by hymexazol, Tsao and Guy (14) showed that when hymexazol is incorporated into the pimaricin-vancomycin-pentachloronitrobenzene (PCNB) agar ($P_{10}VP$) for *Phytophthora* isolation (15), *Mortierella* spp. are also inhibited, thus increasing the value of this medium for direct isolation from soil. During the past four years, a $P_{10}VP$ medium that has added hymexazol but uses half-strength potato-dextrose agar (PDA), not cornmeal agar (CMA), as a base (B. L. Shearer, *personal communication*) has been used extensively in Western Australia in jarrah dieback research for the isolation of *Phytophthora* spp. from soil and plant material (1,2,11,13).

Methods that have been used to detect *P. cinnamomi* Rands quantitatively in soil include baiting (3), sieving and plating residues onto selective media (3,5), and direct plating of soil suspensions onto selective media (5,12). Direct plating of soil suspensions onto $P_{10}VP$ agar with added hymexazol has been used in Western Australia since 1978 to monitor *P. cinnamomi* numbers in forest soils (12; D. E. Schild, *personal communication*).

This study was undertaken to extend the observations of Masago et al (6), Tsao and Guy (14), and Papavizas et al (8) on the effect of hymexazol on the growth of *Phytophthora* spp. and to evaluate the suggestion by Ribeiro (10) that the addition of hymexazol to $P_{10}VP$ agar might allow the medium to be used in a quantitative way. The investigation concentrated on local isolates of *P. cinnamomi* because of the importance of this fungus in jarrah dieback research. Because zoospores are thought to be the main dispersal and infective propagule of the fungus in Western Australian forest soils (7,9,12), particular attention has been paid to zoospore germination and germ tube growth.

MATERIALS AND METHODS

The isolates and origins of the *Phytophthora* spp. used in this study are shown in Table 1; stock cultures were maintained on Difco CMA (17 g per liter). Hyphal extension rate and zoospore germination were compared on half-strength Difco PDA (½PDA) (19.5 g of PDA, 7.5 g of Bacto agar per liter) and ½PDA with added pimaricin, vancomycin, PCNB, and hymexazol ($P_{10}VPH$). The quantities of antibiotics added per liter were 0.4 ml of pimaricin

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(2.5% Pimafucin suspension, Gist-Brocades, N.K., Delft, Netherlands), 200 mg of vancomycin (Vanconin 100%, Eli Lilly [Australia] & Co., Sydney), 100 mg of PCNB (approximately 95%, Sigma Chemical Company, St. Louis, MO), and 50 mg of hymexazol (Tachigaren pure grade, about 99% purity, Sankyo Co., Tokyo, Japan) (14). After the basal medium had been sterilized at 109 C for 45 min, it was cooled to 50 C before the antibiotics were added; then 20 ml of the medium was poured into 9-cm plastic petri dishes with a Teconoma automatic plate pourer. The P_{10}VPH plates were made the day before they were used unless otherwise stated. All plates were kept in the dark at 4 C until required.

Colony extension rate was measured in the following way. A 5-mm disk was cut from just within the margin of a young colony on CMA, and the disk placed centrally, upside-down on the test plate. The plates were incubated at 25 C in the dark, and two measurements of the colony diameter, at right angles, were made daily. Ten replicate plates were made for each treatment. The mean linear extension rate was calculated when colony growth had passed the lag phase.

Several factors affecting zoospore germination were investigated on P_{10}VPH and ½PDA media. Initial experiments were conducted with cultures of *P. palmivora* and *P. nicotianae* because these fungi produce sporangia on agar media and release zoospores readily when the medium is flooded with water. The effect of sterility on zoospore germination was studied by flooding cultures on 10% V-8 juice agar (100 ml of V-8 juice, 1.5 g of $CaCO_3$, 20 g of Bacto agar per liter) with either unsterile soil extract (10 g of soil in 100 ml of deionized water, shaken, then filtered through Whatman No. 1 filter paper) or soil extract that had been sterilized by filtration (0.22-μm Sartorius filter). The zoospore suspensions were checked

TABLE 1. *Phytophthora* spp. used in growth and zoospore germination experiments

Culture collection no.	Name	IMI[a] no.	Source	Isolate from [b]
1	*Phytophthora cactorum* (Lebert & Cohn) Schroeter	129908	DAWA[c]	
2		129909	DAWA	
3			DAWA	
35			WARI	Apple orchard, Vic.
4	*P. cambivora* (Petri) Buisman	131092	DAWA	
5		150819	DAWA	
31	*P. cambivora* A1		WARI	Apple orchard, S.A.
6	*P. cinnamomi* Rands	124492	DAWA	
7		143006	DAWA	
8		148408	DAWA	
25	*P. cinnamomi* A1		WARI	
50		165644	CSIRO	Jarrah forest soil, W.A.
38	*P. cinnamomi* A2		WARI	Pine forest soil, S.A.
41			CSIRO	*Banksia grandis*, W.A.
42			CSIRO	*B. grandis*, W.A.
43			CSIRO	*Leucopogon verticillatus*, W.A.
44			CSIRO	Jarrah forest soil, W.A.
45			CSIRO	Jarrah forest soil, W.A.
46			CSIRO	*Pultenea reticulata*
47			CSIRO	*Xylomelum occidentale*, W.A.
48			CSIRO	*Hovea elliptica*, W.A.
49			CSIRO	*Lomandra* sp., W.A.
51			CSIRO	*Podocarpus* sp., W.A.
52			CSIRO	Jarrah forest soil, W.A.
53			CSIRO	Jarrah forest soil, W.A.
54			CSIRO	Jarrah forest soil, W.A.
55			CSIRO	Jarrah forest soil, W.A.
56			CSIRO	*Leucopogon pulchellus*, W.A.
57			CSIRO	Jarrah forest soil, W.A.
58			CSIRO	Jarrah forest soil, W.A.
59	*P. cinnamomi* A2		CSIRO	*Bossiaea eriocarpa*, W.A.
60			CSIRO	*Hibbertia subvaginata*, W.A.
119			CSIRO	Pot trial, W.A.
9	*P. citricola* Sawada	129904	DAWA	
10		133316	DAWA	
11		134765	DAWA	
20			WARI	*Erica* sp., S.A.
33	*P. cryptogea* Pethybridge & Lafferty A1		WARI	Pine forest, Qld.
34	*P. cryptogea* A2		WARI	Pine forest, S.A.
39			FDWA	Pine forest, W.A.
14	*P. megasperma* Drechsler var. *sojae* Hildebrand	133317	DAWA	
30			WARI	Pine forest, S.A.
40			FDWA	Pine forest, W.A.
18	*P. nicotianae* van Breeda de Haan var. *nicotianae*	129912	DAWA	
16	*P. nicotianae* van Breeda de Haan var. *parasitica* (Dastur) Waterhouse	147252	DAWA	
17		148503	DAWA	
36			WARI	*Pimelea* sp., S.A.
37			WARI	Orchids, S.A.
27	*P. palmivora* (Butler) Butler A1		WARI	
28	*P. palmivora* A2		WARI	

[a] IMI = Imperial Mycological Institute.

[b] Vic. = Victoria, S.A. = South Australia, W.A. = Western Australia, Qld. = Queensland.

[c] DAWA = Department of Agriculture, Western Australia; WARI = Waite Agricultural Research Institute, South Australia; CSIRO = CSIRO, Division of Forest Research, Kelmscott, Western Australia; FDWA = Forests Department, Western Australia.

microscopically and discarded if too dilute. The zoospore concentrations gave a mean of 16 spores (range, 5–30) per field of view, using a ×10 objective. Drops of the zoospore suspension were placed on either ½PDA or P_{10}VPH, and the plates were incubated at 25 C in the dark. Four drops of zoospores were used per plate and two plates per treatment. The plates were examined after either 5 or 23 hr. Zoospores were killed with formalin vapor, and the number of germinated and ungerminated zoospores were counted in at least one field of view in each of the eight drops, using a ×10 objective, until a minimum of 100 zoospores had been assessed. Germ tube lengths of 25 germinated spores were measured.

Effect of the age of the medium on percentage germination of sterile and unsterile zoospores was also measured, using P_{10}VPH agar plates 1 and 4 days old. The experiment was performed as described above.

Percentage germination and germ tube growth of encysted zoospores on ½PDA and P_{10}VPH media was initially studied using isolates of *P. palmivora, P. nicotianae* var. *nicotianae*, and *P. nicotianae* var. *parasitica* for the reasons outlined above. Zoospore suspensions were prepared by flooding colonies on V-8 agar with sterile soil extract, and half of the zoospores released were encysted either by keeping the suspension at 4 C for 30 min or by shaking the zoospore suspension vigorously for 15 min with a wrist-action shaker. Drops of the zoospore suspensions were placed on the test media and assessed as described above.

More extensive investigations on the effect of ½PDA and P_{10}VPH media on the germination and germ tube growth of motile and encysted zoospores were made with *P. cinnamomi* isolates from Western Australia. Sterile zoospores were produced, using the method of Hwang, Ko, and Aragaki (4), except that sporangia were chilled at 4 C for 5 min instead of 16 C for 30 min. Half of the zoospores were encysted by shaking as described above. Drops of motile or encysted zoospore suspension were placed on the test media and assessed as described earlier.

RESULTS

Effect of P_{10}VPH on colony extension rate. The mean colony extension rates of the various *Phytophthora* isolates on ½PDA and P_{10}VPH agar are shown in Fig. 1. In all cases except *P. cambivora*

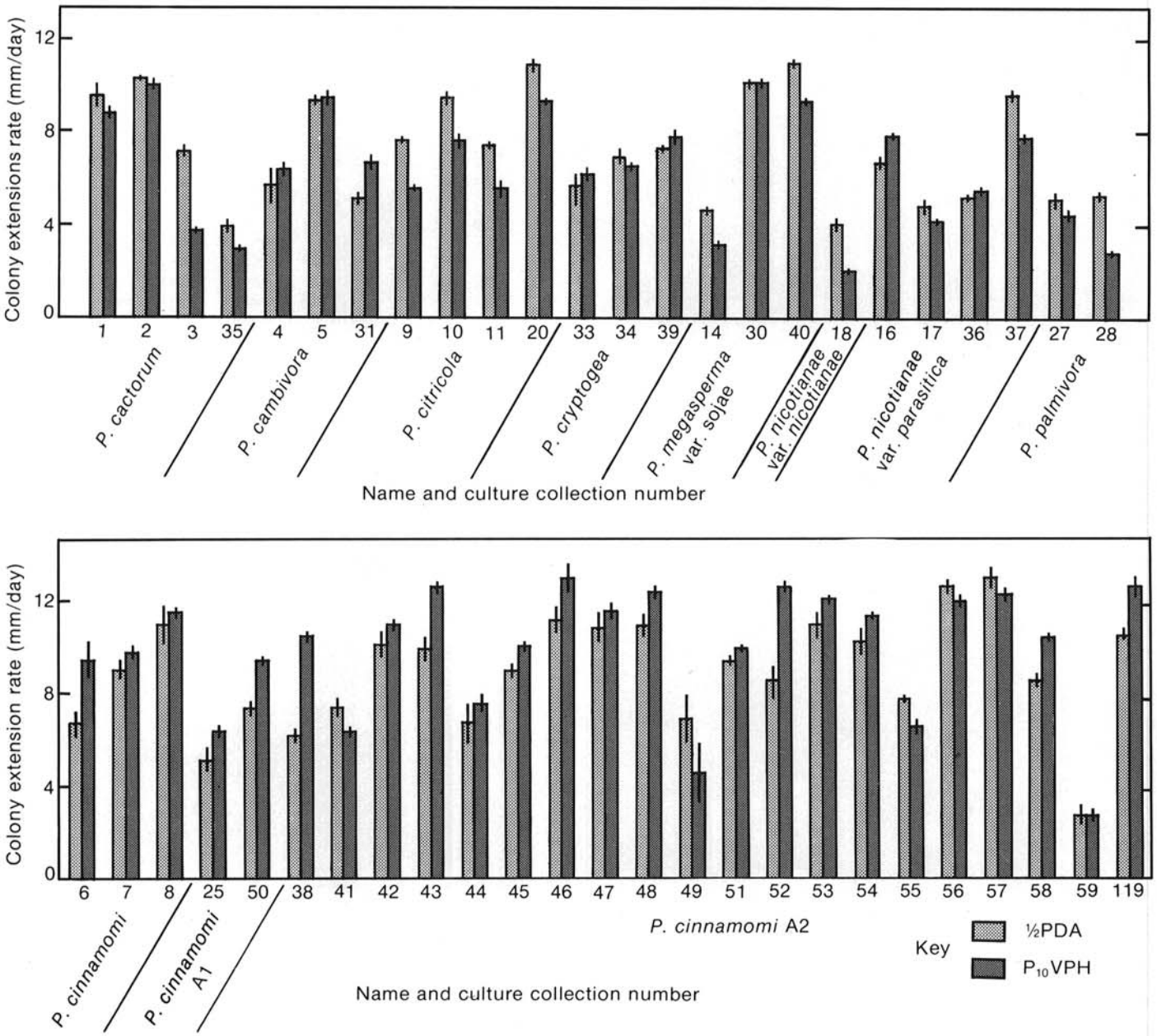

Fig. 1. Colony extension rate of *Phytophthora* isolates on ½PDA and P_{10}VPH. Values are means of 10 replicates. Error bar represents one standard deviation.

isolate 5, *P. cinnamomi* isolate 59, and *P. megasperma* var. *sojae* isolate 30, the colony extension rates were significantly different ($P = 0.05$). All of the isolates examined from Waterhouse's groups I–III and V (16), except *P. nicotianae* var. *parasitica* isolates 16 and 36, showed a reduction in colony extension rate on P_{10}VPH. In most of these isolates, growth on P_{10}VPH was 70–90% of that on ½PDA, but in *P. cactorum* isolate 3, *P. nicotianae* var. *nicotianae* isolate 18, and *P. palmivora* isolate 28 it was approximately 50%. Isolates from Waterhouse's group VI, however, showed the reverse trend; in 25 out of the 31 isolates examined, growth was enhanced. In most cases, the colony extension rate on P_{10}VPH was 105–130% of that on ½PDA, but in *P. cinnamomi* isolate 38, growth was

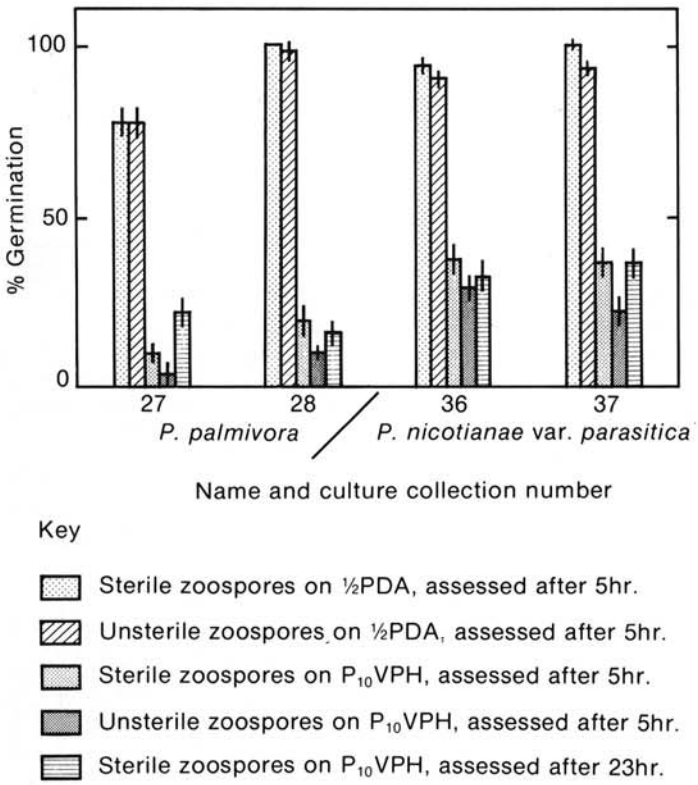

Fig. 2. Percentage germination of sterile and unsterile motile zoospores on ½PDA and P_{10}VPH after incubation for 5 and 23 hr. Error bar represents one standard deviation.

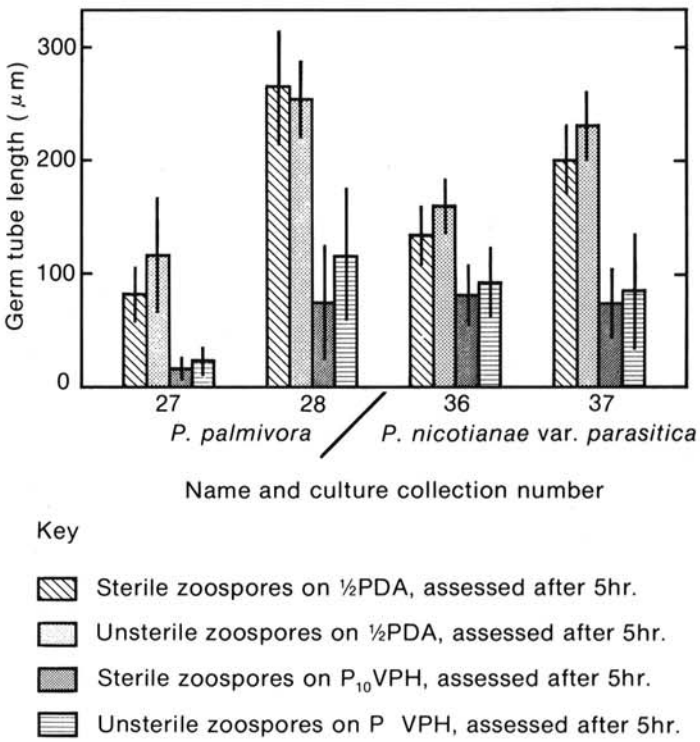

Fig. 3. Germ tube length of sterile and unsterile motile zoospores on ½PDA and P_{10}VPH after incubation for 5 hr. Values are means of 25 measurements. Error bar represents one standard deviation.

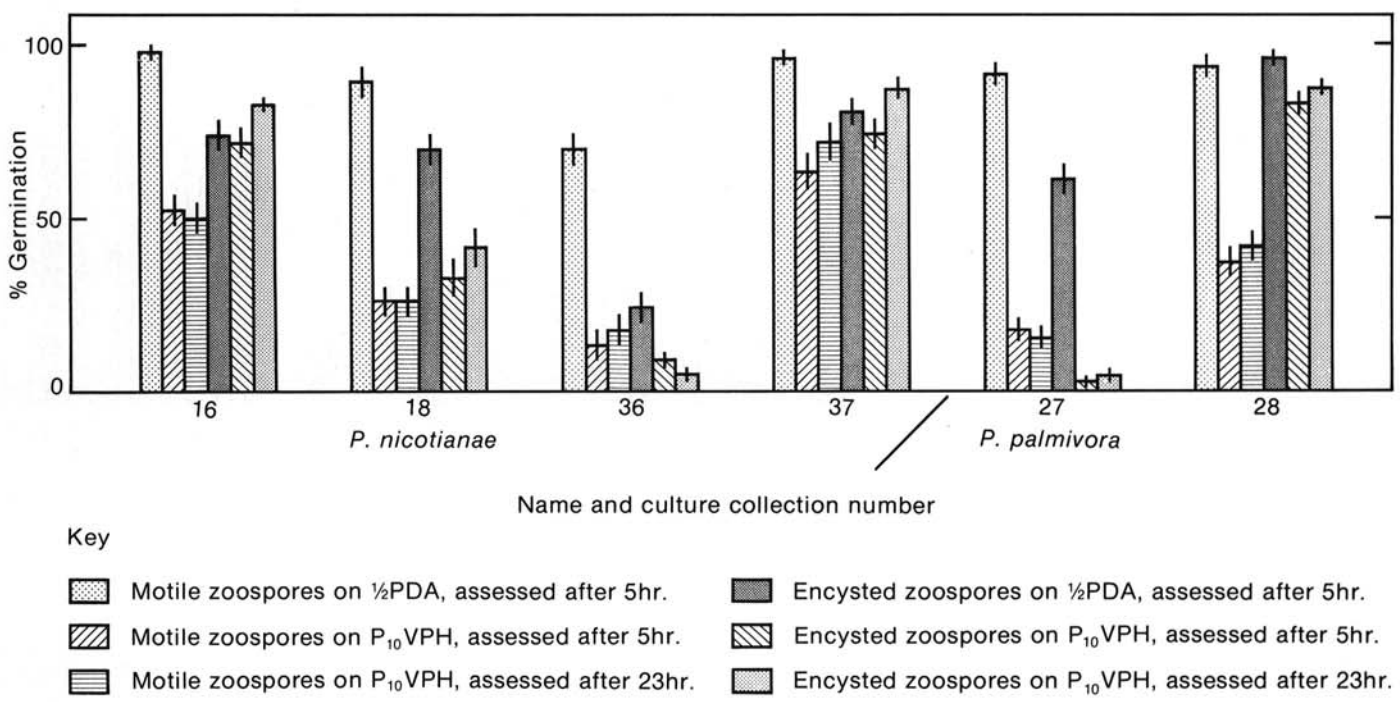

Fig. 4. Percentage germination of motile and encysted zoospores of *P. nicotianae* var. *nicotianae* (isolate 18), *P. nicotianae* var. *parasitica* (isolates 16, 36, and 37), and *P. palmivora* (isolates 27 and 28) on ½PDA and P_{10}VPH after incubation for 5 and 23 hr. Zoospores of isolates 18, 27, and 36 were encysted by chilling; zoospores of isolates 16, 28, and 37 were encysted by shaking. Error bar represents one standard deviation.

about 170% faster on P_{10}VPH.

Effect of P_{10}VPH on zoospore germination. P_{10}VPH affects the percentage germination of motile zoospores of *P. palmivora* and *P. nicotianae* var. *parasitica* in sterile and unsterile soil extract (Fig. 2). The effect of the medium was much greater than the effect of the sterility of the soil extract; germination on P_{10}VPH was between 10 and 35% of that on ½PDA. Four-day-old P_{10}VPH agar showed a similar suppression of zoospore germination. In addition, the P_{10}VPH medium suppressed germ tube growth (Fig. 3).

The effect of P_{10}VPH medium on the percentage germination of motile and encysted zoospores of *P. palmivora, P. nicotianae* var. *parasitica*, and *P. nicotianae* var. *nicotianae* was compared with the effect of ½PDA (Fig. 4). Encystment itself significantly reduced the number of zoospores that germinated on ½PDA. P_{10}VPH reduced the percentage germination of encysted zoospores less than that of the motile ones (Fig. 4). The degree of reduction, however, was not consistent among isolates of the same species, as shown by comparison of *P. nicotianae* isolates 16, 18, 36, and 37 and *P. palmivora* 27 with 28.

P. cinnamomi isolates showed the same trends (Fig. 5). In almost all isolates of this species tested, the percentage germination of motile zoospores was significantly less on P_{10}VPH agar than on ½PDA after 5 hr, but it had increased significantly ($P = 0.05$) on P_{10}VPH in 12 of 19 isolates after 23 hr. Percentage germination of zoospores on ½PDA was impossible to assess after 23 hr because of extensive hyphal development. Zoospores of some isolates (7,43, 50) germinated very poorly after encystment by shaking, whereas in others (6, 45, 54, 55, and 60) the percentage germination was not affected. In most cases, the percentage germination of encysted zoospores on P_{10}VPH agar was reduced 10–20% after 5 hr of incubation, although in isolate 48 it was reduced 60%. In 16 out of the 20 isolates tested, the percentage germination was significantly greater ($P = 0.05$) on P_{10}VPH after 23 hr than after 5 hr.

Although the germ tube length of motile and encysted zoospores of most isolates of *P. cinnamomi* was reduced on the P_{10}VPH agar (Fig. 6), the effect was not as great as that shown by *P. palmivora* and *P. nicotianae* var. *parasitica* (Fig. 3).

Comparison of the effects of P_{10}VPH on colony extension rate, zoospore germination, and germ tube growth. Although some

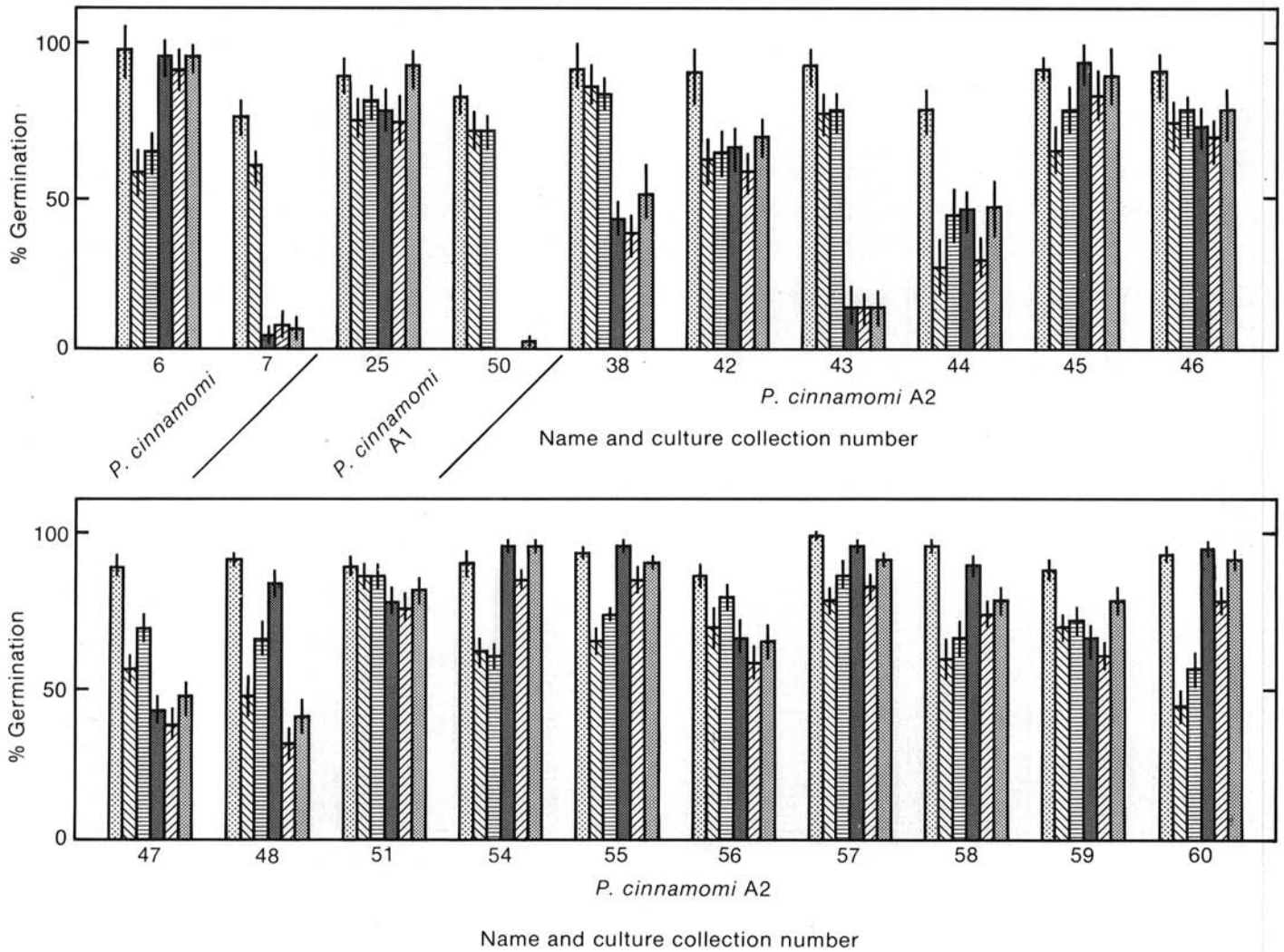

Fig. 5. Percentage germination of motile and encysted zoospores of *P. cinnamomi* on ½PDA and P_{10}VPH after incubation for 5 and 23 hr. Error bar represents one standard deviation.

isolates, such as *P. palmivora* 27 and 28, showed suppression of mycelial growth and percentage germination of motile and encysted zoospores and reduction of germ tube length on P_{10}VPH (Figs. 1–4), other isolates did not behave as consistently. *P. nicotianae* var. *parasitica* isolates 16 and 36 showed enhanced mycelial growth but suppressed germination and germ tube growth of motile zoospores on P_{10}VPH (Figs. 1–3). The germination of encysted zoospores of these two isolates was not reduced on P_{10}VPH (Fig. 4).

Many of the *P. cinnamomi* isolates examined also behaved inconsistently on P_{10}VPH agar. Isolates 56 and 57 showed suppression of mycelial growth and percentage germination of motile and encysted zoospores and reduction of germ tube length on P_{10}VPH (Figs. 1, 5, and 6). However, most of the *P. cinnamomi* isolates showed enhanced mycelial growth on P_{10}VPH (Fig. 1) but either no effect or suppression of percentage germination of motile and encysted zoospores and reduction of germ tube length (Figs. 5 and 6). Percentage of germination and germ tube growth was not enhanced on P_{10}VPH in any of the isolates.

DISCUSSION

The growth of a number of *Phytophthora* spp. on different antibiotic agars with and without hymexazol has been measured by several workers (6,8,14). Measuring colony radius at 2 days, Masago et al (6) found that the growth rate in six out of the 12 species tested was less on PDA with hymexazol than on PDA alone. On a benomyl-nystatin-PCNB-rifampicin-ampicillin (BNPRA) antibiotic agar with hymexazol at different levels, they showed that, compared with that on PDA, the colony radius was reduced in nine out of the 12 species measured. Using two isolates of *P. capsici* Leonian, Papavizas et al (8) showed that on the BNPRA medium with different levels of hymexazol, colony radius after 4 days did not differ significantly. On P_{10}VP agar, however, with different hymexazol levels, colony radius was reduced significantly with increasing hymexazol concentration, and one isolate (S1) was more sensitive than the other. On P_{10}VP agar with hymexazol, the linear growth rate of all the six *Phytophthora* spp. used by Tsao and Guy (14) was reduced.

Our results with the P_{10}VPH medium with *Phytophthora* isolates from Waterhouse's groups I–III and V (16) confirm Tsao and Guy's observations. In most of the isolates we examined, the mean colony extension rate was reduced about 70–90% on the antibiotic agar. We also found that different isolates of the same species were not equally sensitive to the antibiotics, as mentioned by Papavizas et al (8). Our results with group VI isolates differ from the results of previous workers, as they show enhancement of linear growth rate. The mean linear colony extension rate of the *P. cinnamomi* isolates on the P_{10}VPH agar was 113% of that on ½PDA (Table 2), but as noted above, different isolates of the same species showed considerable variation in their response to the antibiotics.

The effect of hymexazol on zoospore germination has not been extensively studied. Masago et al (6) showed that the percentage germination of encysted zoospores of *P. capsici* was less on media containing hymexazol than on PDA or BNPRA.

Our results show that in almost all cases, the germination of motile and encysted zoospores and subsequent germ tube growth is suppressed on P_{10}VPH agar, but the degree of suppression varies widely between isolates of the same species. In addition, the effect of P_{10}VPH agar on zoospores is not necessarily the same as its effect on mycelial growth. The large number of *P. cinnamomi* isolates used in this investigation enable us to summarize some of the effects of P_{10}VPH agar on Western Australian isolates of this species (Table 2). This table clearly illustrates that the various morphological and physiological states of *P. cinnamomi* respond differently to antibiotics. We cannot assume that the effect of P_{10}VPH agar on mycelial growth rate indicates how the antibiotics affect zoospore germination and germ tube growth.

If P_{10}VPH agar is to be used for quantitative assessment of *P.*

TABLE 2. Summary of the effect of P_{10}VPH agar on *P. cinnamomi* isolates

Character measured	No. of isolates examined	Rate on P_{10}VPH agar expressed as percent of rate on ½PDA
Mean linear colony extension rate	25	113 ± 20[a]
Mean germination		
motile zoospores after 5 hr.	20	72 ± 15
Encysted zoospores after 5 hr.	18[b]	85 ± 15
Mean germ tube length		
Motile zoospores after 5 hr.	18[b]	81 ± 14
Encysted zoospores after 5 hr.	18[b]	81 ± 12

[a] Standard deviation.
[b] Isolates 7 and 50 not included.

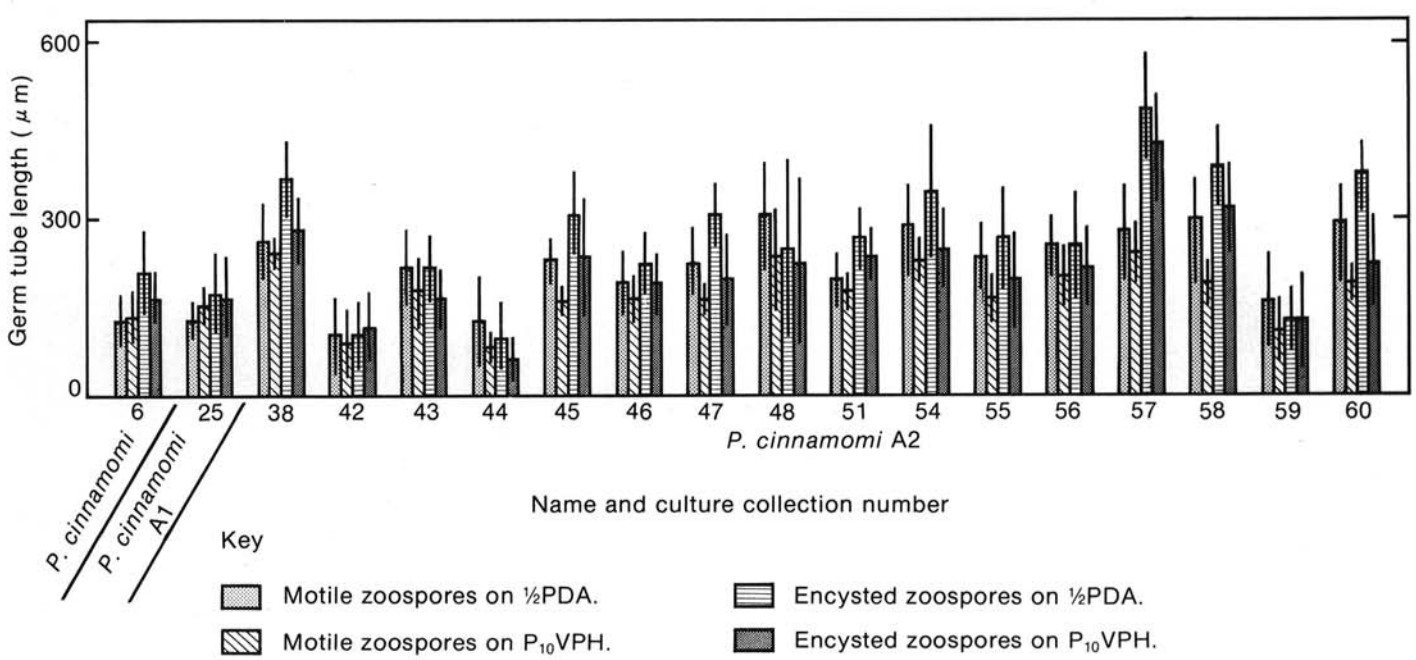

Fig. 6. Germ tube length of motile and encysted zoospores of *P. cinnamomi* on ½PDA and P_{10}VPH after incubation for 5 hr. Values are means of 25 measurements. Error bar represents one standard deviation.

cinnamomi zoospores in soil, determination of the effect that nonsterile soil extract has on the percentage germination of zoospores is important. From our results with *P. nicotianae* var. *parasitica* and *P. palmivora*, we conclude that the sterility of the zoospore suspension has very little effect on the percentage germination on either ½PDA or P_{10}VPH. Thus we would expect *P. cinnamomi* zoospores in a soil extract to behave in the same way as the sterile zoospores used in this study.

If the P_{10}VPH agar is used for the quantitative detection of *P. cinnamomi* zoospores in soil, the numbers of viable propagules will be underestimated due to the antibiotics, which cannot be ignored. The figures for the detection of *P. cinnamomi* in soil given by Shea et al (12) are likely to have been underestimated by about 25%.

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