

Resistance

In Vitro Reactions of *Cladosporium caryigenum* with Pecan Condensed Tannins and Isoquercitrin

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ABSTRACT

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In vitro growth of *Cladosporium caryigenum*, the pecan scab incitant, was significantly inhibited by condensed tannin and isoquercitrin, allelochemicals that had been extracted from freeze-dried pecan leaves (*Carya illinoensis* cv. Van Deman). Nine isolates of *C. caryigenum* varied in their responses to condensed tannin at a concentration of 4,000 $\mu\text{g/ml}$.

Additional keywords: disease resistance, pecan phenolics.

Even the most tolerant isolate was inhibited at concentrations of 4,000 $\mu\text{g/ml}$ and above. Isoquercitrin at 4,000 $\mu\text{g/ml}$ was about two to four times more inhibitory than tannin to growth of three isolates of *C. caryigenum*, and differences were found in the tolerance of the three isolates to isoquercitrin.

Pecan scab, incited by the fungus *Cladosporium caryigenum* (Ellis & Langl.) Gottwald, is the most serious disease of pecan, *Carya illinoensis* Koch. The disease was first reported on hickory in 1885 (25) and is a limiting factor in pecan production. Elucidation of factors responsible for disease resistance, including antifungal chemicals, is a vital prelude to resistance breeding efforts in tree crops such as pecan.

In 1977, Langhans et al (19,20) obtained plant extracts from pecan that were inhibitory to in vitro growth of *C. caryigenum*. Subsequently, Hedin et al (12,14) identified juglone from the active fractions. Juglone had fungitoxic activity toward *C. caryigenum* at 0.05 mg/ml in liquid culture (20). Variable responses among isolates to juglone were observed (24). Juglone has been implicated as a disease resistance factor in both pecan and walnut (3-6). Linalool, a compound previously reported to occur in pecan (22), also exhibited some fungitoxic activity to *C. caryigenum*. The presence of other unidentified pecan fungitoxicants was estab-

lished by Windham and Langhans (20,24).

Tannins and other phenolics have been recognized as possible factors in plant defense against natural enemies (1,7,9). Pecan leaves have been shown to contain extractable condensed tannins at concentrations ranging from 1,700 to 20,000 $\mu\text{g}/\text{ml}$ throughout the growing season (10,18). In 1982, Graves demonstrated the fungitoxic effect of pecan tannins to one isolate of *C. caryigenum* (C. H. Graves, Jr., unpublished). Recently, isoquercitrin, a known flavonoid allelochemical, also was detected in pecan tissue (15).

This study is part of a long-range program to identify potentially important resistance factors in pecan and species related to *C. caryigenum*. Bioassay procedures were used to investigate the influence of condensed tannin and isoquercitrin from pecan on growth of selected isolates of *C. caryigenum*. The objectives were to determine the concentrations of condensed tannin that inhibit *in vitro* growth of a tolerant isolate of *C. caryigenum* and to study responses of several isolates of *C. caryigenum* to these compounds.

MATERIALS AND METHODS

Establishment of inoculum. Inoculum from hyphal-tip cultures of *C. caryigenum* that had been stored in screwcap test tubes under oil at -1 C was grown on potato-dextrose agar in 100- \times 15-mm plastic petri dishes. After sufficient growth had occurred, four 5-mm plugs of fungal stromata were added to each of four 250-ml Erlenmeyer flasks containing 100 ml of potato-dextrose broth (PDB). The cultures were incubated on a rotary shaker at 25 C for 3 wk and used as inoculum sources for subsequent bioassays.

Inoculum of newly acquired, wild-type isolates was prepared as follows: pecan leaflets with dark lesions indicative of pecan scab were surface-sterilized by successive dips in 70% ethyl alcohol and 1% sodium hypochlorite followed by three rinses in sterile distilled water. Leaflet pieces containing a portion of a lesion and tissue in advance of the lesion were plated on potato-dextrose agar supplemented with 2.5% lactic acid. Isolates of wild-types were obtained within 3 wk, and liquid cultures of inoculum were prepared as described previously.

Extraction of condensed tannins and isoquercitrin. Structurally defined condensed tannins and isoquercitrin were extracted preparatively as described by Hedin et al (13,15). Leaf tissue from Van Deman pecan trees collected in July 1983 and 1984 was freeze-dried, ground to a powder, and extracted three times with cyclohexane-ethyl acetate-acetic acid (500:500:1). The residue was extracted with 70% aqueous acetone and separated into aqueous and acetone layers by addition of sodium chloride. The acetone layer was evaporated under vacuum to yield a solid residue. This residue was dialyzed overnight against distilled water and freeze-dried.

Extracted freeze-dried solids were fractionated by column chromatography on Sephadex LH-20 (5 \times 75 cm; solvent, 70% aqueous methanol). Successive elutions of the column were made with 70% aqueous methanol until all flavonoid and tannin fractions were recovered. Aliquots were evaluated for tannin content by thin-layer chromatography on 0.2-mm thick polyamide sheets

(J. T. Baker Co., Phillipsburg, NJ) with 70% aqueous methanol, and chromatograms were visualized by spraying with a complex of diphenyl boric acid and ethanol amine to identify fractions containing condensed tannin, isoquercitrin, or both. The presence of condensed tannin was further confirmed by boiling an aliquot of the fraction for 1 hr with *n*-butanol-HCl (95:5), which results in development of a red-pink chromophore. Pure fractions were pooled as appropriate and freeze-dried. Fractions containing mixtures of condensed tannins and isoquercitrin were concentrated and separated by chromatography on a polyamide column (5 cm \times 20 cm; solvent, 70% aqueous methanol). Elution of the column with 70% aqueous methanol yielded isoquercitrin, which could be rechromatographed under the same conditions to give the compound in about 95% purity. Subsequent elution with 70% aqueous acetone yielded tannin. Samples were subjected to thin layer chromatography as previously described, then pooled and freeze-dried.

Bioassay of condensed tannins. Seven isolates of *C. caryigenum* (Isolates 1-7, Table 1) were grown in aqueous PDB or PDB containing condensed tannin at 4,000 $\mu\text{g}/\text{ml}$. The isolates included three that, in previous studies (23), were pathogenic to excised nuts of many pecan cultivars, three isolates that were pathogenic to only a few, and one isolate that had been used in previous studies (12,19,20). Isolate StCpP₆, which was the least sensitive in the above assays, was further tested on substrates of PDB and PDB with 2,000, 4,000, 6,000, 8,000, and 12,000 μg of condensed tannin per milliliter.

Inoculum of each isolate was comminuted in a Waring microblender for 15 sec at high speed. Sterile, 125-ml Erlenmeyer flasks containing 50 ml of PDB plus condensed tannin treatments were seeded with 5 ml of inoculum. Controls consisting of 50 ml of PDB without condensed tannin, but adjusted to the varying pH values associated with the tannin treatments, were also inoculated. Cultures were incubated on a rotary shaker at about 25 C for 14 days. Mycelia were collected by vacuum filtration on pre-weighed Whatman filter paper, dried at 65 C for 48 hr, and weighed. A completely random experimental design with three replications of treatments was employed for statistical analysis. Mean differences were detected by Duncan's multiple range test. Experiments were conducted twice.

Bioassay comparison of condensed tannin and isoquercitrin. Isolate StCpP₆ and two wild-type isolates of *C. caryigenum* (Isolates 8-9, Table 1) were grown on PDB and PDB containing either condensed tannin or isoquercitrin at 4,000 $\mu\text{g}/\text{ml}$. This bioassay of fungal growth, with pH controls, was performed as previously described. A completely random experimental design using a two-factor factorial arrangement of isolate \times substrate was employed for statistical analysis. Mean differences were detected by Duncan's multiple range test. Experiments were conducted twice.

RESULTS

Bioassay of condensed tannins. Significant differences were found in the growth of seven isolates of *C. caryigenum* on PDB with no condensed tannin (Fig. 1), as previously reported for

TABLE 1. *Cladosporium caryigenum* isolates employed in a bioassay of condensed tannin and/or isoquercitrin extracted from pecan leaves

Isolate no. ^a	Isolate code	Level of virulence ^b	Host cultivar	Location of host tree
1	StCpP ₆	low	Stuart	Lumberton, MS
2	SuCr	high	Success	Shreveport, LA
3	B-10	high	Seedling 10	Brownwood, TX
4	MoSprt	low	Moore	Shreveport, LA
5	SuCpPl	high	Success	Shreveport, LA
6	B-13	low	Seedling 13	Brownwood, TX
7	Dehill	high	Delmas	Shreveport, LA
8	Wild-type	ND	Van Deman	Mississippi State, MS
9	Wild-type	ND	Stuart	Starkville, MS

^aIsolates 1-7 were from cultures used in previous studies, stored under oil at -1 C . Isolate 3 was used in studies by Langhans and Hedin (12,19,20). Isolates 8 and 9 (wild-type) were newly acquired from Van Deman, a highly susceptible cultivar, and Stuart, a somewhat resistant one.

^bLevel of virulence as determined by Street (23); ND = not determined.

other isolates of this fungus (8). Growth of all isolates was reduced by the presence of pecan leaf condensed tannin (Fig. 1) at a concentration of 4,000 $\mu\text{g}/\text{ml}$, but growth of isolate StCpP₆ in the presence of tannin was significantly greater than that of all other isolates. Isolate SuCpP1 was intermediate in sensitivity to tannin, and isolates SuCr, B-10, MoSprrt, B-13, and DeHill were greatly inhibited at this concentration.

When isolate StCpP₆ was tested at five concentrations of condensed tannin, the dry weight of mycelia decreased significantly and progressively with increasing concentrations from 2,000 to 6,000 $\mu\text{g}/\text{ml}$ (Fig. 2). Changes in pH equivalent to those caused by increasing tannin concentrations had little influence on the growth of this isolate (Fig. 2). The pH of filtered substrates for all treatments was about 5 at the end of the experiment.

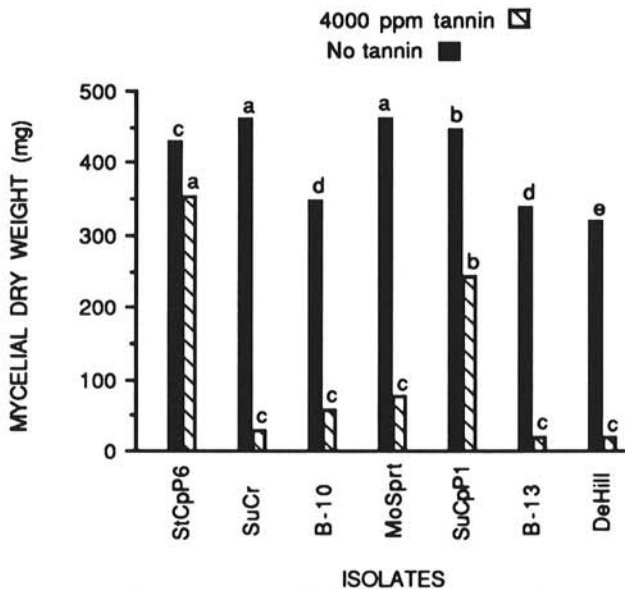


Fig. 1. Mycelial growth after 14 days of isolates of *Cladosporium caryigenum* on two substrates, potato-dextrose broth (PDB) or PDB with 4,000 μg of condensed tannin extracted from pecan leaves per milliliter. Data are an average of three replications. Different letters above bars within a substrate indicate significant differences at $P = 0.05$ according to Duncan's multiple range test.

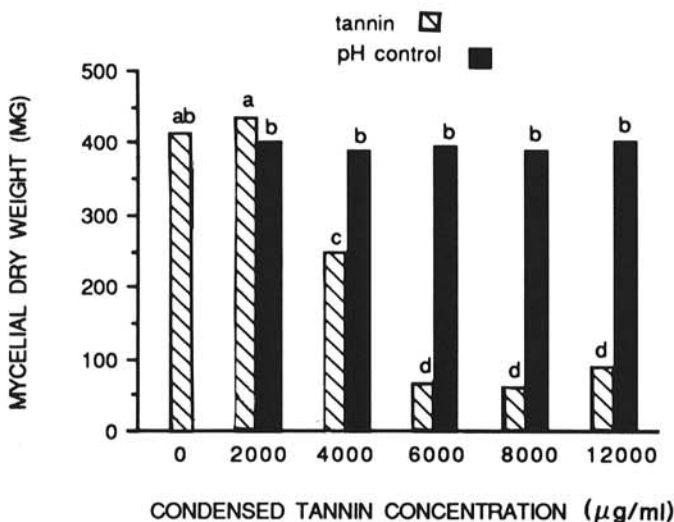


Fig. 2. Effect of increasing concentrations of condensed tannin and associated pH changes on the growth of isolate StCpP₆ of *Cladosporium caryigenum*. Associated pH values (pH controls) were 4.7, 4.2, 4.0, 3.9, 3.7, and 3.5, respectively. Data are an average of three replications. Different letters above bars indicate significant differences for all treatments, including pH controls, at $P = 0.05$ according to Duncan's multiple range test.

Bioassay comparison of condensed tannins and isoquercitrin.

Means of mycelial dry weights for each isolate in the five substrates are given in Table 2. Growth of the Van Deman wild-type isolate (Isolate 8, Table 1) was not inhibited (when compared to the control) by condensed tannin at 4,000 $\mu\text{g}/\text{ml}$, but isoquercitrin severely inhibited the growth of this isolate. Growth of the Stuart wild-type isolate (Isolate 9, Table 1) was inhibited by both compounds, but isoquercitrin was about four times more inhibitory than condensed tannin (Table 2). Growth of the StCpP₆ isolate was also inhibited by both tannin and isoquercitrin, and isoquercitrin was twice as inhibitory as condensed tannin. Some decrease in isolate growth was noted among the pH controls; however, growth in isoquercitrin and condensed tannin treatments was significantly lower than that in any pH control except in the case of the Van Deman isolate on condensed tannin. The pH of filtered substrates for all treatments was about 5 at the end of the experiment.

A significant interaction occurred between isolates and substrates (Table 2). Isolates rank the same relative to each other in PDB and in the pH control treatments (pH 4.0 and 3.6) for mycelial dry weight production. However, isolates differ in ranking in the tannin and isoquercitrin treatments, indicating that isolates respond distinctively to these phenolics. When the medium contained 4,000 μg of isoquercitrin per milliliter, the Stuart isolate grew less than the StCpP₆ isolate and the Van Deman isolate was intermediate. When the medium contained 4,000 μg of condensed tannin per milliliter, the Stuart isolate grew less than the Van Deman isolate and the StCpP₆ was intermediate.

DISCUSSION

The fact that condensed tannin and isoquercitrin inhibit the *in vitro* growth of *C. caryigenum* suggests that these compounds may be involved in resistance of pecan to this pathogen. Both condensed tannin and isoquercitrin have been positively correlated with resistance of young cotton leaves and stems to *Verticillium dahliae* (16,21). Cotton leaf extracts containing both compounds have been shown to limit *in vitro* growth of this pathogen (2). Hunter (17), also working with cotton seedlings, reported that *Rhizoctonia solani* was significantly inhibited *in vitro* at catechin levels of 2,900–4,600 $\mu\text{g}/\text{ml}$, and selected isolates of *R. solani* gave differential responses to catechin. The isolate of *R. solani* most virulent to cotton also exhibited the greatest tolerance to catechin. Other investigators (2) have implicated condensed tannin and isoquercitrin as factors for resistance to plant pathogens in cotton.

The similarities and differences in tolerances of isolates of *C. caryigenum* to juglone (24) and condensed tannin and the relationship to isolate virulence, which was established by Street (23), is of interest. In the present study, isolates seemed to have differential tolerance to inhibitory levels of tannin and isoquercitrin. For example, the Stuart isolate, from a tree with little scab, was least tolerant to both compounds, whereas the Van Deman isolate, from a tree with severe scab, was tolerant to tannin but inhibited by isoquercitrin. The StCpP₆ isolate, from a tree with severe scab,

TABLE 2. The effect of condensed tannin, isoquercitrin, and associated pH changes on the growth of three isolates of *Cladosporium caryigenum*

Substrate	Isolates ^a		
	Van Deman wild-type	Stuart wild-type	StCpP ₆
Potato dextrose broth (PDB)	381 a ^b	407 a	427 a
PDB + condensed tannin (4,000 $\mu\text{g}/\text{ml}$)	333 a	176 c	290 c
PDB + isoquercitrin (4,000 $\mu\text{g}/\text{ml}$)	80 c	46 d	143 d
PDB at pH 4.0 (tannin)	308 a	330 b	340 b
PDB at pH 3.6 (isoquercitrin)	213 b	306 b	333 b

^aGrowth response was measured as milligrams of mycelial dry weight after 14 days.

^bEach value is the mean of three replications. Values in a column followed by the same letter are not significantly ($P = 0.05$) different according to Duncan's multiple range test.

was inhibited by both compounds but exhibited the highest tolerance to isoquercitrin of any isolate. These relationships suggest that these phenolic compounds could play a role in resistance.

Concentrations of juglone among pecan cultivars and hickory species (3-5,19,20) and methods of histochemical localization (11) have been studied, but this has not been done for tannins or isoquercitrin. Cultivars of pecan and/or hickory species might have different levels of each phenolic compound, and the differential capability of isolates to tolerate phenolic compounds might dictate isolate prevalence on a given host genotype. Further, the respective levels of each of these allelochemicals in combination might determine the quality of resistance.

For disease resistance in a host-pathogen relationship to be attributed to chemical factors within tissues of the host requires more than identification of the factor and establishment of its antimicrobial activity in vitro. Considerations of concentration, location, and availability of the chemicals in tissues invaded by the pathogen are equally important. Studies on resistance of pecan to *C. caryigenum* are continuing.

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