Compatible and Incompatible Responses in Alfalfa Cotyledons to Races 1 and 2 of *Colletotrichum trifolii*

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ABSTRACT

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Specific race interactions between *Colletotrichum trifolii* and cultivars of resistant and susceptible alfalfa were examined in nonwounded excised cotyledons. Cotyledons in incompatible and compatible interactions exhibited visible differential reactions within 7–10 days after inoculation. *C. trifolii* infected alfalfa by directly penetrating the epidermis via a penetration peg from the appressorium. Appressoria matured within 20 h on resistant and susceptible cotyledons, but primary hyphae did not develop on resistant cotyledons. Phenylalanine ammonia-lyase and the pterocarpan phytoalexin medicarpin began to increase in resistant tissues after 24 h. Cotyledon tissues apparently became resistant prior to significant increases in medicarpin. Medicarpin concentration in resistant cultivars was highest at 72 h and declined to baseline levels by 120 h. Secondary spores were produced in susceptible tissues after 96 h. Cotyledons appear to be ideal tissues to use in investigations of defense expression in the anthracnose disease of alfalfa.

Additional keywords: defense compounds, fungitoxicity, induced resistance, lucerne, *Medicago*, race specificity, resistance mechanism, tolerance.

Anthracnose, caused by *Colletotrichum trifolii* Bain & Essary, is an important disease of alfalfa (*Medicago sativa* L.) that limits growth and forage yield and decreases plant vigor (2,8,9,13). The disease has been controlled primarily through the use of resistant cultivars developed through multiple cycles of phenotypic recurrent selection for resistance (2,11). In 1978, two physiological races of *C. trifolii* were identified (11,28,35). Race specificity may be detected by evaluating alfalfa seedlings or mature stems inoculated with spores (11,29). Resistance to races 1 and 2 is conditioned independently by different, single dominant genes, designated An_1 and An_2, respectively; these genes are inherited tetrasomically (10,12). Alfalfa is genotypically heterogeneous within a cultivar because it is an autotetraploid and is cross-pollinated. Therefore, disease resistance is typically characterized as a percentage of the seed carrying resistance genes. Ostazeski and Elgin (30) determined that certain alfalfa clones and plant populations were protected from disease caused by race 2 by simultaneous or prior inoculation with race 1. O'Neill et al (27) found a positive correlation between virulence of the inducing isolate and protection from subsequent challenge by race 2 in alfalfa seedlings. They also found that higher inducing inoculum concentrations provided alfalfa seedlings greater protection from race 2 challenge inoculations, regardless of the race 2 inoculum concentration.

The mechanisms for resistance or induced resistance to anthracnose are unknown. However, in some alfalfa diseases, resistance to pathogens has been attributed to the production of postinfection compounds, principally the phytoalexin medicarpin (1,16,17,25, 33,34). The number, quantity, and toxicity of the induced phytoalexins are not well documented and vary depending on the specific interaction and host tissues studied. Postinfection fungitoxic fluorescent and phenolic compounds increase in stems of alfalfa clones with genes for resistance to anthracnose caused by either race 1 or 2 (1). Medicarpin effectively inhibits spore germination and growth of pregerminated spores of races 1 and 2 of *C. trifolii* in vitro; however, hyphal growth is not affected (N. R. O'Neill, *unpublished data*). Histological studies indicate that the fungus penetrates the epidermis of resistant plants from appressoria but fails to form infection hyphae and ramify throughout the tissue (4,24). Incompatibility in primary infections is not associated with the lack of conidial germination or failure to form appressoria with penetration pegs, and resistance is most likely expressed prior to internal cell-wall penetration and ramification of infection hyphae (4).

If resistance is due to the toxicity of medicarpin, effective concentrations of medicarpin should be present at the time of epidermal penetration and, possibly, during challenge inoculations in induced resistance interactions. Precise determinations of medicarpin concentrations induced in resistant tissues from primary and secondary infections are necessary to determine whether it accumulates to inhibitory concentrations at appropriate times in plant tissues to cause the observed cessation of fungal growth.

Detailed biochemical and genetic analysis of the phenylpropanoid pathway was recently reported for alfalfa suspension cells (6,7,18). Although the timing of induction of phytoalexin biosynthetic enzymes correlated directly with the resistance of alfalfa callus cultures to fungal pathogens *Verticillium albo-atrum* and *Phytophthora megasperma* f. sp. *medicaginis* (21,22), similar correlations have not been shown for the *C. trifolii*-alfalfa interaction in callus or suspension cells.

Cotyledon tissues were chosen for our defense-response studies for several reasons. Alfalfa cotyledons have a relatively simple architecture, consisting of mesophyll parenchyma cells, the epidermis, and a few vascular elements. This arrangement facilitates quantitative inoculation and sampling of tissues. Also, cotyledons are easily manipulated and have been one of the classical gene-specific tissues used for elicitor and phytoalexin studies in legumes (5,14,15,19,20,32).

The present study was initiated to investigate anthracnose defense responses in alfalfa cotyledons by determining phenylalanine ammonia-lyase (PAL) enzyme activity and medicarpin accumulation and to determine whether these parameters correlate

with histopathological events during compatible and incompatible plant-fungus interactions.

MATERIALS AND METHODS

Fungal isolates and inoculum preparation. Field isolates of *C. trifolii* race 1 (isolate 2sp2) and race 2 (isolate SB-2) were obtained and maintained as described previously (27). Cultures were derived from single spores and stored in sterile soil at 4 C. Race identity was confirmed in mature plants of six clones each of three differential alfalfa resistance phenotypes by stem-injection inoculations, as described previously (27,29). Isolate virulence, ability to induce resistance in interactions with alfalfa cultivars possessing specific genes for resistance, and preparation of spore suspension inoculum were reported previously (27).

Host differentials and cotyledon inoculation. Cultivars Saranac (SS phenotype, susceptible to both races), Arc (RS phenotype, resistant to race 1 and susceptible to race 2), Saranac AR (RR phenotype, resistant to both races), and population B-46 were used in these experiments. Experimental population B-46 has the RS phenotype, which indicates the presence of the race 1 resistance gene An_1 and the absence of the race 2 resistance gene An_2 (N. R. O'Neill and T. A. Campbell, *unpublished data*). The percentage of seed with anthracnose resistance in these cultivars was determined earlier by standard test procedures (26). Seedlings were grown in Pro-mix BX (Premier Brands, Inc., Stamford, CT) in a growth chamber at 25 C under a 16-h photoperiod and a photosynthetic photon flux density of 68 μmol m^{-2} s^{-1}. Cotyledons were excised from 10-day-old seedlings and placed on moist, sterile filter paper (Whatman No. 1, Whatman International, Ltd., Maidstone, England) in glass petri dishes. The abaxial surfaces of cotyledons were sprayed with a Chromist sprayer (Gelman Scientific, Inc., Ann Arbor, MI) held at a 45-degree angle 10 cm from the plate. Approximately 1 ml of spore suspension of the appropriate race was delivered per plate, until a fine mist covered each cotyledon. Plates were sealed with Parafilm and returned to the growth chamber after inoculation. For analysis of PAL and medicarpin, samples consisted of 30 cotyledons for each replication of each treatment from Saranac or Arc inoculated with race 1 isolate 2sp2 or with water. Each sample of 30 cotyledons was bulked and assayed for PAL or medicarpin at each interval from 0 to 168 h after inoculation. Each 30-cotyledon sample for PAL or medicarpin analyses was replicated twice in one experiment and three times in a second experiment. Results from the second experiment are presented.

Anthracnose resistance expressed in cotyledons of alfalfa cultivars and lines. Excised cotyledons of Arc, Saranac, Saranac AR, and B-46 were inoculated with either race 1 or 2 of *C. trifolii*, as described above, and incubated for 10 days. Control treatments received distilled water only. Treatments consisted of four replications of 25 cotyledons each. Cotyledons observed under a dissecting microscope were rated susceptible or resistant 4, 7, 8, and 10 days after inoculation. Cotyledons exhibiting sporulating acervuli or setae were rated susceptible. Cotyledons exhibiting mild hypersensitive flecking but no acervuli or sporulation were rated resistant. The experiment was repeated twice with similar results. Data from the second experiment are presented.

Histopathology of infection in cotyledons. Cotyledons from five Saranac or Arc seeds were inoculated with race 1 or with sterile distilled water and examined histologically 4, 8, 20, 31, 49, 54, 68, 95, 140, 168, and 195 h after inoculation. One of two cotyledons from each seedling was inoculated and evaluated after 10 days to identify cotyledons not exhibiting the typical Arc RS or Saranac SS phenotypes. The corresponding cotyledon was excluded from the histological study. Each remaining cotyledon was placed on a slide, immersed in 0.05% Trypan blue in lactophenol, covered with a coverslip, and boiled gently over a bunsen burner until the sample cleared. Cotyledons were examined with a Zeiss universal compound microscope (Carl Zeiss, Oberkochen, Germany) at 500× for the presence of ungerminated and germinated spores, immature and mature appressoria, appressoria with pores, and primary and secondary hyphae, each of which was considered a single event. Events were recorded in a random order along a transect and included all spores in the field of view. At least 50 spores were evaluated and recorded for each of five replicate cotyledons per cultivar.

Extraction and analysis of medicarpin. After each incubation period, each 30-cotyledon sample was weighed, placed in 4.5 ml of 80% (v/v) acetonitrile, and frozen at 20 C for later extraction. The extraction method was modified from an earlier method used to reduce the chlorophyll content of samples and to achieve high efficiency (>94% compared with samples spiked with pure medicarpin) and consistency in handling large sample numbers (1). Thawed cotyledon samples from each treatment were homogenized at 4 C with a Tekmar tissumizer (Tekmar Co., Cincinnati), and 10 ml of distilled water was added to each vial to reduce the acetonitrile concentration to 25%. The homogenate was passed through a 3-ml Baker-10 SPE octadecyl (Cl8) disposable extraction column (T. S. Baker, Inc., Phillipsburg, NJ) that had been preconditioned with methanol followed by 25% acetonitrile at 2 volumes/1.5 ml. The eluate was discarded, and the medicarpin-containing fraction was eluted with 4 ml of 100% acetonitrile. Each sample was evaporated under nitrogen and resuspended in 100 μl of acetonitrile.

Twenty microliters of each sample was separated in a Perkin Elmer high-pressure liquid chromatography (HPLC) system (Perkin Elmer Corp., Norwalk, CT) on a reverse phase Cl8 column (10-μm particle size; 250 × 4.6 mm column). Separations were achieved with a 20-min linear gradient of 50–100% (v/v) acetonitrile at a flow rate of 1 ml/min. The effluent was monitored for absorbance at 210 nm, and medicarpin was quantified as peak area units (millivolts per second) by Baseline chromatography software (Waters Chromatography Division, Millipore Corporation, Milford, MA) based on an internal standard of pure medicarpin. Crystalline medicarpin was provided by D. Gustine, and its purity was confirmed by gas chromatography-mass spectrophotometry analysis.

Assay for PAL activity. After each incubation period, each 30-cotyledon sample was blotted, weighed, and thoroughly ground with a Ten Broeck homogenizer (Fisher Scientific, Pittsburgh, PA) in 2 ml of 100 mM sodium borate, pH 8.8, 0.05% (v/v) β-mercaptoethanol, and 0.05% (w/v) disodium EDTA. The homogenate was centrifuged at 3,500 *g* for 15 min at 4 C, and the supernatant was used as the enzyme source. The 4-ml reaction mixture contained 2.7 ml of 100 μM sodium borate buffer, pH 8.8, 100 μl of 2 mM L-phenylalanine, 200 μl of ^{14}C-(U)-L-phenylalanine (0.2 μCi) specific activity 493 mCi/mmol (New England Nuclear, Wilmington, DE), and 1 ml of the enzyme source. The reaction was incubated at 37 C for 1 h and was stopped with the addition of 200 μl of a saturated solution of *trans*-cinnamic acid in 25 mM NaOH, followed by an additional 200 μl of 6 N HCL. The reaction mixture was vigorously mixed with 4.5 ml of toluene and centrifuged at 3,000 *g* for 10 min, and 3 ml of the toluene phase was counted in a scintillation counter (model 5303, TM Analytic, Branden, FL). PAL activity was calculated as the production of nanomoles of cinnamate per hour per milligram of protein. Protein assays were based on the method of Bradford (3), using the Pierce Coomassie protein reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard.

RESULTS

Cotyledon reactions of alfalfa cultivars and lines. The cultivar responses in compatible and incompatible interactions with race 1 or 2 clearly differentiated the resistance responses expected from those cultivars (Fig. 1). As reported previously (5), the percentage of cotyledons exhibiting setae or acervuli increased rapidly in susceptible cultivars, with little change after 10–14 days. The percent resistance recorded in cotyledons after 8–10 days was characteristic of the cultivar's genetic population. The results of preliminary experiments, in which seedlings of cultivars were evaluated for resistance by standard test procedures, were similar to results with cotyledon evaluations. The resistance evaluations with seedlings of cultivars revealed the following percentages of

resistance to races 1 and 2, respectively: Saranac, 1 and 6%; Arc, 70 and 0%; Saranac AR, 60 and 66%; B-46, 78 and 4% (data not shown). Although resistance percents vary slightly in response to inoculum levels and the specific fungal isolate used (5,27,29), high inoculum levels and the most virulent isolates enhance the stringency of the selection.

Histopathology of infection in cotyledons. The time course of race 1 spore germination and appressorial development on the surfaces of Saranac (SS) and Arc (RS) cotyledons was similar (Fig. 2). Immature appressoria that stained pale blue rapidly developed into mature, light- to dark-brown pigmented appressoria. By 20 h, 73% of the conidia on Saranac and 67% of the conidia on Arc had germinated and developed into mature appressoria. Cotyledons were penetrated directly through the cuticle and epidermal cells via a penetration peg from the appressorium. Spore germination by formation of hyphae was rarely observed, and direct penetration by hyphae was not seen. Primary hyphae were visible in Saranac cotyledons after 48 h but not in Arc cotyledons. Both inter- and intracellular infection hyphae were present in Saranac, whereas in Arc subepidermal infection hyphae were not observed. Acervuli with setae and conidia developed in Saranac cotyledons and ruptured the epidermis by 95 h after inoculation. By 168 h, secondary conidia began germinating to form appressoria. Macroscopic symptoms resembled the typical hypersensitive response on Arc and were visible after 95 h as small, sunken, light-brown lesions giving a pitted appearance to the cotyledon surface. The pits remained restricted for the duration of the experiment. In contrast, lesions on Saranac became progressively water-soaked and were light tan and chlorotic. Evaluations of cotyledons incubated for 195 h were not included because Saranac tissue had completely collapsed.

Production of medicarpin and PAL. The principal phytoalexin quantified by HPLC was medicarpin, which was detected in greater quantities in cotyledons from the incompatible interaction between *C. trifolii* and the resistant alfalfa population Arc (RS) than in susceptible Saranac (SS) or uninoculated controls (Fig. 3). The quantity of medicarpin began increasing after 24 h, reaching a maximum of 18 μg/g of fresh weight of tissue 72 h after inoculation. Levels of medicarpin in the compatible interaction with Saranac and uninoculated treatments remained relatively low throughout the experiment.

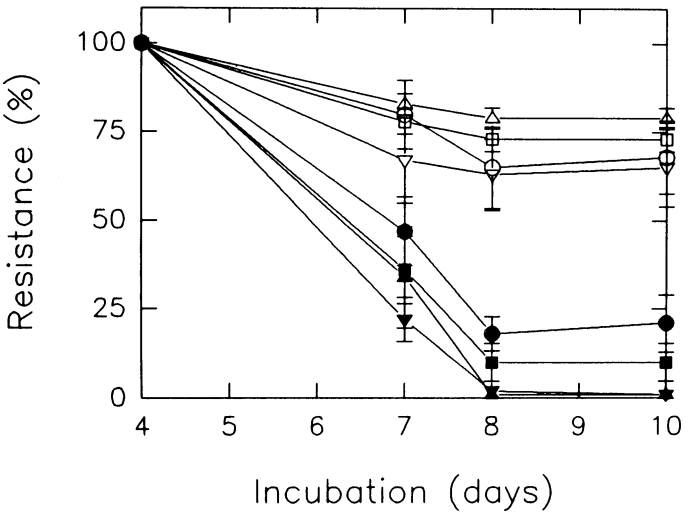

Fig. 1. Percent resistance over a 10-day incubation period of excised alfalfa cotyledons inoculated with races 1 or 2 of *Colletotrichum trifolii*. Percent resistance is based on microscopic observations of each cotyledon for the presence or absence of sporulating acervuli. Race-cultivar interactions: △ = Arc, race 1; □ = B46RS, race 1; ○ = Saranac AR, race 2; ▽ = Saranac AR, race 1; ● = Saranac, race 2; ■ = B46RS, race 2; ▲ = Arc, race 2; and ▼ = Saranac, race 1. Open and closed symbols represent the presence or absence, respectively, of a gene for resistance to the race used as inoculum in each host-parasite interaction. Symbols represent the mean of three samples of 25 cotyledons each. Vertical lines through the data points represent standard errors.

Large increases in the extractable levels of PAL were observed in the incompatible interaction between Arc and race 1 of *C. trifolii* (Fig. 4). PAL levels, determined by activity assays, increased rapidly after 24 h and declined after 144 h. The kinetics of PAL induction were consistent with a role in medicarpin synthesis. Levels of PAL in the compatible interaction increased slightly after 48 h to a specific activity of approximately 10 nmol cinnamate per milligram of protein. PAL activity of cotyledons increased 1–2 days later in the compatible interaction than in the incompatible interaction. PAL activity remained at basal levels in uninoculated cotyledons.

DISCUSSION

The results of this report and others (4,23) indicate that the development of *C. trifolii* in resistant cotyledons and hypocotyls is inhibited when appressoria mature, in some cases with observable epidermal penetration but no observations of primary hyphae. The time course for fungal growth limitation in incompatible interactions varies among studies. In one study, Porto et al (31) reported penetration of susceptible stems between 12- and 20-h postinoculation. Churchill et al (4) found 71–93% of

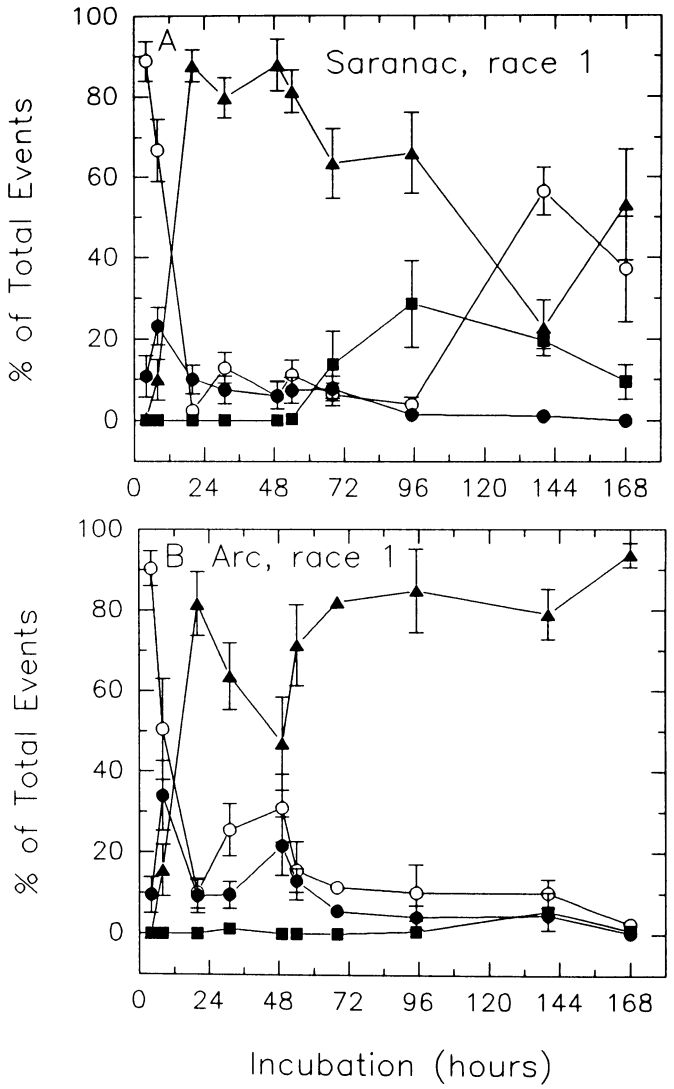

Fig. 2. Time course for *Colletotrichum trifolii* race 1 spore development during **A**, the compatible interaction with alfalfa cultivar Saranac and **B**, the incompatible interaction with alfalfa cultivar Arc. Fungal growth events were monitored on spray-inoculated cotyledons. Symbols indicate the percentage of spores exhibiting each event: ○ = ungerminated spores; ● = germinated spores; ▲ = immature and mature appressoria; ■ = primary hyphae. Vertical lines through the data points represent standard errors. A minimum of 250 spores was evaluated for each time period and cultivar.

conidia on resistant and susceptible plants had germinated and developed into mature appressoria by 16 h. This rate is faster than that reported by Mould et al (23), who observed that 85–93% of germinated spores formed appressoria by 48 h and colonized the first cell in resistant and susceptible plants from 60- to 72-h postinoculation. In our studies, 67 and 73% of appressoria in resistant or susceptible tissues, respectively, matured by 20 h, and by 48 h, primary hyphae were visible in susceptible plants, but fungal growth had ceased in resistant plants. Mould et al (23) also noted that there were timing differences between reports for these events and suggested that they may be attributable to isolate, cultivar, inoculation, and/or incubation conditions. Our results in the timing of events in excised cotyledons were similar to those reported for excised hypocotyls (4).

At the time the primary mature appressoria are at a maximum (i.e., 20-h postinoculation), PAL activity and medicarpin accumulation are no greater than in uninoculated controls. PAL activity

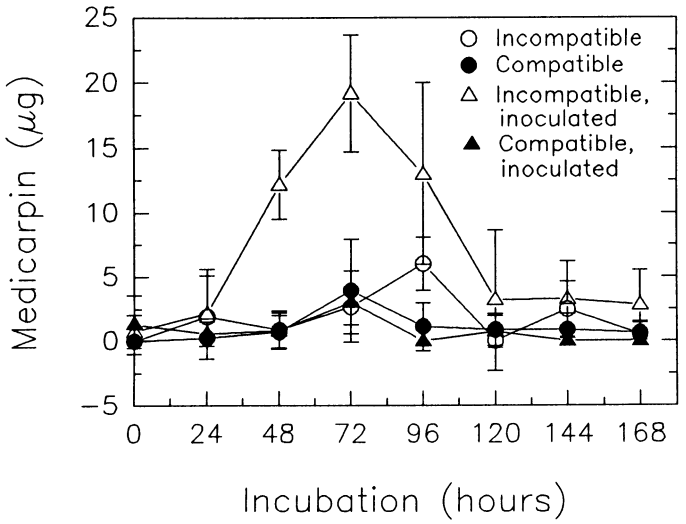

Fig. 3. Time course for production of medicarpin in resistant and susceptible alfalfa cotyledons inoculated with *Colletotrichum trifolii* race 1. Open symbols represent the race 1-resistant cultivar Arc, and closed symbols represent the susceptible cultivar Saranac. Vertical lines through the data points represent standard errors. Symbols represent the mean of three samples of 30 cotyledons each.

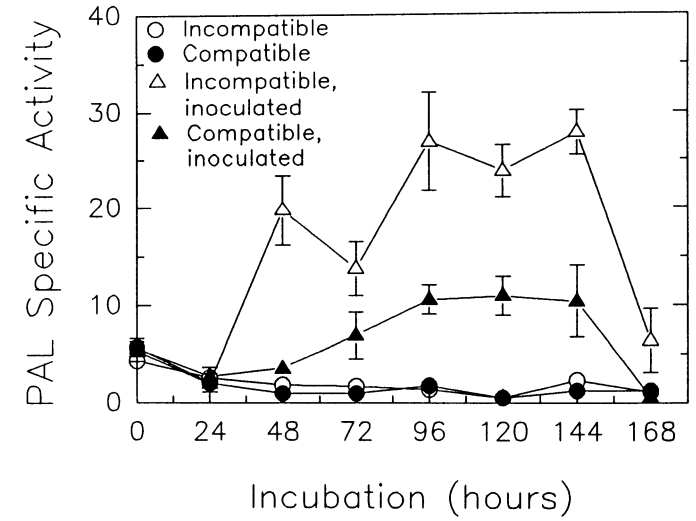

Fig. 4. Time course for production of phenylalanine ammonia-lyase (PAL) in resistant and susceptible alfalfa cotyledons inoculated with *Colletotrichum trifolii* race 1. PAL-specific activity was calculated as the production of nanomoles of cinnamate per hour per milligram of protein. Open symbols represent the race 1-resistant cultivar Arc, and closed symbols represent the susceptible cultivar Saranac. Vertical lines through the data points represent standard errors. Symbols represent the mean of three samples of 30 cotyledons each.

and accompanying medicarpin levels reach maximal levels only well after primary fungal inocula have stopped growing. The relative insensitivity of races and isolates of *C. trifolii* to medicarpin when measured by hyphal growth (N. R. O'Neill, *unpublished data*) and the comparatively low levels of medicarpin found in infected seedlings and cotyledons suggest that medicarpin may not be limiting primary hyphal growth during infection of resistant tissues. Instead, the increase in PAL activity and the concomitant accumulation of medicarpin may function more significantly as deterrents of germination of secondary spores produced from secondary or challenge spore-infection attempts. Such secondary spores appear on the cotyledonary surface of susceptible tissues after 96 h. This could be a very important mechanism for resistance in alfalfa fields in which only a portion of the plants are resistant and spore inoculum from susceptible plants is continuously present during favorable environmental conditions. On the other hand, medicarpin levels were not determined at the epidermal surface or cellular level in cells in contact with developing or mature appressoria.

Further histological as well as metabolic detoxification studies are needed to conclusively demonstrate the role of medicarpin in restricting *C. trifolii* in alfalfa. The cotyledon assay appears to be a valuable tool for further analyses of defense responses in alfalfa because the tissue accurately reflects race-host specificities. However, results from excised tissues from later time periods should be interpreted with caution until the effects of increasing senescence in these tissues is known. Further studies are needed to determine whether the timing of the pathogenesis events vary with host tissue type and age.

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