Correlation of Medicarpin Production With Resistance to *Phytophthora megasperma* f. sp. *medicaginis* in Alfalfa Seedlings

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ABSTRACT

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The resistance of 18 alfalfa cultivars to *Phytophthora megasperma* f. sp. *medicaginis* (Pmm), as determined in greenhouse tests, was correlated with the accumulation of medicarpin, one of three antifungal compounds produced in seedling assays. Two of the induced compounds were isolated and identified as 3-hydroxy, 9-methoxypterocarpan (medicarpin) and 4',7-dihydroxyflavanon (liquiritigenin), on the basis of ultraviolet and mass spectral data. The third compound was not identified. Medicarpin was the major antifungal compound formed. Inoculated seedlings initially contained traces of medicarpin, which increased rapidly by 12 hr, and reached maximum concentrations at about 72 hr. Medicarpin was produced following inoculation of both resistant and susceptible cultivars, but the concentration was always less in susceptible cultivars (correlation coefficient = −0.95 for the degree of susceptibility determined in the greenhouse with medicarpin concentrations produced in the seedling assay). Bioassays showed that Pmm was sensitive to medicarpin (ED_{50} for inhibition of mycelial growth = 97 $\mu g\ ml^{-1}$).

Several factors have been proposed to account for the resistance of alfalfa (*Medicago sativa* L.) to Phytophthora root rot caused by *Phytophthora megasperma* Drechs. f. sp. *medicaginis* Kuan and Erwin (Pmm). Chi and Sabo (1) reported that the degree of chemotaxis of Pmm zoospores toward the roots of alfalfa seedlings was correlated with the susceptibility of various cultivars. Marks and Mitchell (11,12) reported that wound periderm appeared to form around the infection court on the tap root and suggested that cultivar resistance might be associated with the central stele. A hypersensitive-type reaction and accumulation of an orange-yellow, granular material in the cortical cells of the root tip of a resistant cultivar also was observed by Marks and Mitchell (12). They suggested that this material could contribute to resistance. We have also observed a hypersensitive-type reaction in the cortical cells of the roots of resistant cultivars.

Many plants respond to invasion by pathogenic or nonpathogenic organisms by a hypersensitive defense reaction typified by the accumulation of low-molecular-weight antimicrobial substances called phytoalexins (2,3,10,15). Since phytoalexins have been isolated from alfalfa leaves and might be involved in the resistance of alfalfa to several pathogens (4–6,9,13), we investigated the possibility that they may contribute to resistance to Pmm.

MATERIALS AND METHODS

Greenhouse evaluation of alfalfa cultivars for resistance to root rot. Seeds of 18 alfalfa cultivars and polycrosses (Table 1) were planted in two rows in pots (10 × 10 × 10 cm) containing steamed UC mix (peat moss:sand, 1:1, v/v). Plants were generally inoculated after 3 wk in the greenhouse. They were fertilized with Osmocoat® (18-16-12, N-P-K; 4 g per pot) 2 wk after planting.

The inoculum was prepared from 7-day-old cultures of Pmm isolate P1057 (Phytophthora culture collection, Department of Plant Pathology, University of California, Riverside) grown on petri plates (9 cm in diameter) containing 10 ml of V8C agar (200 ml of cleared Campbell's V-8 juice and 2 g $CaCO_3$ per liter). Mycelia obtained from two petri plates were blended for 15 sec in a Waring Blendor in 1 L of distilled water. Three-week-old plants were inoculated by pouring 50 ml of inoculum into trenches made between the rows of plants in each pot. Saucers were placed under the pots, which were watered by flooding the surface of the soil and filling the saucers under each pot with water for 4 days. The saucers were then inverted and placed beneath the pots and plants were watered only as necessary for 3 days. The cycle was repeated until the end of the experiment.

TABLE 1. Relationship between the amount of medicarpin extracted from seedlings of alfalfa cultivars with varying degrees of resistance 48 hr after inoculation with a mycelial suspension of isolate P1057 of *Phytophthora megasperma* f. sp. *medicaginis* and the disease indices of 3-wk-old plants from the same varieties inoculated in the greenhouse

Cultivar	Seed sources[w]	Disease index[x]	Medicarpin (μg/g fresh wt)
A77-10B	UCR	2.10 a[y]	9.61 a[y]
PX76-101	NK	2.92 b	6.25 b
Valador	NAPB	2.96 b	5.83 b
PX76-84	NK	3.10 bc	5.91 b
Trident	NAPB	3.10 bc	4.35 c
Washoe	UNR	3.32 bcd	...[z]
As-13R	FM	3.45 cde	3.45 cd
K3 651-Fd71	NK	3.55 def	2.87 cd
K3 650-Fd77	NK	3.59 def	3.37 d
Agate 4082	UM	3.56 defg	3.53 de
Lahontan	UC,IVFS	3.66 defg	...[z]
Answer	NAPB	3.79 efg	2.46 ef
UC Salton	UC,IVFS	3.98 fgh	2.13 ef
UC 66C	UC,IVFS	4.07 ghi	...[z]
AZ-Ron	UA	4.29 hi	2.01 ef
Moapa 69	UC,IVFS	4.30 hi	2.15 ef
DeKalb 185	RS	4.41 hi	1.64 f
African	UCR	4.45 i	...[z]

[w] UCR = University of California, Riverside; NK = Northrup King Co.; NAPB = North American Plant Breeders; UNR = University of Nevada, Reno; FM = Ferry Morse Seed Co.; UM = University of Minnesota, St. Paul; UC, IVFS = University of California, Imperial Valley Field Station; UA = University of Arizona, Tucson; and RS = Ramsey Seed Inc.
[x] Rated on a scale of 0–5, in which 0 = completely healthy and 5 = dead.
[y] Mean values in each column followed by the same letter are not significantly different at $P = 0.01$ according to Duncan's multiple range test.
[z] Unable to extract due to poor germination and/or contamination.

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Two weeks after inoculation, the severity of root rot was scored visually: 0 = no disease; 1 = small lesions on lateral roots; 2 = slightly larger lesions encompassing not over 20% of the circumference of the taproot; 3 = lesions covering the circumference of the taproot in a narrow band; 4 = large lesions covering 100% of the root but the plant still living; and 5 = the entire root rotted and the plant dead. All treatments were replicated four times with 20 plants per replication and the experiments were repeated twice. Experiments were performed in a greenhouse at 20–28 C.

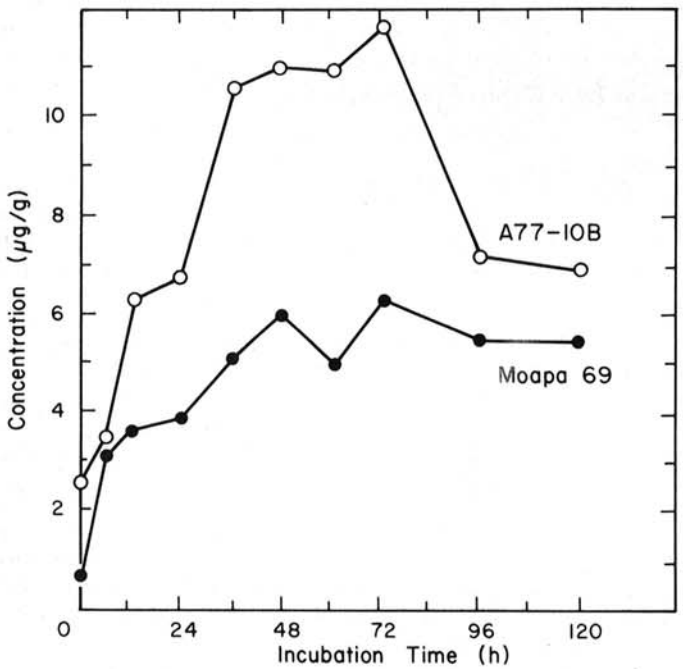

Fig. 1. Time course of medicarpin production by the alfalfa cultivars Moapa 69 (susceptible) and A77-10B (resistant) following inoculation with *Phytophthora megasperma* f. sp. *medicaginis*.

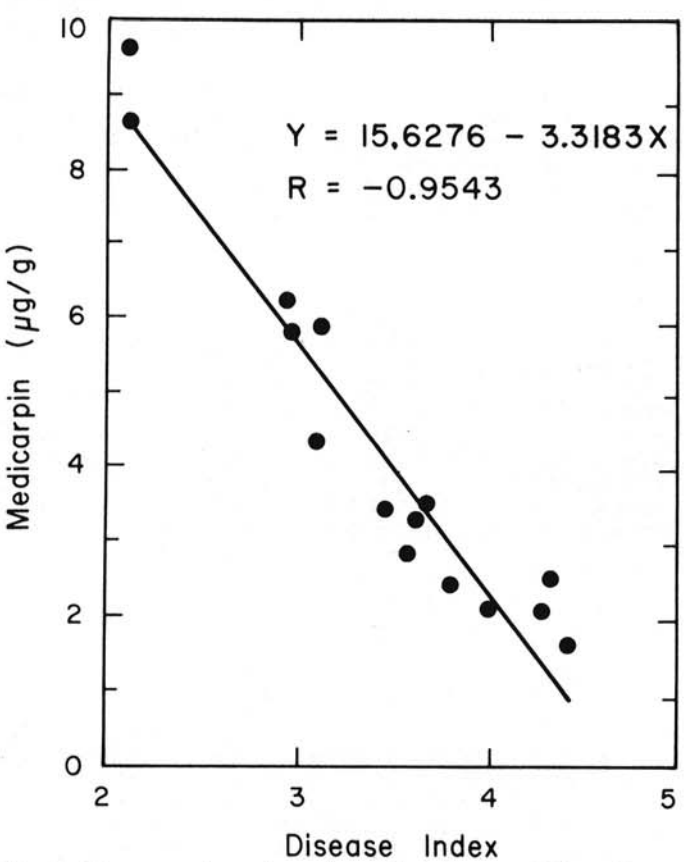

Fig. 2. Disease severity ratings of greenhouse-grown alfalfa cultivars inoculated with *Phytophthora megasperma* f. sp. *medicaginis* and the amount of medicarpin produced in seedling assays. Disease severity was rated on a scale of 0–5, in which 0 = completely healthy and 5 = dead.

Evaluation of phytoalexin production by inoculated alfalfa seedlings. Seeds of cultivars tested in the greenhouse for resistance (Table 1) were germinated on autoclaved Whatman No. 1 filter paper moistened with 10 ml of sterilized deionized water in sterile glass petri dishes (90 × 50 mm). Seeds (3.3 g per petri dish) were surface sterilized in a solution of 95% ethanol, sodium hypochlorite, and water (4:1:5, v/v) for 15 min and rinsed twice with sterilized distilled water prior to incubation at 25 C. Three petri dishes were used for each cultivar in each experiment. Ten milliliters of sterilized deionized water was added to each dish after 2 days.

Roots of 4-day-old seedlings in each petri dish were inoculated with 25 ml of a hyphal suspension of Pmm prepared by mincing a 7-day-old culture as described above except that one petri plate culture was blended in 100 ml of sterilized deionized water. Seedlings were incubated on a laboratory bench at 25–27 C with a 12-hr photoperiod (Vitalite, Power-Twist, Double Cathode 40-W fluorescent lamps, Duro-Test Corp., North Bergen, NJ 07047; 1,305 $\mu W\ cm^{-2}$).

Extraction and assay of phytoalexins. Replicate plates of seedlings were harvested at different time periods after inoculation. The whole plants were weighed, homogenized with 95% ethanol (2 ml/g of plant tissue) in a Sorvall Omnimixer at full speed for 2 min and filtered through Whatman No. 41 filter paper. Ethanolic extracts were reduced to about 1/10 of the original volume in vacuo at 50 C. These were partitioned twice with equal volumes of ethyl acetate the the organic fractions were dried in vacuo at 50 C. The residues were dissolved in ethyl acetate (4 μl/g fresh weight of original plant tissue) and 20 μl applied to thin-layer chromatography (TLC) plates of Silica Gel GF (precoated Redi/Plate, 0.250 mm thickness, Fisher Scientific Co., Pittsburgh, PA 15219). The plates were developed with either hexanes:ethyl acetate:methanol (120:80:3, v/v; solvent 1) or chloroform: acetone:concentrated ammonium hydroxide (50:50:1, v/v; solvent 2). Unless otherwise stated, reported R_f values were obtained by using solvent 1.

Areas on the TLC plates containing antifungal compounds were detected by the TLC bioassay (8) and could also be detected as absorbing spots when the plates were irradiated with ultraviolet light (254 nm). The spot corresponding to medicarpin on plates developed with solvent 1 was eluted by centrifuging the silica gel in 1.5 ml of 95% ethanol. The eluates were then scanned over the range of 250 to 320 nm in a spectrophotometer and the concentration of medicarpin was determined by the absorption at 287 nm, assuming log Σ = 3.90 (18). No corrections were made for losses during extraction or TLC.

Bioassays. Antifungal compounds on TLC plates were detected by the method previously described (8), using *Cladosporium cucumerinum* spores.

Purified preparations of medicarpin were also bioassayed for inhibition of the radial growth of Pmm by modification of the method of Pierre and Bateman (16). Known amounts of medicarpin in ethanol were added to 3 ml of V8C agar in sterile petri dishes (5 cm in diameter) to give final concentrations of 0 to 167 $\mu g\ ml^{-1}$. Ethanol was used at the rate of 42 $\mu l\ ml^{-1}$ of medium. Five-mm mycelial disks cut from the growing edge of 7-day-old Pmm agar cultures were placed on the assay plates and mycelial growth was assessed daily by measuring the diameter of each colony grown in the dark at 25 C.

RESULTS

Extraction of phytoalexins. Thin-layer chromatographic bioassays of extracts from resistant inoculated seedlings disclosed the presence of one major and two minor antifungal spots, none of which were detected in extracts from noninoculated plants.

The compound with an R_f value of 0.75 had an ultraviolet

absorption spectrum indistinguishable from 3-hydroxy,9-methoxypterocarpan (medicarpin) (λ_{max} EtOH = 279 [s] and 284 nm). The mass spectrum (M^+ = 270; prominent fragments at m/e 255, 239, 237, 227, 226, 209, 197, 181, 161, 148, and 147) was also indistinguishable from that of an authentic sample of medicarpin, thus establishing the identity of this compound.

The second compound with an R_f value of 0.44 gave spectral data identical to that of a synthetic sample of 4',7-dihydroxyflavanone (14) (λ_{max} EtOH = 276 and 314 nm; mass spectrum [M^+ = 256; prominent fragments at m/e 255, 239, 228, 227, 213, 163, 150, 137, 120, 110, and 91]). The third compound with an R_f value of 0.47 and γ_{max} = 286 nm was not identified.

Time course of medicarpin production in inoculated alfalfa seedlings. At 0 hr after inoculation only 0.7 $\mu g\ g^{-1}$ medicarpin was detected in the susceptible Moapa 69 compared to 2.5 $\mu g\ g^{-1}$ in the resistant cultivar A77-10B (Fig. 1). Medicarpin accumulated rapidly in extracts of both cultivars until 48 to 72 hr and then declined. Accumulation of medicarpin in the susceptible cultivar, Moapa 69, was always less than that in the resistant cultivar A77-10B (Fig. 1).

Medicarpin production and the resistance of alfalfa cultivars to Pmm. The amount of medicarpin which accumulated in inoculated seedlings of various alfalfa cultivars (Table 1) was inversely correlated with their susceptibility to Pmm ($r = -0.95$, Fig. 2).

Biological assays. Growth of Pmm was only slightly inhibited by medicarpin at 50 $\mu g\ ml^{-1}$ or less, but increasing concentrations led to inhibition of fungal growth. Growth was completely inhibited at 133 $\mu g\ ml^{-1}$ (Fig. 3) and the median effective dose (ED_{50}) was 97 $\mu g\ ml^{-1}$ (Fig. 4).

DISCUSSION

Our experiments were conducted under axenic conditions in the laboratory using a system designed to quantitate phytoalexin production resulting from infection by Pmm. This system eliminated many of the problems of studying phytoalexin production in roots of soil-grown plants such as bacterial contamination and root injury resulting from removal of plants from soil. Our experimental approach thus provided a system in which phytoalexin production could be reliably studied; however, we did not attempt to determine the degree of resistance expressed by seedlings in our experiments.

Three antifungal compounds were produced by alfalfa seedlings in response to infection by Pmm. Two of these compounds were medicarpin (3-hydroxy,9-methoxypterocarpan) and liquiritigenin (4',7-dihydroxyflavanone). The predominant one, as judged by the amount produced and antifungal activity was medicarpin. This compound was first identified by Smith et al (18) as a fungus-induced product from alfalfa and has since been isolated in response to several different pathogens (4,5,9). Confirming Ingham (6), liquiritigenin also accumulated in alfalfa seedlings, but its contribution toward resistance is uncertain because of its slight antifungal activity. However, it is a probable intermediate in the biosynthesis of medicarpin (19). We were not able to isolate vestitol and sativan, which have been reported from fungus-infected alfalfa leaves (6).

Time course studies (Fig. 1) showed that inoculated seedlings of the susceptible cultivar Moapa 69 produced considerably less medicarpin than the resistant A77-10B. The observed high correlation coefficient ($r = -0.95$) between the susceptibility of cultivars and the concentration of medicarpin produced in inoculated seedlings (Table 1, Fig. 2) supports previous work (7,12,17) indicating that levels of resistance can be reliably detected by the use of alfalfa seedlings and further indicates that medicarpin accumulation may be an important factor in the expression of resistance. Because cultivated alfalfa is heterozygous and tetraploid, the medicarpin concentrations that were extracted from plant tissue (Table 1) are an average value dependent upon the relative numbers of resistant and susceptible genotypes within each cultivar. Yoshikawa et al (20) also demonstrated that much higher concentrations of a phytoalexin may be present at localized infection sites in resistant plants than in the whole mass of plant tissue. Accordingly, extraction of whole seedlings in our

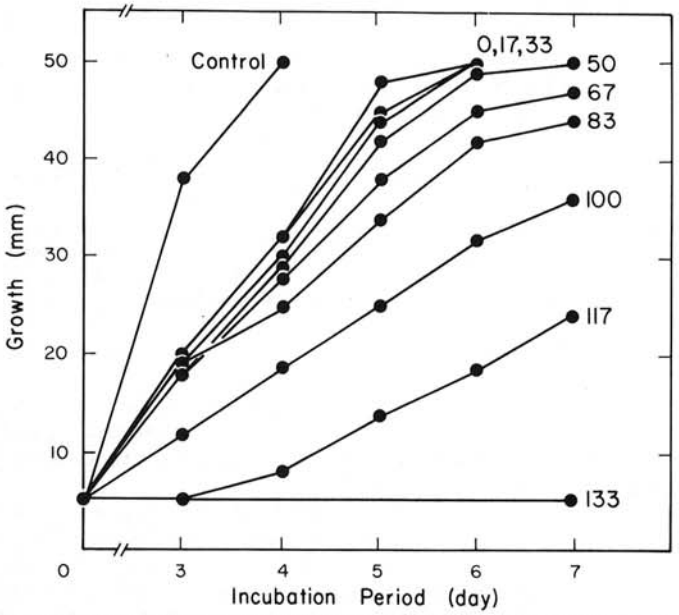

Fig. 3. Radial growth of *Phytophthora megasperma* f. sp. *medicaginis* in the presence of various concentrations of medicarpin. Controls did not contain medicarpin or ethanol. Values included the 5 mm diameter of the inoculum disks.

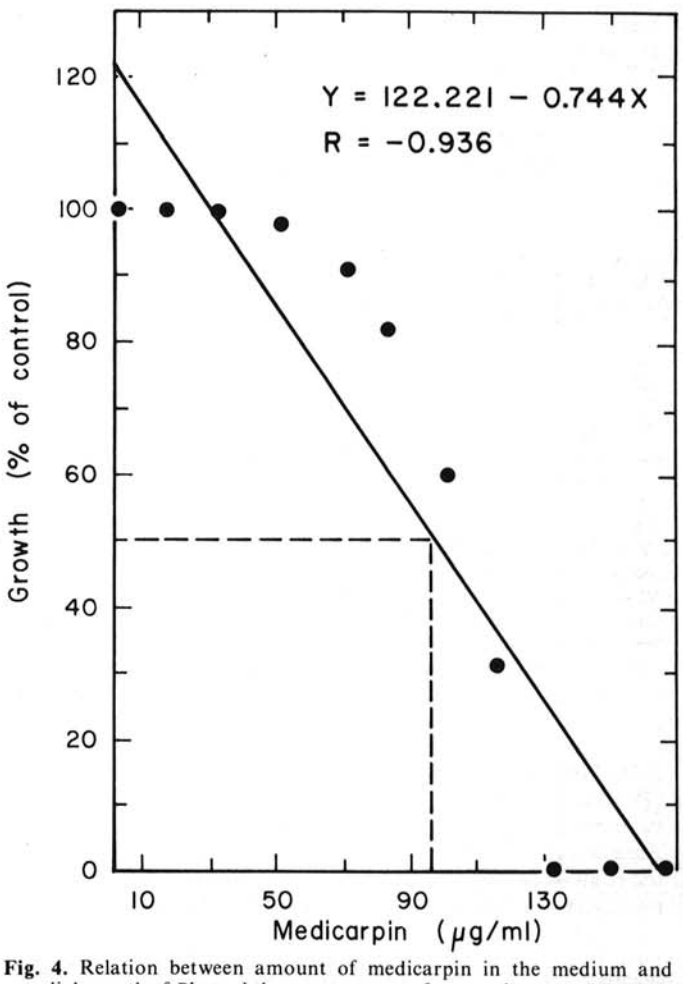

Fig. 4. Relation between amount of medicarpin in the medium and mycelial growth of *Phytophthora megasperma* f. sp. *medicaginis*. All values calculated relative to fungus growth in medium supplied 42 $\mu l\ ml^{-1}$ ethanol but no medicarpin and the length of the growth period was 6 days. ED_{50} = 97 μg/ml.

experiments probably led to underestimation of the medicarpin concentrations at localized infection sites. More work is required to adequately assess the localized concentrations accumulating in infection sites of resistant and susceptible alfalfa seedlings.

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