Stimulation of Oospore Production in *Phytophthora megasperma* f. sp. *medicaginis* by Medicarpin

Ahmad Vaziri, N. T. Keen, and D. C. Erwin

Graduate research assistant and professors, respectively, Department of Plant Pathology, University of California, Riverside 92521.
Accepted for publication 4 January 1983.

ABSTRACT

Vaziri, A., Keen, N. T., and Erwin, D. C. 1983. Stimulation of oospore production in *Phytophthora megasperma* f. sp. *medicaginis* by medicarpin. Phytopathology 73:730-734.

Medicarpin inhibited mycelial growth of *Phytophthora megasperma* f. sp. *medicaginis* (*Pmm*) isolates from alfalfa but stimulated oospore production (correlation coefficient = 0.98 for the number of oospores produced as a function of medicarpin concentration in the medium). However, medicarpin (75–125 $\mu g \cdot ml^{-1}$) did not stimulate oospore production in isolates of *P. megasperma* from other hosts or in *Phytophthora cinnamomi*. Oospore production was not stimulated by the soybean phytoalexins, glyceollin and glycinol, in either *Pmm* or *P. cinnamomi*.

Additional key words: Glycine max, Medicago.

During the course of an investigation of the effect of medicarpin on the growth of *Phytophthora megasperma* Drechs. f. sp. *medicaginis* Kuan and Erwin (*Pmm*) (19), we observed that the phytoalexin stimulated oopsore production. We report here that medicarpin specifically stimulates oospore production in several isolates of *Pmm*, but not in other *P. megasperma* formae speciales or in *Phytophthora cinnamomi*.

MATERIALS AND METHODS

General. Purified medicarpin was prepared from alfalfa seedlings (19) or from infected jack bean (*Canavalia ensiformis*) cotyledons (9). Glyceollin and glycinol (6a,3,9-trihydroxypterocarpan) were obtained from cotyledons of soybean (*Glycine max* [L.] Merr.) (7). Medicarpin was further purified and glyceollin isomers were separated by high performance liquid chromatography as described by Holliday et al (6) on a 9.4 mm × 50-cm column of Partisil 10/50 D320. Under the conditions employed, medicarpin eluted at 12.5 min, and glyceollin isomers I, II, and III eluted at 17.0, 18.1, and 19.7 min, respectively. Medicarpin was collected as a single symmetrical peak as determined by UV absorption (280 nm) and refractive index detectors, therefore indicating a high degree of purity.

Effect of medicarpin, glyceollin, and glycinol on mycelial growth and oospore production of several isolates of *Pmm* and of some other *Phytophthora* species. In different experiments, acetone solutions of medicarpin (75, 100, or 125 $\mu g \cdot ml^{-1}$), glyceollin (25, 50, or 100 $\mu g \cdot ml^{-1}$), or glycinol (100 or 200 $\mu g \cdot ml^{-1}$) were added to cooled V8-C agar (200 ml of cleared Campbell's V-8 juice and 2 g $CaCO_3$ per liter) or to a synthetic medium (SM) (4) modified to include 1 ml of β-sitosterol (30 mg dissolved in 30 ml of dichloromethane) and 14 g of Difco Noble agar per liter. Three milliliters of medium were used in sterile plastic petri dishes (5 cm in diameter). The final concentration of acetone in the medium was 1% v/v. Ethanol (final concentration, 1% v/v) was substituted for acetone in one experiment. The solvents had no significant effect on mycelial growth or oospore production when used at these concentrations.

Inocula consisted of 5-mm-diameter plugs cut from the growing edge of 7-day-old cultures that were placed in the center of the assay plates and incubated at 24 ± 1 C for 6 days. Colonies were measured at 24 hr intervals. Changes in net colony diameter (mean diameter of colonies minus the diameter of the inoculum plug) were plotted against incubation time. Oospore production was assessed by removing the colony from a plate, comminuting it in a Sorvall Omni-mixer in 17 ml of sterile deionized distilled water for 15 sec at full speed and counting the oospores in a Hawksley eelworm-counting chamber. Three counts were made for each treatment. Since medicarpin at higher concentrations was completely inhibitory to *P. megasperma* f. sp. *glycinea* (*Pmg*) and *P. cinnamomi*, a lower concentration (25 $\mu g \cdot ml^{-1}$) was used for these isolates. Quadruplicate plates were used for each treatment, and the experiments were repeated twice.

Pathogenicity of *Phytophthora* isolates on soybean. Seven-day-old plants (cultivars Harosoy and Harosoy 63) were grown in the greenhouse, and hypocotyls were wounded (5). Small pieces of mycelium grown on V8-C agar were inserted into shallow (through the epidermis and cortex only) or deep (approximately one half of the hypocotyl diameter) wounds. Twenty plants were inoculated in each pot. Pots were placed in a moist chamber and symptoms were recorded after 2 days. Root inoculations were performed by pouring 50 ml of a mycelial suspension into trenches made between the plants in each pot (19).

RESULTS

Effect of medicarpin on oospore production by *Pmm* and other *Phytophthora* spp. Oospore production by several isolates of *Pmm* was stimulated by medicarpin in V8-C agar medium.

Oospores per plate of isolate P1057 and 0 medicarpin was 81,500; at 75 $\mu g \cdot ml^{-1}$, 245,500; at 100, 314,000; and at 125, 437,000. These values differed significantly ($P = 0.01$) according to Duncan's multiple range test. The control treatment (0 medicarpin) was done with and without 1% ethanol (solvent for medicarpin), but there was no significant difference in numbers of oospores. The number of oospores produced was highly correlated ($r = 0.98$) with medicarpin concentrations up to 125 $\mu g \cdot ml^{-1}$. Values were not recorded at higher phytoalexin concentrations, because little or no fungal growth occurred above 125 $\mu g \cdot ml^{-1}$, as previously observed (19). None of the *Pmm* isolates produced oospores on SM only, but most were induced to make them when medicarpin was added to the medium (Table 1). Isolates P347 and P1253, believed to be *Pmm*, did not produce oospores in either SM or V8-C, with or without medicarpin. Isolates 234, 254, and 260 of *Pmm* did not produce oospores in SM supplemented with medicarpin, but production was stimulated in V8-C agar supplemented with medicarpin (Table 1).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

Medicarpin did not stimulate oospore production by *P. cinnamomi* on either tested medium (Table 1). However, the phytoalexin inhibited oospore formation by *P. megasperma* f. sp. *glycinea* and the Douglas fir (DF) group 1 isolates of *P. megasperma*. The latter isolates were unique in forming oospores on synthetic medium not supplemented with medicarpin.

Effect of glyceollin and glycinol. Glyceollin and glycinol are pterocarpan phytoalexins from soybean and the latter compound is structurally similar to medicarpin. It was therefore of interest to test whether these compounds also stimulated oospore production by *Pmm* or other *Phytophthora* species. Despite the fact that glyceollin inhibited the growth of most tested isolates (Table 2), neither it nor glycinol stimulated oospore production in any tested *Phytophthora* isolate on either V8-C or SM. Glycinol had no inhibitory effect on the growth rate of the isolates tested up to 200 $\mu g \cdot ml^{-1}$.

Growth inhibition of *Phytophthora* species by phytoalexins. The growth curves obtained by plotting the colony diameters against time were of three types on V8-C and SM (Fig. 1) (17). Growth type A, produced by controls without phytoalexins, was characterized by growth on the first day, which was linear throughout the incubation period. In type B, there was a lag period, after which growth was linear, similar to type A. This response was the most common with media containing a phytoalexin. In type C, there was a lag period, after which the growth rate increased progressively throughout the incubation period. This pattern was observed with a few isolates on media supplemented with phytoalexins (Table 2). No consistent pattern was apparent regarding the effect of medicarpin and glyceollin on the growth of several isolates of *Pmm* and *Pmg* (Table 2).

Pathogenicity of various *Phytophthora* isolates on soybean. Isolates 304 and 306 from Douglas fir (DF) group 1 were reported to be pathogenic on soybean when inoculated into hypocotyl wounds (5). Accordingly, these isolates were thought to be indistinguishable from *Pmg*. We repeated the inoculation tests with soybean and also tested the response of the DF isolates to

TABLE 1. Effect of medicarpin on oospore production by several Phytophthora isolates

Phytophthora isolate	Source	Source host	Pathogenicity[a] to alfalfa	Number of oospores per plate			
				Medium			
				Synthetic		V8-C	
				Without medicarpin	With[b] medicarpin	Without medicarpin	With[b] medicarpin
P. megasperma f. sp. *medicaginis*							
P1057	California	Alfalfa	P	0	42,600	93,600	192,800
P844	California	Alfalfa	P	0	129	4,500	4,800
P410	Minnesota	Alfalfa	P	0	25,700	36,000	96,000
P1138	Wisconsin	Alfalfa	P	0	3,800	1,250	10,200
P182	Ohio	Alfalfa	P	0	8,700	6,400	12,000
P176	California	Alfalfa	P	0	400	0	580
P127	Australia	Alfalfa	P	0	59,500	4,000	39,400
DZ-1-B2	California	Alfalfa	P	0	1,300	23,200	35,400
P339	Mississippi	Alfalfa	P	0	82,600	59,200	196,000
P347	Mississippi	Alfalfa	P	0	0	0	0
P1253	Australia	Chickpea	P	0	0	0	0
234	Wisconsin	Alfalfa	P	0	0	26,000	67,700
254	Wisconsin	Alfalfa	P	0	0	0	2,000
260	Wisconsin	Alfalfa	P	0	0	12,000	13,800
P. megasperma (large spored)							
508	Oregon	Alfalfa	S	0	0	0	0
509	Oregon	Alfalfa	S	0	0	28,600	34,600
P. megasperma cv. HTI							
P240	California	Alfalfa	P	0	0	0	0
P. megasperma f. sp. *glycinea*							
$P900_5$	Ohio	Soybean	N	0	0	46,800	0
P405 a	Mississippi	Soybean	N	0	0	18,700	562
race 1	Illinois	Soybean	N	0	0	13,900	0
race 4	Illinois	Soybean	N	0	0	28,800	9,600
race 5	Illinois	Soybean	N	0	0	22,400	4,800
race 6	Illinois	Soybean	N	0	0	51,800	8,600
race 7	Indiana	Soybean	N	0	0	16,000	10,000
P. megasperma Douglas fir group 1							
304	Oregon	Douglas fir	N	43,800	0	38,900	0
306	Oregon	Douglas fir	N	27,000	0	22, 800	0
Douglas fir group 2							
336	Washington	Douglas fir	N	0	0	0	0
C-17-2D-2	Oregon	Douglas fir	N	0	0	0	0
P. megasperma f. sp. *trifolii*							
102	Mississippi	Arrowleaf clover	N	0	0	0	0
105	Mississippi	Arrowleaf clover	N	0	0	0	0
P. cinnamomi							
P110 (A2)	Type culture	Cinnamon	N	0	0	0	0
P138 (A1)	California	Avocado	N	0	0	0	0

[a] P = pathogenic; S = slightly pathogenic; N = nonpathogenic.
[b] 75 $\mu g \cdot ml^{-1}$ for *P. megasperma* f. sp. *medicaginis*, 25 $\mu g \cdot ml^{-1}$ for *P. megasperma* f. sp. *glycinea* and *P. cinnamomi* isolates.

phytoalexins. Confirming Hamm and Hansen (5), both of the DF group 1 isolates as well as races 1 and 7 of *Pmg* attacked and killed soybean seedlings when inoculated into deep hypocotyl wounds on cultivar Harosoy. The group 2 isolates, *P. cinnamomi*, and isolates of *Pmm* did not attack soybean when inoculated into deep wounds. However, neither DF group 1 isolate produced a susceptible plant reaction when inoculated into cultivars Harosoy or Harosoy 63 through shallow hypocotyl wounds. Instead a hypersensitive reaction occurred. The *Pmg* isolates produced typical race-specific reactions under the same conditions. Further, no plant reactions were observed when the DF group 1 isolates were applied by root drench to soybean seedlings. Consistent with results of the inoculation experiments, the DF group 1 isolates produced oospores on unsupplemented SM, unlike any other tested isolate, and addition of medicarpin to this medium or V8-C entirely abolished oospore production (Table 1). These responses were unlike any tested *Pmg* isolate.

DISCUSSION

Medicarpin stimulated oospore production in 12 of the 14 tested isolates of *Pmm* on either synthetic or V8-C medium. Especially significant was the fact that no isolate of *Pmm* produced oospores on SM only, but nine of the 14 isolates produced oospores when this medium was supplemented with medicarpin. Isolates P347 (in culture since 1965) and P1253 (from chickpea) (8) of *Pmm* did not respond to medicarpin, but these isolates may be anomalies, since they did not produce any oospores on V8-C or in inoculated alfalfa seedlings incubated in water. Thus, they may lack the capability to produce oospores under any condition. The large-spored isolates of *P. megasperma* originated from mildly diseased alfalfa roots in Oregon (Table 1). Since greenhouse pathogenicity tests showed them to be far less aggressive to alfalfa than the small-spored isolates commonly found on alfalfa, they must not be *Pmm* as suggested by their general lack of response to medicarpin. The high

TABLE 2. Effects of medicarpin and glyceollin on the growth of several *Phytophthora* isolates

Phytophthora isolate	Percent inhibition[a]			
	Medium			
	Synthetic		V8-C	
	Medicarpin[b]	Glyceollin[c]	Medicarpin[b]	Glyceollin[c]
P. megasperma				
f. sp. *medicaginis*				
P1057	30	49	64	46
P844	66	45	10*[d]	42
P410	44	72	42	30
P1138	51	83	58*	42
P182	65	52	64	46
P176	59	72	32	23
P127	72	56	72	52
DZ-1-B2	76	68	78	56
P339	52	43	41	29
P347	45	73	50	36
P1253	64	58	51	37
234	43*	10	0	39
254	10	15	32	48
260	41	48	10	37
P. megasperma				
(large spored)				
508	57	57	53	43
509	47	30	47	33
P. megasperma				
cultivar HTI				
P240	38	52	42	45
P. megasperma				
f. sp. *glycinea*				
P900s	78	100	81	100
P405 a	63	39	20	37
race 1	59	45	71	30
race 4	21	44	58	39
race 5	20	43	59	35
race 6	15*	30*	43*	17
race 7	28	40	18	23
P. megasperma				
Douglas fir group 1				
304	79	63	79	65
306	79	63	76	65
Douglas fir group 2				
336	90	57	66	58
C-17-2D-2	55	63	33	43
P. megasperma				
f. sp. *trifolii*				
102	40	79	45	75
105	51	86	46	80
P. cinnamomi				
P110 (A2)	35	30	49	51
P138 (A1)	50	55	48	60

[a] Calculated as $[(C-T)/C] \times 100$, where C = rate of growth in $mm \cdot h^{-1}$ on control medium and T = rate on medium containing phytoalexin (1).
[b] 75 $\mu g \cdot ml^{-1}$, except 25 $\mu g \cdot ml^{-1}$ for the *P. megasperma* f. sp. *glycinea* and *P. cinnamomi* isolates.
[c] 50 $\mu g \cdot ml^{-1}$.
[d] Growth curves were type B for all the treatments except fot those marked with a * on which the curves were of type C; see Fig. 1 for examples of different growth curves.

temperature isolate P240 (*P. megasperma* cultivar HTI) (16) was isolated from alfalfa in the Imperial Valley of Southern California in 1963. High temperatures (29–32 C and above) are required for this isolate to cause severe root rot. This fungus has never produced oospores under any experimental conditions, and it is morphologically distinct from *Pmm* because of its large hyphal swellings.

The observed specificity of oospore production stimulated by medicarpin in the isolates classified as *Pmm*, but not other isolates of *Phytophthora megasperma* or *P. cinnamomi* suggests that it may be a useful identification criterion for *Pmm*. It is possible that the fungus could have evolved to accelerated production of oospores when confronted by the hostile environment presumably constituted by medicarpin in the host (19). The response could accordingly be significant in the survival of *Pmm*.

Oospore production has been induced in heterothallic *Phytophthora* species by certain chemicals (13,21), bacteria (11), or *Trichoderma* species (1,2,14,15). The diversity of these agents suggests that the stimulatory effect is relatively nonspecific and that it results from chemical and/or physical irritation. Mode of action studies have also indicated that pterocarpanoid phytoalexins inhibit fungal growth by inducing relatively nonspecific membrane damage (18). Several factors, however, suggest that the stimulation of oospore production caused in *Pmm* by medicarpin does not result from injury accompanying inhibited growth. First, medicarpin induced the effect in most of the tested isolates of *Pmm*, but in no other *Phytophthora* species, even though they were all sensitive to the phytoalexin (Tables 1 and 2). Second, the related pterocarpanoid phytoalexins, glyceollin and glycinol, did not stimulate oospore production in *Pmm* or other *Phytophthora* species. The stimulatory effect of medicarpin on *Pmm* also seems unrelated to sterol-induced oospore production reported by several workers in pythiaceous fungi (10,12,20), since the medicarpin preparations were judged free of contaminants by HPLC and the SM employed included β-sitosterol. All of these factors indicate that the stimulatory effect of medicarpin on oospore production by *Pmm* does not result from simple chemical irritation.

Hamm and Hansen (5) believed that DF group 1 isolates might be indistinguishable from *Pmg* isolates from soybean, since hypocotyl inoculation of the former isolates gave susceptible symptoms on soybeans. Our results indicate that, while the DF group 1 isolates indeed attack soybean when plants are inoculated in massive wounds, they cannot be considered identical with *Pmg* isolated from soybean. When shallow hypocotyl wounds were employed, *Pmg* was again pathogenic but the DF group 1 isolates induced only a pronounced hypersensitive defense reaction and a large accumulation of the soybean phytoalexin glyceollin. The uniqueness of *Pmg* and the DF group 1 isolates was confirmed by the oospore production experiments. None of the *Pmg* isolates produced oospores on SM with or without medicarpin (Table 1), but both of the DF group 1 isolates did. Further, most isolates of *Pmg* produced oospores on V8-C in the presence of medicarpin, but both DF isolates did not.

Cruickshank (3) observed that certain compatible pathogens were more tolerant of the phytoalexins from their host plant than were nonpathogens. This relationship was not observed when several isolates of *Pmm* and *Pmg* were tested against glyceollin from soybean and medicarpin from alfalfa, since as much variation in phytoalexin sensitivity was noted within as between the groups (Table 2).

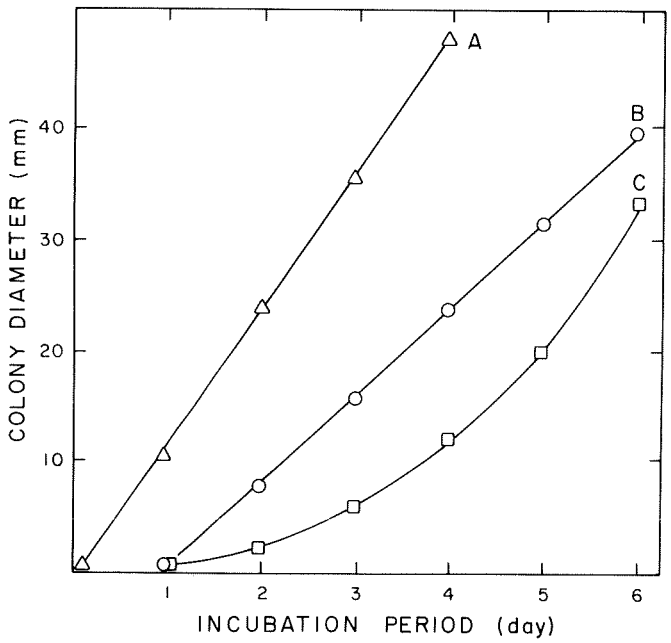

Fig. 1. Graph illustrating the types of growth curves plotted from the colony diameter of *Phytophthora megasperma* f. sp. *medicaginis* on V8-C or SM agar containing phytoalexins. **A,** Growth of isolate P1057 on V8-C without a phytoalexin. **B,** Growth of isolate P1057 on V8-C with 75 $\mu g \cdot ml^{-1}$ medicarpin. **C,** Growth of isolate P844 in V8-C containing 75 $\mu g \cdot ml^{-1}$ medicarpin. A, B, and C refer to the type of growth obtained (see text).

LITERATURE CITED

1. Bailey, J. A., Carter, G. A., and Skipp, R. A. 1976. The use and interpretation of bioassays for fungitoxicity of phytoalexins in agar media. Physiol. Plant Pathol. 8:189-194.
2. Brasier, C. M. 1971. Induction of sexual reproduction in single A^2 isolates of *Phytophthora* species by *Trichoderma viride*. Nature 231:283.
3. Cruickshank, I. A. M. 1977. A review of the role of phytoalexins in disease resistance mechanisms. Pontific. Acad. Scient. Scripta Varia 41:509-569.
4. Erwin, D. C., and Katznelson, H. 1961. Studies on the nutrition of *Phytophthora cryptogea*. Can. J. Microbiol. 7:15-25.
5. Hamm, R. B., and Hansen, E. M. 1981. Host specificity of *Phytophthora megasperma* from Douglas fir, soybean and alfalfa. Phytopathology 71:65-68.
6. Holliday, M. J., Keen, N. T., and Long, M. 1981. Cell death patterns and accumulation of fluorescent material in the hypersensitive response of soybean leaves to *Pseudomonas syringae* pv. *glycinea*. Physiol. Plant Pathol. 18:279-287.
7. Ingham, J. L., Keen, N. T., Mulheirn, L. J., and Lyne, R. L. 1981. Inducibly-formed isoflavonoids from leaves of soybean. Phytochemistry 20:795-798.
8. Irwin, J. A. G., and Dale, J. L. 1982. Relationships between *Phytophthora megasperma* isolates from chickpea, lucerne and soybean. Aust. J. Bot. 30:199-210.
9. Keen, N. T. 1972. Accumulation of wyerone in broadbean and demethylhomopterocarpin in jack bean after inoculation with *Phytophthora megasperma* var. *sojae*. Phytopathology 62:1365-1366.
10. Mellano, H. M., Munnecke, D. E., and Sims, J. J. 1970. Relationship of pectic enzyme activity and presence of sterols to pathogenicity of *Pythium ultimum* on roots of *Antirrhinum majus*. Phytopathology 60:943-950.
11. Mukerjee, N., and Roy, A. B. 1962. Microbial influence on the formation of oospores in culture by *Phytophthora parasitica* var. *sabdariffae*. Phytopathology 52:583-584.
12. Nes, W. D., Hanners, P. K., Bean, G. A., and Patterson, G. W. 1982. Inhibition of growth and sitosterol-induced sexual reproduction in *Phytophthora cactorum* by steroidal alkaloids. Phytopathology 72:447-450.
13. Noon, J. P., and Hickman, C. J. 1974. Oospore production by a single isolate of *Phytophthora capsici* in the presence of chloroneb. Can. J. Bot. 52:1591-1595.
14. Pratt, B. H., Sedgley, J. H., Heather, W. A., and Shepherd, C. J. 1972. Oospore production in *Phytophthora cinnamomi* in the presence of *Trichoderma koningii*. Aust. J. Biol. Sci. 25:861-863.
15. Reeves, R. J., and Jackson, R. M. 1974. Stimulation of sexual reproduction in *Phytophthora* by damage. J. Gen. Microbiol. 84:303-310.
16. Ribeiro, O. K., Erwin, D. C., and Khan, R. A. 1978. A new high-temperature *Phytophthora* pathogenic to roots of alfalfa. Phytopathology 68:155-161.
17. Skipp, R. A., and Bailey, J. A. 1977. The fungitoxicity of isoflavanoid phytoalexins measured using different types of bioassay. Physiol. Plant Pathol. 11:101-112.
18. VanEtten, H. D., and Pueppke, S. G. 1976. Isoflavonoid phytoalexins. Pages 239–289 in: Biochemical Aspects of Plant-Parasite

Relationships. J. Friend and D. R. Threlfall, eds. Academic Press, London. 354 pp.
19. Vaziri, A., Keen, N. T., and Erwin, D. C. 1981. Correlation of medicarpin production with resistance to *Phytophthora megasperma* f. sp. *medicaginis* in alfalfa seedlings. Phytopathology 71:1235-1238.
20. Zentmyer, G. A. 1980. *Phytophthora cinnamomi* and the diseases it causes. Phytopathological Monograph 10, The American Phytopathological Society, St. Paul, MN.
21. Zentmyer, G. A., and Erwin, D. C. 1970. Development and reproduction of *Phytophthora*. Phytopathology 60:1120-1127.