Expression of Field Resistance in Callus Tissue Inoculated with *Phytophthora cinnamomi*

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ABSTRACT

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Callus cultures initiated from Australian native and horticultural species with varying degrees of field resistance or tolerance to the fungal pathogen *Phytophthora cinnamomi* were screened for expression of resistance to the pathogen. The extent of hyphal growth on callus correlated with the susceptibility of the plant from which the callus was derived. The optimum temperature for fungal growth on the calli was 30 C. This was the temperature at which the difference was greatest between hyphal growth on callus derived from resistant and susceptible plants. The amount of callose formed in the cultured cells in response to the pathogen correlated with the degree of field resistance.

Additional key words: Acacia, Eucalyptus, Lupinus, Lycopersicon, Nicotiana, Triticum, Zea.

Phytophthora cinnamomi Rands, which causes root rot and dieback in horticultural crops and ornamental species, is an economically important pathogen in all tropical and mild temperate climates (22). In Australia, the fungus has also caused widespread destruction of native forests in the southwestern and southeastern states. In Western Australia the fungus is associated with dieback of large areas of jarrah forest (*Eucalyptus marginata*) and other components of native flora. Little information is available on how tolerant plants survive contact with this extremely pathogenic fungus. The response at the cellular level in whole plants is complex, as each cell type responds differently to contact with the pathogen (9). In some host-pathogen interactions, resistance expressed in intact plants is also expressed in tissue culture (3,6,7,10,12,16,20) providing that the media composition and environmental conditions are appropriate (5).

In this study we examined first whether the degree of resistance or tolerance expressed by Australian native species and horticultural plants in the field correlates with growth of the fungus on callus cells and whether the responses of these cells and cells in intact plants are similar. Second, we explored whether tissue culture might provide a simplified model system for examining the expression of resistance to *P. cinnamomi* as it does for some other *Phytophthora* species (11,17) and whether growth of the pathogen

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on callus might be used as a bioassay of the field resistance of individual jarrah trees.

MATERIALS AND METHODS

Initiation and maintenance of callus cultures. Callus cultures were established from several species using media (Table 1) that preliminary experiments on callus induction and growth has shown to produce firm, dry callus. Hormones were manipulated in experiments in which the nature of the callus surface was to be altered. Shoot cultures of mature *Eucalyptus* trees and seedlings showing a range of responses to the pathogen were set up using methods described previously (15) and callus initiated from leaves or roots of this in vitro material.

All stock calli were maintained in 9-cm petri dishes in the dark at 25 C and subcultured every 4 wk. One to two weeks before inoculation with the fungus, callus pieces were transferred to fresh medium in 5-cm petri dishes. Pieces of callus 1–2 cm in diameter were transferred intact. Smaller pieces were placed together to provide a continuous surface over which the fungus could grow.

Selection of *Eucalyptus* lines. In dieback areas, virtually all jarrah trees are killed. Among the few survivors, trees with a healthy crown and good growth form were selected for in vitro propagation. Sources for shoot cultures included mature jarrah (*E. marginata* F. Muell.) trees surviving in dieback sites at Carinyah, Western Australia (tree 45, Table 1), and at Karnet, Western Australia (trees 3, 37, and 53, Table 1). Two of these (trees 37 and 45) had been inoculated in 1971. From glasshouse experiments with seedlings inoculated with *P. cinnamomi*, there are also rare survivors and these too were taken into culture (seedlings 20 and 21, Table 1). These lines have been designated resistant, although further work on clones from these lines is under way to determine whether they are resistant or disease escapes. The susceptible line was taken at Dwellingup, Western Australia, from a mature jarrah tree inoculated with *P. cinnamomi*, which subsequently died (tree 14, Table 1). Seedlings of jarrah are generally susceptible to *P. cinnamomi*, so callus from unselected seed was designated susceptible. Both mature trees and seedlings of marri (*E. calophylla* Lindl.) are resistant to the disease, and callus from marri seedlings was designated resistant.

Growth and maintenance of the pathogen. *Fungal inoculum. P. cinnamomi* (A_2 isolate) from the Brisbane Ranges, Victoria, was maintained on V8 agar (10%) at 24 C. Ten small blocks of agar and attached hyphae were cut with a sterile scalpel from the margin of 5- to 6-day-old cultures and placed on a sterilized, moist Miracloth (Calbiochem, Behring Corp., La Jolla, CA) disk overlaying a 10% V8 agar plate. The plates were incubated at 24 C for 5 days. Small sections (3 × 2 mm) of Miracloth with attached hyphae were cut,

TABLE 1. Source of material and culture conditions for callus production, production of callose in callus inoculated with hyphae of *Phytophthora cinnamomi*, and growth of hyphae of *P. cinnamomi* on calli

Media[a]	Species and designation (source of explant[b])	Field resistance[c]	Callose response[d]	Mean hyphal growth (mm)[e] 18 hr	24 hr	48 hr
A	*Acacia pulchella* R. Br., prickly Moses (SR, C)	R	++	0.7 (0.10)	2.4 d (0.19)	6.3 j (0.14)
A	*Lycopersicon esculentum* Mill. 'Grosse lisse,' tomato (S)	S	···			
A	*L. peruvianum* Mill., genotype S_1S_3, wild tomato[f] (S)	R	++	1.2 (0.10)	2.5 d (0.16)	5.0 i (0.36)
A	*Nicotiana alata* Link and Otto, genotype S_1S_3, ornamental tobacco[g] (S)	S	···	2.1 (0.12)	4.7 f (0.12)	9.4 l (0.04)
B	*Lupinus angustifolius* L., lupin					
	(SR)	S	···			
	(H)	S	···	2.2 (0.14)	3.4 e (0.10)	7.5 k (0.50)
B	*Triticum aestivum* L., 'Hard Condor,' wheat (SR)	R	++			
B	*Zea mays* L. 'Flat Red,' maize (SR)	R	++	1.2 (0.12)	1.4 c (0.10)	3.5 h (0.17)
C	*Eucalyptus calophylla* Lindl., marri					
	(SR, C)	R	+			
	(C)	R	+		1.3 (0.09)	2.7 D (0.19)
C	*E. marginata* F. Muell., jarrah					
	(SR, C, L)	S	+			
	seedlings (C)	S			1.6 (0.10)	6.6 E (0.49)
	seedling 20 (L)	R			1.6 (0.21)	3.1 D (0.33)
	seedling 21 (L)	R			1.0 (0.10)	4.8 DE (0.93)
	tree 14 (L)	S			1.8 (0.14)	6.9 E (0.36)
	tree 45 (L)	R			1.8 (0.27)	7.6 E (0.84)
	tree 37 (L)	R			1.2 (0.10)	2.8 D (0.47)
	tree 53 (R)	R	+	1.2 (0.17)	1.8 cd (0.08)	5.2 i (0.44)
	tree 53 (L)	R			1.3 (0.10)	3.7 D (0.74)
	tree 3 (R)	R	+	1.8 (0.16)	4.2 f (0.40)	10.0 l (0.50)

[a] A = Medium of Gibson et al (4), 0.25 μM kinetin, 10 μM dichlorophenoxyacetic acid (2,4-D). B = Medium of Murashige and Skoog (17), 15 μM 2,4-D. C = Medium of Murashige and Skoog (17), 5 μM 2,4,-D, 15% coconut milk.

[b] S = stem of glasshouse-grown plants. SR, H, C = root, hypocotyl, or cotyledon of axenic seedlings. R, L = roots or leaves of in vitro cultures.

[c] R = resistant. S = susceptible.

[d] ++ = strong fluorescence, + = weak fluorescence, ··· = no fluorescence.

[e] Standard error in parentheses. Data for marri cotyledons are pooled from five genotypes (seedlings) and for jarrah cotyledons from 13 genotypes. Means with the same letter in columns were not significantly different using Newman-Keuls procedures ($P = 0.05$). A lowercase letter indicates material from an experiment with calli 1–1.5 cm in diameter, and an uppercase letter indicates material from an experiment with calli 2–2.5 cm in diameter.

[f] Seed supplied by the Victorian State Department of Agriculture.

[g] Seed provided by K. K. Pandey, Grasslands Research Institute, D.S.I.R., Palmerston North, New Zealand.

under sterile conditions, to inoculate the callus. Care was taken to avoid drying of the fungal inoculum.

Inoculation. Calli 1–2 cm in diameter were inoculated with the hyphae-Miracloth sections (one section per callus) with the fungal surface uppermost. At various time intervals, hyphal growth was measured from the edge of the section using an eyepiece graticule in a dissecting microscope. Hyphae were used as inocula in preference to fungal zoospores because of the difficulties in identifying initial inoculation sites. Inoculated and control calli (three to four replicates per treatment) were incubated at 15, 20, 25, 28, 30, and 35 C (± 1 C) in the dark. The mean hyphal growth values (in millimeters) on the calli were analyzed using one way analysis of variance. The Newman-Keuls multiple comparison procedure was used to compare these means.

Assessment of resistance. Expression of resistance in infected calli was assessed by measuring the extent of pathogen growth and by observing both the amount of callose formed and the extent of browning of the calli.

Microscopy. At various times after infection, callus cells were examined for callose formation by staining with decolorized aniline blue (9). Maize, wild tomato, and lupin calli were fixed 24–48 hr after infection for 30 min in 1% paraformaldehyde, 0.25% glutaraldehyde in piperazine -*N*-*N*'-bis[2 ethanol-sulfonic acid] (PIPES; Sigma) buffer (15 mM, pH 6.8), dehydrated in an ethanol series, and embedded in JB-4 resin (Polysciences Inc., New Jersey). Sections 2 μm thick were cut on a Porter Blum ultramicrotome and stained with either decolorized aniline blue and/or toluidine blue (0.05% in benzoate buffer, pH 4.5) to monitor the pattern of fungal growth.

RESULTS

The optimum temperature for fungal growth on the medium or on calli was 30 C; the greatest difference in hyphal growth on callus derived from various species was observed at this temperature (Fig. 1).

On small pieces of callus (1–1.5 cm diameter), the extent of hyphal growth (at 30 C) correlated with the degree of susceptibility of the species from which the callus was derived. This was true up to 24 hr after inoculation, but after that time, hyphae sometimes extended onto the medium and growth was extensive on both resistant and susceptible callus types. If larger pieces of callus were used, differential growth of the fungus could be measured up to 48 hr.

Calli derived from resistant species, *Zea mays* L., *Acacia pulchella* R. Br., *Lycopersicon peruvianum* Mill., and *E. marginata* (no. 53) supported limited fungal growth (Table 1). In contrast, prolific aerial and radial growth occurred on callus of *L. angustifolius* L., *E. marginata* (no. 3), and *Nicotiana alata* Link and Otto. This difference between hyphal growth rates at both 24 and 48 hr on resistant and susceptible species was highly significant ($P = 0.05$, Table 1).

A detailed analysis of the extent of hyphal growth on two eucalypt species is summarized in Table 1. For these comparisons, pieces of callus 2–2.5 cm in diameter were used, and 24 hr after inoculation, there was no difference between growth on callus from resistant and susceptible species. At 48 hr, the differences were quite clear and significant ($P = 0.05$): Growth on calli derived from lines known to be resistant to the disease was limited (marri cotyledons, jarrah trees 53 and 37, seedlings 20 and 21). In contrast, growth on calli derived from lines known to be susceptible to the disease (jarrah cotyledons and tree 14) was greater than that on the resistant calli. The only exception was callus derived from jarrah tree 45, which supported extensive growth. The differences in hyphal growth on different calli were even more pronounced by 96 hr after inoculation (Fig. 2).

The concentration of the phytohormones in the growth medium altered the morphology of the calli, as well as the degree of fungal growth. In some cases, callus from different organs of the same plant had a different texture when grown on the same hormonal regime; for example, hypocotyl callus of *Acacia* was always softer and wetter than root callus and supported greater fungal growth (Table 2). Root callus was soft and moist at 0.5 μM 2,4-D and 0.1 μM K but became firmer with increasing concentrations of either 2,4-D or kinetin.

The production of callose in callus cells in response to the pathogen correlates with the degree of field resistance of the plant from which the callus was derived except in the lines of *Eucalyptus*

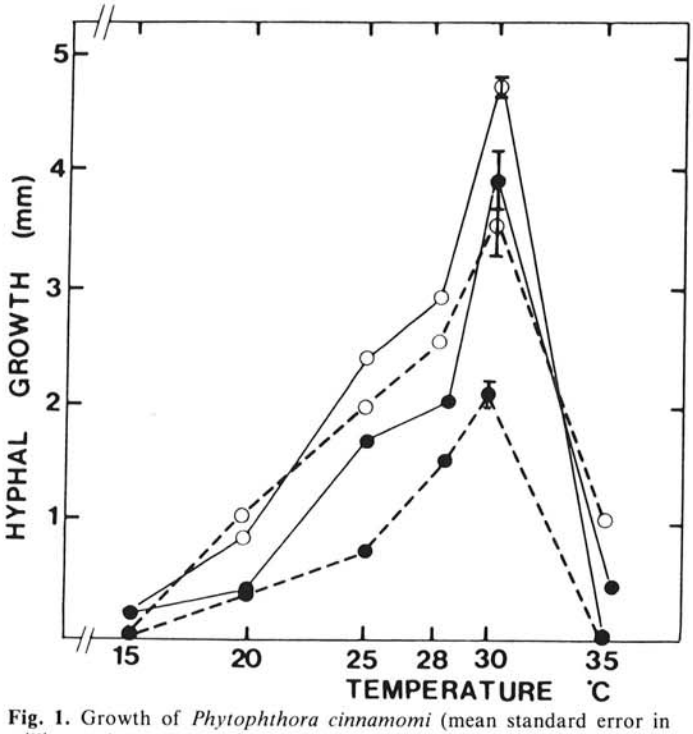


Fig. 1. Growth of *Phytophthora cinnamomi* (mean standard error in millimeters) at a range of temperatures on calli derived from plants that show varying degrees of field resistance, 24 hr after inoculation. Ornamental tobacco (susceptible), —o—; jarrah seedling root callus (susceptible), —•—; fungal growth on tissue culture media, - - - o - - -; seedling root callus of *Acacia pulchella* (resistant), - - - • - - - .

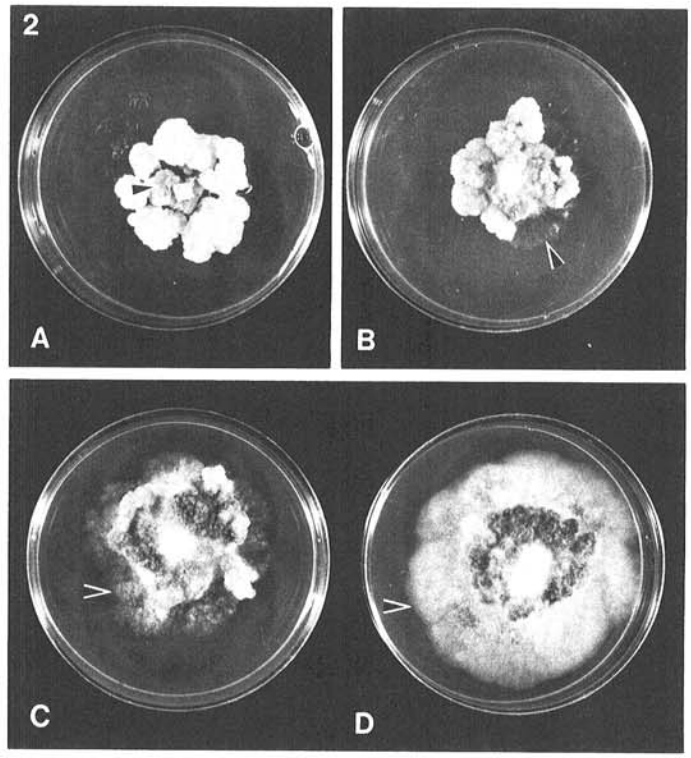


Fig. 2. Growth of *Phytophthora cinnamomi* at 30 C on calli derived from eucalypts with varying degrees of field resistance 96 hr after inoculation. Arrows indicate the growing edge of the fungus. **A,** Marri cotyledon callus (resistant). **B,** Jarrah tree no. 37 leaf callus (resistant). **C,** Jarrah tree no. 53 leaf callus (resistant). **D,** Jarrah cotyledon callus (susceptible).

in which little callose was detected in either resistant or susceptible callus (Table 1). Callose, detected by fluorescence with decolorized aniline blue, was present as large deposits in callus derived from the resistant cereals, maize, and wheat and from *Acacia* and wild tomato. No callose was observed in infected callus from susceptible plants such as tobacco or lupin.

In both maize and wheat callus, many of the callose deposits encircled the hyphae and appeared as fluorescent rings (Figs. 3, 4). In callus of wild tomato, large numbers of cells responded to the presence of the pathogen by producing callose. Each cell was capable of producing several callose deposits in response to contact with several invading hyphae (Fig. 5). Callose was also formed as deposits in the lumena of the fungal hyphae and at their coralloid tips when grown on each of the callus lines. Sections of infected callus showed intercellular hyphae growing through air spaces between the large parenchymatous cells (Fig. 6). The calli were largely undifferentiated, but seedling eucalypt root callus had some tracheids. In sections of infected maize callus, cells some distance from the hyphae showed small callose deposits that presumably blocked the plasmodesmata (Fig.7).

A hypersensitive-like reaction expressed as a reddish-brown cell discoloration in the immediate vicinity of the hyphae was observed in wild tomato. This response was first observed 24 hr after inoculation and was intense by 46 hr and fungal growth was severely limited (Fig. 8). Browning was also observed in callus from resistant lines of marri and jarrah and less extensively in callus from susceptible jarrah.

DISCUSSION

Resistance and susceptibility to the fungal pathogen *P. cinnamomi* was expressed in callus tissue. Resistance was detected by limited, sparse fungal growth on callus, in contrast to the prolific, aerial growth seen on media or on callus derived from susceptible species. Restricted pathogen growth was also observed on calli of jarrah trees or seedlings that survived in dieback sites or field trials (lines 20, 21, 37, and 53), whereas calli derived from jarrah trees that were dying in infected sites supported prolific growth. Two jarrah trees (lines 3 and 45) appeared to be resistant on the basis of field trials, but pathogen growth on derived callus indicated that either the mechanism of resistance is not expressed in cultured cells or that these trees are "disease escapes." Experiments using clonal plants micropropagated from jarrah line 3 indicate that they are susceptible (Bennett and McComb, *unpublished*) so that the latter hypothesis is likely for this tree at least. Although fungal growth was limited on calli from all resistant species, it was never completely inhibited. However, in infected roots of whole plants of *A. pulchella* (18), resistant eucalypts (14), and maize (9), fungal growth is never completely inhibited, but growth is slower than that observed in susceptible hosts.

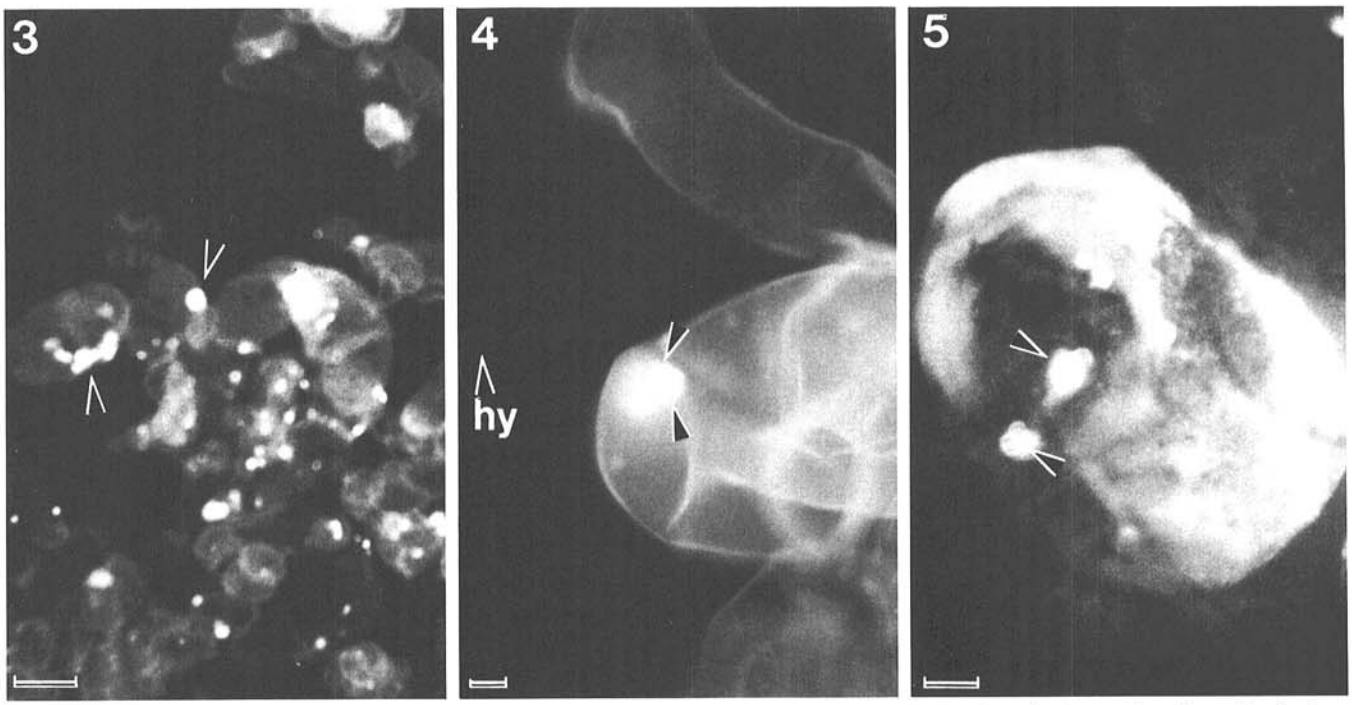
In evaluating fungal growth on calli, it is important to use calli with comparable texture. Both the source of the callus and the culture conditions affect fungal growth; similar findings have been reported for other fungal-callus interactions (3,5,11,16). In

TABLE 2. Hyphal growth on callus from *Acacia pulchella* (a resistant species) growing on media with different phytohormones

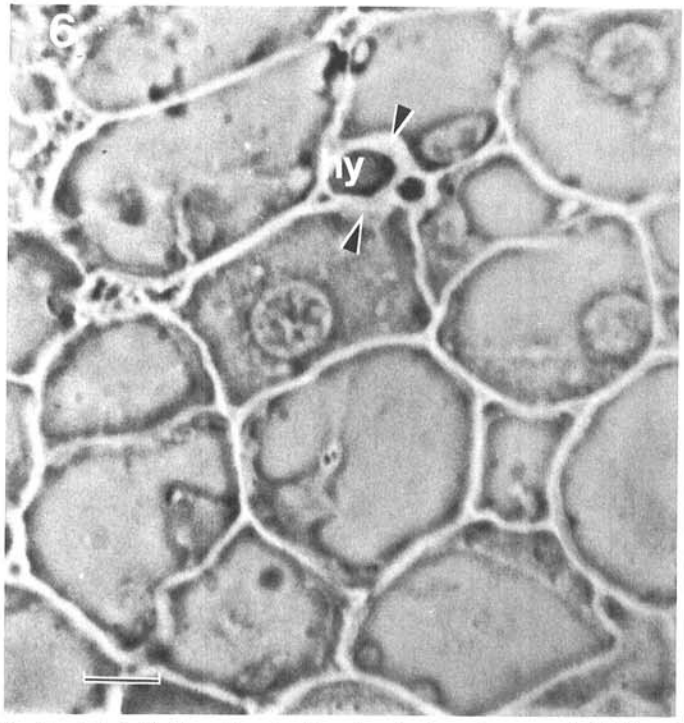
Kinetin (μM)	Extent of hyphal growth (mm)[a]					
	0.5 μM 2,4-D[b]		10 μM 2,4-D		15 μM 2,4-D	
	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl
0.1	3.1 (0.62)	3.3 (0.38)	2.5 (0.66)	2.5 (0.29)	1.8 (0.33)	2.9 (0.22)
1.0	2.7 (0.08)	2.9 (0.22)	1.8 (0.38)	2.4 (0.65)	1.5 (0.22)	2.3 (0.33)
10.0	1.3 (0.14)	1.7 (0.16)	1.6 (0.08)	2.5 (0.29)	1.1 (0.22)	2.7 (0.33)

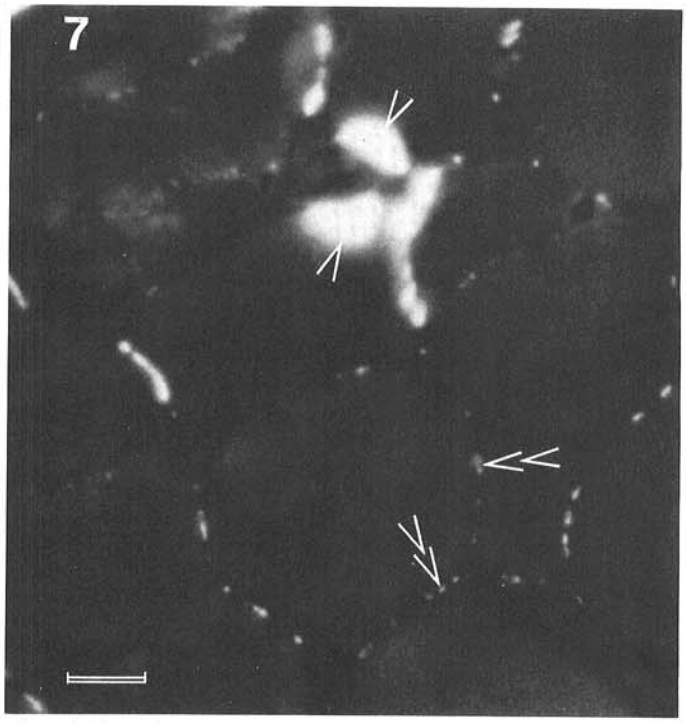
[a]Means of three replicates with standard errors in parentheses.

[b]2,4-D = dichlorophenoxyacetic acid.



Figs. 3–5. Fluorescent micrographs of whole mount squashed callus cells stained with aniline blue. **3,** Callose deposits (arrows) in callus cells of resistant maize infected with hyphae of *Phytophthora cinnamomi* 21 hr after inoculation. Bar = 100 μm. **4,** A callose deposit (arrows) in response to hyphae (hy) have penetrated callus cells of wheat, a resistant species, 17 hr after inoculation. Bar = 10 μm. **5,** A wild tomato (resistant) callus cell with several callose deposits (arrows) in response to invading hyphae, 24 hr after inoculation. Bar = 20 μm.





Figs. 6 and 7. 6, Section of root callus of maize (a resistant species) 24 hr after inoculation with hyphae of *Phytophthora cinnamomi*. Hyphae (hy) that penetrate between callus cells and adjacent callus cells form wall appositions or papillae (arrows). Nomarski optics. Bar = 20 μm. **7,** Fluorescent micrograph of section shown in Figure 6 stained with decolorized aniline blue. Papillae (single arrow) fluoresce when stained with aniline blue indicating the presence of callose. Small callose deposits (double arrows) are also seen between cells some distance from a hypha. Bar = 20 μm.

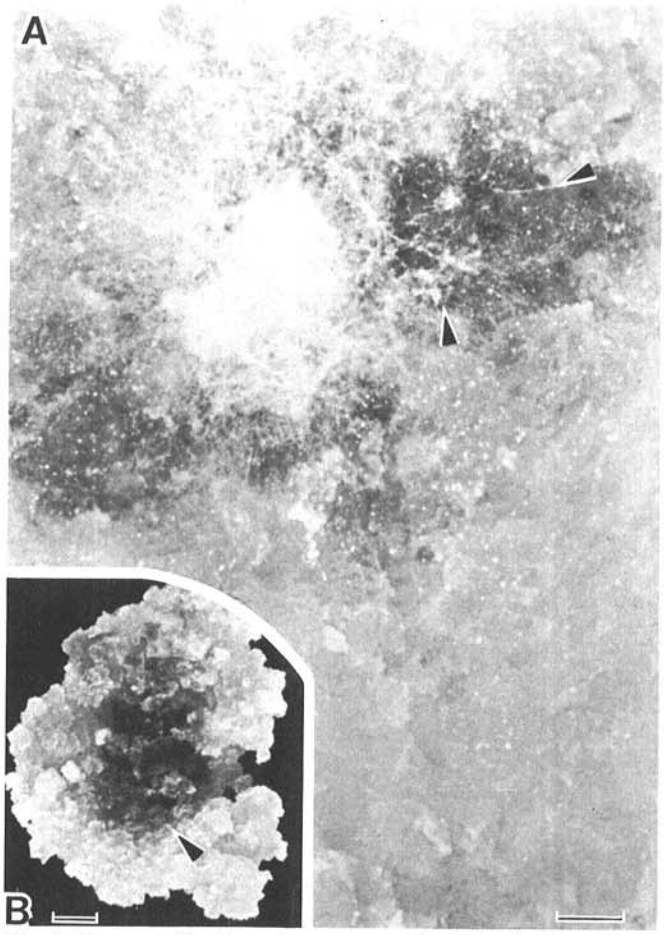


Fig. 8. Hypersensitive browning response of callus of wild tomato (a resistant species): **A,** 24 hr after inoculation, **B,** 46 hr after inoculation. Arrows show the limit of hyphal growth in relation to the browning. Scale bars, A = 2 mm, B = 3 mm.

comparisons within species such as jarrah, callus with a similar dry texture from the same plant organ was used. For comparisons among species, although callus from different organs was used, care was taken to use only callus with a firm dry surface.

Nonhost resistance (polygenic resistance) has not been extensively examined in tissue culture systems. Miller et al (16) found that nonhost resistance in alfalfa callus to the soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* was expressed in tissue culture but only at kinetin concentrations of 2.0–4.0 mg/L in the growth medium that gave dry friable callus. On the growth media used in our study, all the callus lines were dry, firm, or friable, and this was important for determining differences in hyphal growth rates.

Apart from the relative growth of the fungus, the production of callose in the callus also reflected the relative resistance of the plant from which the callus was derived. The form of the callose deposits was similar to that produced in intact roots. For example, maize callus and root tissue (8,9) both respond to the presence of the pathogen by producing large callose deposits surrounding the hyphae. Several of the species that produce callose in response to the pathogen in the tissue culture system, viz. *A. pulchella*, jarrah, marri, and wheat also produce callose in roots of infected plants (2,18). However, there was no correlation in eucalypt calli between the amounts of callose formed and the resistant or susceptible nature of the source of explant. Intact plants of marri and jarrah also showed no appreciable difference in callose production in response to infection with *P. cinnamomi* (14). Although the role of callose in plant defense is as yet unknown, the ability to produce callose quickly and in large amounts correlates with resistance in many cases (1). Callose formation in callus cells has been recorded for soybean suspension cultures in response to a fungal cell wall polymer (13) and in callus from *Nicotiana tabaccum* in response to the fungal pathogen *Peronospora tabacina* (19).

Another response commonly observed in infected resistant whole plants is browning at the site of infection. This response is also expressed in the callus-fungal interaction where the callus is derived from a resistant host; however, this response is not consistently expressed and several resistant calli did not respond this way.

The similarities of cellular response exhibited in infected cultured cells and root tissue to the broad host range pathogen *P. cinnamomi* indicate that a tissue culture system may be useful for investigating the basis of mechanisms associated with nonhost resistance. The advantage over using intact plants is that in undifferentiated callus the cell responses are more uniform than those of the intact root, in which the various cell layers behave differently in response to contact with the fungus (21). In addition, large amounts of callus at comparable stages of infection can be obtained for experimental purposes. The correlation between response in callus and the field resistance of jarrah suggests that a callus-fungal screening method might be used to select from disease-free areas, superior timber trees that are likely to be resistant to *P. cinnamomi*. However, further work is necessary to determine whether in all cases, the resistance mechanism expressed in trees can be expressed in callus cultures.

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