Expression of Genetic Susceptibility, Host Resistance, and Nonhost Resistance in Alfalfa Callus Tissue Inoculated with *Phytophthora megasperma*

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ABSTRACT

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An alfalfa (*Medicago sativa*) callus tissue culture system was developed in which host resistance and susceptibility to the alfalfa pathogen, *Phytophthora megasperma* f. sp. *medicaginis* (Pmm), and nonhost resistance to the soybean pathogen, *P. megasperma* f. sp. *glycinea* (Pmg), were expressed. Hyphae of Pmm completely covered 4- to 6-wk-old calli derived from a plant susceptible to Pmm (M269) within 5 days after inoculation, but growth was limited on calli derived from a plant resistant to Pmm (M194). Colonization of both callus types by Pmg was more restricted than colonization of M194 by Pmm. Calli in all interactions became discolored within 3 days after inoculation, but resistant and nonhost calli were more discolored and darker brown than susceptible ones. Both callus morphology and expression of disease resistance were influenced by the concentration of kinetin in the growth medium: as the kinetin concentration decreased, the consistency of the calli appeared more fluid and they were more susceptible to both Pmm and Pmg. A kinetin concentration of at least 2.0 mg/L of growth medium was necessary for a clear differentiation of resistant and susceptible genotypes.

The use of tissue culture systems to study various aspects of host-pathogen interactions has certain advantages over studies utilizing intact plants. Foremost among these are the simplicity offered by the presence of one or a few plant cell types, and the ability to control environmental and nutritional factors (1,6–10). Although results from studies of this type are usually interpreted with caution (1,15), several studies have demonstrated that with proper control of medium composition and environment, the same host-pathogen interaction type expressed in intact plants is expressed in callus tissue (4,7,9,14). In tobacco, both quantitatively and qualitatively inherited resistance to the black shank pathogen, *Phytophthora parasitica* var. *nicotianae*, are expressed in callus tissue (4,7). In fact, Helgeson et al (6) demonstrated that the same dominant gene determining resistance to race O of the pathogen is expressed in intact tobacco plants and in callus tissue.

We have investigated the possibility of utilizing tissue culture to study host and nonhost resistance in alfalfa to the alfalfa pathogen, *Phytophthora megasperma* f. sp. *medicaginis* (Pmm), and to the soybean pathogen, *P. megasperma* f. sp. *glycinea* (Pmg), respectively. Use of callus tissue could provide a simplified system to examine several aspects of host-pathogen interactions in alfalfa, including a comparison of host and nonhost resistance to two related plant pathogens.

This paper describes an alfalfa tissue culture system in which susceptibility, host resistance, and nonhost resistance to *P. megasperma* are expressed. A portion of this work was published previously (15).

MATERIALS AND METHODS

Initiation and maintenance of callus cultures. Calli were initiated from immature ovaries (17) of two alfalfa plants, one resistant (M194), the other susceptible (M269) to Pmm (11). Flower buds 2–4 mm long were surface sterilized by submerging them for 3 min in 95% ethanol, then 3 min in 1.1% sodium hypochlorite (a 1:5 dilution of commercial bleach). Buds were rinsed twice in sterile distilled water, and ovaries 1–2 mm long were removed aseptically and transferred to bottles containing modified Schenk-Hildebrandt (SH) medium (18), supplemented with (per liter) 2.0 mg 2,4-dichlorophenoxyacetic acid (2,4-D), 2.0 mg α-naphthaleneacetic acid (NAA), and 0.5, 1.0, 2.0, or 4.0 mg kinetin. Cultures were incubated in the dark at 21 ± 1 C for 4–6 wk, then subdivided into calli 3–5 mm in diameter. These were transferred to fresh media for an additional 4–6 wk until they were ~2 cm in diameter.

Growth and maintenance of pathogens. A single-zoospore isolate of Pmm (5b4, ATCC no. 42154) that was highly aggressive on alfalfa was used in all of the experiments. The isolate of Pmg (race 1, isolate 16) was obtained from C. R. Grau, University of Wisconsin at Madison. Both Pmm and Pmg were maintained on V-8 agar at 22 ± 2 C in ambient light, and pathogenicity was assured by testing periodically on alfalfa and soybean, respectively (12,13).

Inoculation procedures. Calli of M269 or M194 growing on modified SH medium were inoculated with hyphae of Pmm or Pmg. Hyphal inoculum was used instead of zoospores in order to localize the inoculation and prevent the inoculum from reaching the culture medium. Blocks of inoculum 1 mm^2 were taken from edges of 4- to 6-day-old colonies on V-8 agar medium. Excess agar was removed, and one block of inoculum was placed on the top of each callus piece. Calli were maintained at 21 ± 1 C in the dark for 3 days, and rated each day for mycelial development and tissue discoloration. Uninoculated calli served as controls.

Rating system for whole callus pieces. Calli were evaluated daily for hyphal colonization and tissue discoloration according to the following 0–4 rating system: 0 = no aerial hyphae visible and no tissue discoloration; 1 = aerial hyphae (or tissue discoloration) on the upper 25% of the callus; 2 = aerial hyphae (or tissue discoloration) on the upper 50% of the callus; 3 = aerial hyphae (or tissue discoloration) on the upper 75% of the callus; and 4 = aerial hyphae (or tissue discoloration) on the entire callus. Each treatment contained three replicates and each replicate was composed of three subsamples (calli).

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Determination of hyphal development in callus tissue. To determine the extent of colonization by Pmm or Pmg, calli were cut horizontally into four equal slices; each slice was mounted on a glass slide and stained with lactophenol cotton blue (10 g phenol, 10 ml glycerine, 10 ml lactic acid, 0.02 g aniline blue, and 10 ml distilled water). Slices were examined by using a dissecting microscope (×20), and colonization was rated subjectively on a scale of 0–5. A value of 0 was assigned to tissue slices with no visible hyphae, and a value of 5 to slices in which the hyphae had formed a thick mat. Three calli were evaluated per treatment.

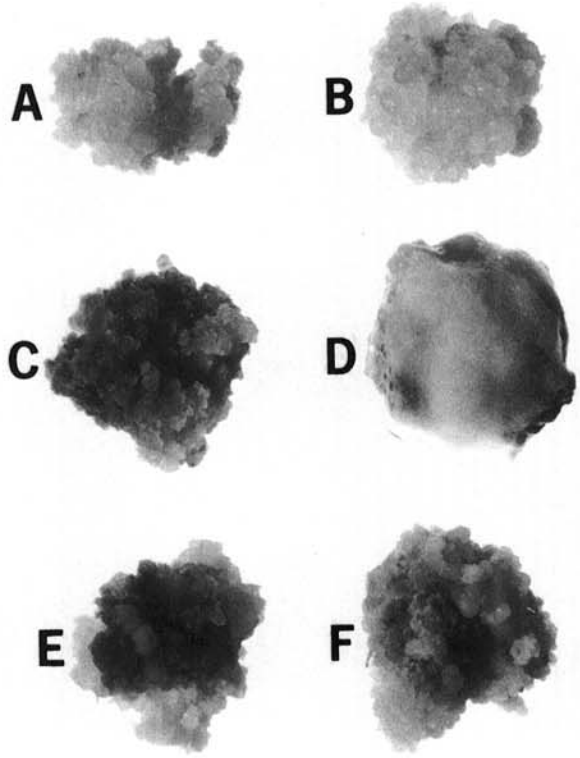

Fig. 1. Responses of two alfalfa callus tissue lines to *Phytophthora megasperma* f. sp. *medicaginis* (Pmm) and *P. megasperma* f. sp. *glycinea* (Pmg) 5 days after inoculation. Line M269 is susceptible to Pmm, line M194 is resistant, and both are nonhosts of Pmg. Calli were grown on modified Schenk-Hildebrandt medium (18) with a kinetin concentration of 2.0 mg/L. **A,** M194/uninoculated control; **B,** M269/uninoculated control; **C,** M194/Pmm; **D,** M269/Pmm; **E,** M194/Pmg; and **F,** M269/Pmg. Note the aerial hyphal development on M269/Pmm and the darkening reaction of M269/Pmg, M194/Pmm, and M194/Pmg.

TABLE 1. Aerial hyphal development and callus discoloration in two alfalfa callus tissue lines after inoculation with *Phytophthora megasperma* f. sp. *medicaginis* (Pmm) or *P. megasperma* f. sp. *glycinea* (Pmg). Line M269 is susceptible to Pmm, line M194 is resistant, and both are nonhosts of Pmg[a]

	Aerial hyphal development[b,c,d]			Tissue discoloration[b,c,d]		
Treatment	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
M269/Pmm	0.0	1.6	2.7	2.0	3.3	3.0
M194/Pmm	0.0	0.3	0.3	1.3	2.7	4.0
M269/Pmg	0.0	0.0	0.3	1.3	1.7	4.0
M194/Pmg	0.0	0.0	0.0	1.3	1.3	3.7
M269/Control	0.0	0.0	0.0	0.0	0.0	0.0
M194/Control	0.0	0.0	0.0	0.0	0.0	0.0

[a] Calli were grown on modified Schenk-Hildebrandt medium (18) with a kinetin concentration of 2.0 mg/L.
[b] Mean of three replications; each replicate contained three calli. The experiment was a 2 × 2 factorial with uninoculated controls that were not included in the analysis.
[c] Aerial hyphal development and callus tissue discoloration were rated on a 0 to 4 scale, in which 0 = no aerial hyphae or discoloration and 4 = aerial hyphal development or discoloration on entire callus (see Materials and Methods).
[d] Statistical analysis is presented in Table 2.

RESULTS

Susceptibility and host resistance to Pmm, and nonhost resistance to Pmg were expressed by alfalfa calli (Fig. 1, Table 1). Differences between resistant and susceptible interactions in the development of aerial hyphae on inoculated calli were evident within 2 days after inoculation (Table 1). Within 3 days, aerial hyphae of Pmm covered more than 50% of each susceptible (M269) callus piece; 2 days later, susceptible calli were completely covered with hyphae of Pmm (Fig. 1). In host resistant (M194/Pmm) and nonhost resistant (M269/Pmg or M194/Pmg) interactions, aerial hyphae were limited to the top of the callus, usually near the point of inoculation. Host genotype and pathogen type were highly significant ($P = 0.005$) in influencing the degree of hyphal development on calli (Table 2). The interaction term was also highly significant, further demonstrating the dependence of hyphal development on these two factors.

Inoculated callus tissue became brown in all the interactions. Initially, inoculated susceptible calli were more discolored than resistant ones (Table 1), but by the third day resistant calli were darker brown and more discolored than susceptible calli. Statistically, the influence of host genotypes on tissue discoloration was obscured by significant host × pathogen and host × pathogen × time interactions. The most significant component of the browning reaction was time after inoculation, although pathogen type was also highly significant (Table 2). No tissue discoloration or hyphal development was observed with uninoculated controls.

Microscopic examination of tissue slices revealed that both Pmm and Pmg colonized M269 and M194 calli. Colonization was greatest in the susceptible interaction (M269/Pmm), least in the nonhost resistant interactions (M269/Pmg or M194/Pmg), and intermediate in the host resistant interaction (M194/Pmm) (Fig. 2).

The concentration of kinetin in the growth medium affected alfalfa callus tissue morphology and expression of disease resistance or susceptibility (Table 3). Calli grown on medium with a high concentration of kinetin (4.0 mg/L) were usually very friable and dry in appearance; as the concentration of kinetin in the medium decreased, callus tissue appeared increasingly more fluid. The morphology of callus tissue of the line resistant to Pmm (M194) was generally less influenced by the concentration of kinetin in the growth medium than callus tissue of the Pmm-susceptible line, M269. Calli of both M194 and M269 grown on medium containing 4.0 mg of kinetin per liter were smaller and darker yellow than callus pieces grown on media containing lower concentrations of kinetin.

The influence of kinetin concentration in the growth medium on development of aerial hyphae on calli inoculated with Pmm or Pmg

TABLE 2. Analysis of variance for aerial hyphal development and tissue discoloration in alfalfa calli inoculated with *Phytophthora megasperma* f. sp. *medicaginis* or *P. megasperma* f. sp. *glycinea* (data from Table 1)

	Aerial hyphae		Tissue discoloration	
Source	df	MS[a]	df	MS[a]
Replication	2	0.07	2	0.30
Callus line (A)	1	5.51***	1	0.44
Pathogen type (B)	1	7.59***	1	0.78**
A × B	1	3.76***	1	0.0
Time after Inoculation (C)	1	0.84	2	14.78***
A × C	1	0.84	2	5.29*
B × C	1	0.26	2	14.35***
A × B × C	1	0.26	2	6.79**
Error	14	0.22	22	0.15
Total df	23		35	

[a] Asterisks (*, **, and ***) indicate differences significant at $P = 0.05$, $P = 0.01$, and $P = 0.005$, respectively.

was very highly significant ($P = 0.005$) (Table 4). In general, aerial hyphal development by both Pmm and Pmg was more extensive on calli grown on media with low (0.5 and 1.0 mg/L) rather than high (2.0 and 4.0 mg/L) kinetin concentrations. However, there was a significant ($P = 0.05$) interaction between kinetin concentration and callus lines (Table 4), indicating that the effect of kinetin was dependent on callus genotype.

DISCUSSION

An alfalfa callus tissue culture system has been described in which susceptibility, host resistance, and nonhost resistance to *P. megasperma* are expressed. Hyphae of Pmm quickly colonized calli derived from an alfalfa plant susceptible to Pmm, but were restricted in calli derived from a plant resistant to Pmm. Growth of the soybean pathogen, Pmg, was restricted in both alfalfa callus lines. Limitation of fungal growth was more obvious in the nonhost resistant interactions than in the host resistant interaction, but in no case was fungal growth absolutely inhibited. However, this pattern of hyphal development by Pmm and Pmg is quite similar to that which occurs in alfalfa seedlings resistant to Pmm (16). In seedlings, Pmm penetrates the epidermis, cortex, and stele of roots resistant to Pmm, but fungal growth is slower and less extensive than in roots susceptible to Pmm. Hyphae of Pmg also penetrate alfalfa seedling roots, but fungal growth is more restricted than the growth of Pmm in resistant roots. Thus, the response of alfalfa callus tissue to Pmm and Pmg appears to mimic that of alfalfa seedlings to these fungi. This is in agreement with the results of other workers (3,9) who have demonstrated a striking cytological similarity between the responses of calli and plants to compatible or incompatible pathogens.

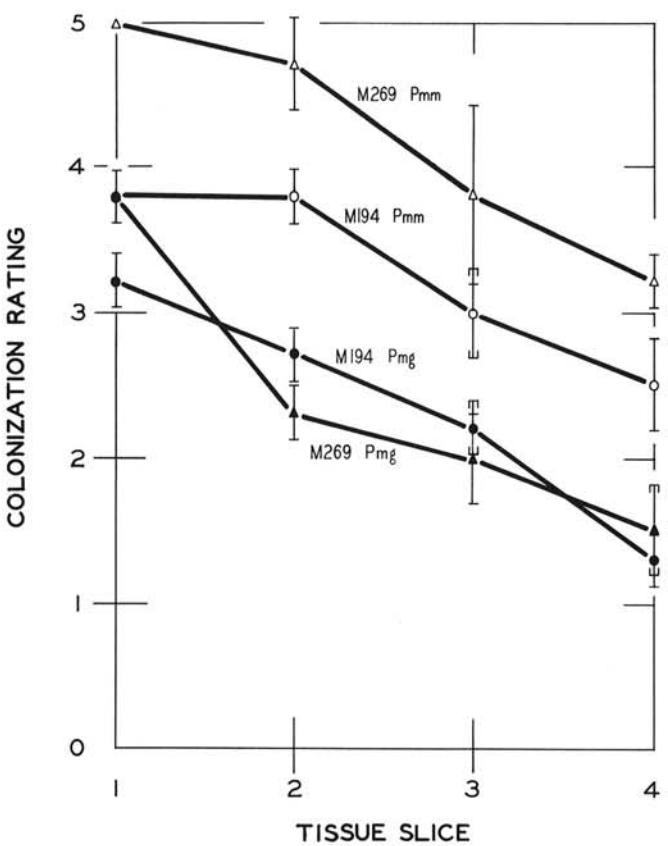

Fig. 2. Colonization of alfalfa callus tissue 72 hr after inoculation with *Phytophthora megasperma* f. sp. *medicaginis* (Pmm) or *P. megasperma* f. sp. *glycinea* (Pmg). Calli were sliced horizontally into four equal slices (numbered 1 to 4 from top to bottom) which were examined microscopically and rated for colonization on a 0 to 5 scale. Inoculum had been applied to the top of the callus. A value of 0 indicates that no hyphae were visible in the slice, while a value of 5 was assigned to slices in which the hyphae formed a thick mat. Alfalfa line M269 is susceptible to Pmm, line M194 is resistant, and both are nonhosts of Pmg. No hyphae were observed in uninoculated controls. Calli were grown on modified Schenk-Hildebrandt medium (18) with a kinetin concentration of 2.0 mg/L. Bars represent standard errors.

Both callus morphology and the expression of disease resistance were influenced by kinetin concentration in the growth medium; calli appeared more fluid and fungal development more extensive as the concentration of kinetin decreased. The ability of plant growth hormones to alter expression of disease resistance has been reported for other plant tissue culture systems (2,5,7,8). In the system involving tobacco and *Phytophthora parasitica* var. *nicotianae*, increasing the concentration of kinetin or benzyladenine relative to the concentration of indoleacetic acid in the growth medium effectively eliminated resistance to *P. parasitica* var. *nicotianae* (5). In the system involving alfalfa and *P. megasperma* the apparent increase in susceptibility of calli of M269 and M194 to Pmm and Pmg may have been related to an increase in available nutrients on the surface of these calli compared to the dry, friable calli grown in the presence of higher concentrations of kinetin. A kinetin concentration of at least 2.0 mg/L was necessary for the expression of the M194 (resistant to Pmm) and M269 (susceptible to Pmm) genotypes in callus tissue.

This study indicates that the system involving alfalfa callus tissue and *P. megasperma* is suitable for the examination of factors that may influence the expression of disease resistance in alfalfa. As a model system in which resistance and susceptibility are clearly differentiated, it should provide a simplified means of elucidating many of the molecular events that occur during the interaction of alfalfa with Pmm and Pmg.

The results of this study show that with carefully controlled

TABLE 3. Aerial hyphal development of *Phytophthora megasperma* f. sp. *medicaginis* (Pmm) and *P. megasperma* f. sp. *glycinea* (Pmg), on Pmm-resistant (M194) and Pmm-susceptible (M269) alfalfa calli, grown on modified Schenk-Hildebrandt (18) medium with various levels of kinetin, 3 days after inoculation

	Aerial hyphal development at indicated kinetin concentration (mg/L growth medium)[a,b,c]			
Interaction	0.5	1.0	2.0	4.0
M269/Pmm	3.3	3.0	3.0	2.7
M194/Pmm	2.0	2.0	1.0	0.3
M269/Pmg	1.7	1.3	1.0	0.7
M194/Pmg	1.0	2.0	0.3	0.3
M269/Control	0.0	0.0	0.0	0.0
M194/Control	0.0	0.0	0.0	0.0

[a] Mean of three replicates; each replicate contained three calli.
[b] Aerial hyphal development was rated on a 0 to 4 scale, in which 0 = no aerial hyphae and 4 = aerial hyphal development on entire callus (see Materials and Methods).
[c] Statistical analysis presented in Table 4.

TABLE 4. Analysis of variance for the effect of kinetin concentration on the development of aerial hyphae of *Phytophthora megasperma* f. sp. *medicaginis* or *P. megasperma* f. sp. *glycinea* on two alfalfa callus lines, 3 days after inoculation (data from Table 3)

	Aerial hyphal development	
Source	df	MS[a]
Replication	2	0.34
Kinetin concentration (A)	3	3.30***
Pathogen type (B)	1	15.19***
A × B	3	0.19
Callus line (C)	1	11.02***
A × C	3	0.91*
B × C	1	6.01***
A × B × C	3	0.25
Error	30	0.28
Total df	47	

[a] Asterisks (* and ***) indicate differences significant at $P = 0.05$ and $P = 0.005$, respectively.

culture conditions, genetic resistance to Pmm is expressed in alfalfa callus tissue. The similarities described in the response of callus tissue and seedling roots to Pmm and Pmg indicate that the tissue culture system may be suitable for the examination of factors that influence the expression of disease resistance in alfalfa. However, as very little is known about the molecular basis of expression of resistance genes in either intact plants or tissue cultures, the results of studies of this type should still be interpreted cautiously.

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