Cytochemical Responses of Pecan to *Cladosporium caryigenum*: In Situ Localization and Quantification of Fungitoxic Phenols

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ABSTRACT

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Selective indicators and a microspectrophotometer were used to quantify juglone, isoquercitrin, and condensed tannin in fresh tissue sections of pecan (*Carya illinoensis*). Some significant differences were observed among seven pecan cultivars and two other hickory species (nutmeg [*C. myristicaeformis*] and mockernut [*C. tomentosa*]) for all three compounds in leaves. Concentrations of juglone and isoquercitrin in nut husks were not significant among three pecan cultivars, but Schley pecan contained significantly less condensed tannin. Juglone and isoquercitrin concentrations were significantly different among nutmeg, shagbark hickory (*C. ovata*), and black walnut (*Juglans nigra*) nut husks, whereas condensed tannin concentrations were significantly less in black walnut. Pecan leaves and nut husks infected with *Cladosporium caryigenum* contained significantly more juglone, isoquercitrin, and condensed tannin than uninfected tissues. This increase in phenolic content was confirmed by transmission electron microscopy, suggesting a physiological response by the plant to the presence of *C. caryigenum*. In addition, hickory species other than pecan consistently contained greater concentrations of all three phenols in infected and uninfected material. These higher phenolic concentrations may be responsible for the greater resistance of hickories to *C. caryigenum*.

Additional keywords: disease resistance, histochemical localization.

Hickories (*Carya* spp.) contain high levels of phenolic compounds, including juglone, isoquercitrin, and condensed tannin; these compounds have been implicated in disease resistance (9,11,13). Previous studies on quantity and seasonal distribution of these compounds were based on extractions from whole leaf or nut samples (6,9). Little is known about the location and quantity of phenols within various organs, including the epidermal sites of fungal infection. Native pecan (*Carya illinoensis* (F. A. Wagenheim) K. Koch) populations exhibit a high level of the scab disease caused by the fungal pathogen *Cladosporium caryigenum* (Ellis & Langl.) Gottwald, whereas other hickory species rarely have this disease, suggesting that these species possess resistance factors not prevalent in pecan. This has led to enumeration of fungitoxic constituents in pecan and other hickories. These constituents include the phenolics, juglone, condensed tannins, and isoquercitrin; the monoterpenoid, linalool; and other unidentified compounds (9,11,13).

Previous studies of pecan and hickories have shown that juglone is associated with resistance to scab (9,13,23). Graves et al (7) developed a histochemical procedure for selectively identifying juglone and its related compounds in pecan tissues. The greatest concentrations of juglone were found in the leaf midribs and lateral vascular bundles. In nuts, juglone was abundant in all areas of the husk except in the sclerenchyma cells. Condensed tannins and isoquercitrin have also been found in pecan (10). The fungitoxic effect of tannins from pecan has been demonstrated against several isolates of *C. caryigenum* (11). Condensed tannins (in vitro concentration = 4,000 μg/ml) significantly inhibited the growth of *C. caryigenum*. Isoquercitrin was two to four times more inhibitory against this pathogen than condensed tannins (11). Inhibitory tannin levels are within the concentrations found in pecan leaves (1,700–20,000 μg/ml) during the course of the growing season (12), whereas in vivo isoquercitrin concentrations are unknown.

Wetzstein and Sparks (21) correlated pecan leaf resistance to *C. caryigenum* with the presence of fewer glandular trichomes, a greater frequency of collapsed trichomes, and abundant phenolic compounds. Condensed tannins were found in the palisade parenchyma and bundle sheath cells of pecan leaves by transmission electron microscopy (TEM). Tannins were localized in the vacuoles of immature, resistant pecan cultivars and were rare or absent in susceptible cultivars. Wood et al (23) suggested that scab susceptibility is partially dependent on the phylloplane composition.

Ultrastructural localization of phenols within plant cells is difficult because the chemical reactivity of these compounds and their tendency to leach from vacuoles cause darkening of the cytoplasm and obscure cellular detail. Phenols react intensely with osmium tetroxide and, thus, appear electron-opaque when viewed with a transmission electron microscope (1,14). Leakage is most apparent in mature cells in which a large central vacuole occupies a large portion of the cell volume (15). If the tissue is properly fixed, then phenolic leakage from vacuoles into the cytoplasm should only occur in cases of cell senescence or diseased tissues. Phenolic leakage into healthy cytoplasm may be a fixation artifact (15). Many authors refer to these electron-opaque phenolic cells as tannin cells, even though the true nature of the phenolic compound is unknown (14).

It is likely that these various phenols, either individually or in combinations, play a role in providing resistance to diseases such as scab and to some insect pests. The purpose of this study was to compare quantitatively variations of juglone, isoquercitrin, and condensed tannin in selected pecan cultivars, hickories, and black walnut; analytical procedures previously described were used (4). These included determination of sampling variations within and among tissues, location of storage sites of phenolic compounds, host response to pathogen challenge, and the presence of phenols at or near the infection site. In addition, TEM was employed for confirmation comparisons of the total phenolic cellular response of two pecan cultivars to scab infection.

MATERIALS AND METHODS

Determination of variation among samples. We collected leaves of Stuart and Schley pecans and shagbark hickory, as previously described (4), in May 1989 to estimate the required sample size for detection of differences in phenols among cultivars. Collections

were made from two trees per cultivar, three leaves per tree, two leaflets per leaf (the terminal and basal leaflets), and three sections per leaflet. Plant samples were frozen, sectioned with an IEC cryostat microtome (Model CTI; International Equipment Co., Needham Heights, MA), and processed as previously described (4). Sections 30 μm thick were stained for juglone and isoquercitrin with Hoepfner-Vorsatz stain and for condensed tannin by heating in a closed water bath at 90 C for 40 min (4). Absorbances were an average of two readings from two different areas within a section. Concentrations were estimated from standard curves generated with a microspectrophotometer (Nanometrics Docuspec II, Sunnyvale, CA) adjusted by Beer's law (22) for differences in light path.

Nuts were collected in early August 1989 from nutmeg and shagbark hickories and black walnut. Collections were made from two trees per cultivar, three nuts per tree, three samples per nut, and three sections per sample. Nut husks were sectioned to 20 μm thick, and tissue just beneath the epidermis was assayed. Absorbance readings were an average of two readings from two different areas within a section. Concentrations were estimated as described above, and data were analyzed with the nested analysis of variance (ANOVA) and Duncan's multiple range test of SAS, PC (SAS Institute Inc., Cary, NC).

Determination of differences between infected and uninfected tissues. Leaves containing scab lesions were collected in July 1989 from Stevens, Stuart, Odom, Pabst, Success, Schley, and Van Deman pecan cultivars and from nutmeg and mockernut hickories. Nuts with scab lesions were collected in August 1989 from Stuart, Success, and Schley pecan, but no infection was found on the nuts of the hickories. An area of tissue containing infected and uninfected material was dissected from five different leaves or nuts from each cultivar. When possible, samples were collected from more than one tree. Tissue was collected and processed as previously described (4). Three sections per leaf or nut sample were processed, and the absorbances were averaged for a section from two areas within that section. Absorbances were recorded from infected and uninfected areas. Concentrations were estimated as described above, and data were analyzed with the nested ANOVA and Duncan's multiple range test of SAS and Scott-Knot means separation test (18).

Verification of *C. caryigenum* infection. Scab lesions were removed from leaf and nut tissues, surface sterilized, plated on potato-dextrose agar, and maintained in the dark at 28 C until colony growth occurred. Isolations obtained from lesions on leaves and nuts confirmed the presence of *C. caryigenum*.

TEM preparation of infected and uninfected tissues. Initially, two fixatives (2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 6.8, with or without 0.5% caffeine) were tested on mature leaflets from a greenhouse-grown Stuart × shagbark hickory cross. Pecan leaves and nut husks of Stuart and Schley cultivars naturally infected with the scab fungus were collected from the orchard at Mississippi State University. Slices (0.5–1 mm) consisting of infected or uninfected tissues were placed into fixative without caffeine, postfixed in 2% osmium tetroxide, then dehydrated in an ethanol series (30, 50, 70, 95, 100%) followed by propylene oxide and gradual incorporation of Spurr's low viscosity resin (19). Thick sections were made from 10 blocks per material and were stained with 1% aqueous toluidine blue; from these, at least four infected and four uninfected blocks of each cultivar were selected for further study. Ultra-thin sections were stained with uranyl acetate for 5–10 min and with lead citrate for 4–7 min (16) before being viewed with a Zeiss 109 transmission electron microscope at 80 kV.

TABLE 1. Nested analysis of variance for juglone, isoquercitrin, and condensed tannin concentrations in leaves of shagbark hickory and Stuart and Schley pecan in determining variation among samples

Source of variation	Degrees of freedom	Sum of squares	F value	Pr > F
Juglone				
Among cultivars	2	313.108	103.616**[a]	0.002
Between trees	3	4.533	0.740	0.973
Among leaves	12	245.029	1.869	0.112
Between leaflets	18	196.673	15.547**	0.001
Error	72	50.600		
Isoquercitrin				
Among cultivars	2	153.178	129.962**	0.001
Between trees	3	1.768	0.135	0.937
Among leaves	12	52.193	2.058	0.081
Between leaflets	18	38.039	4.194**	0.001
Error	72	36.277		
Condensed tannin				
Among cultivars	2	262.592	12.353*	0.036
Between trees	3	31.886	0.314	0.815
Among leaves	12	406.496	0.612	0.806
Between leaflets	18	996.712	22.386**	0.001
Error	72	178.097		

[a]* = Significance at the 0.05 level; ** = significance at the 0.01 level.

RESULTS

Variation among leaf samples. The concentrations of all three phenolic compounds, juglone, isoquercitrin, and condensed tannin, were significantly different among two pecan cultivars and one hickory species (P = 0.002, 0.001, 0.036, respectively) and between leaflets (terminal versus basal; P = 0.001; Table 1). There were no significant differences between trees of a given cultivar or among leaves of an individual tree. Shagbark hickory contained a significantly greater concentration of juglone (P = 0.05) and isoquercitrin (P = 0.05) followed by Stuart and Schley pecan (Fig. 1). Shagbark hickory also contained a significantly greater concentration of condensed tannin (P = 0.05) than either Stuart or Schley pecans (Fig. 1).

Variation among nut samples. Juglone concentrations were significantly different among three species (P = 0.027), between trees within species (P = 0.045), and among areas on a single nut (P = 0.001; Table 2). Significantly different (P = 0.05) juglone concentrations among nutmeg, shagbark, and black walnut varied from a low of 11.4 μg/μl in black walnut to a high of 26.2 μg/μl in nutmeg hickory (Fig. 2).

Isoquercitrin concentrations were significantly different between trees (P = 0.013), among nuts (P = 0.020), and among areas on a single nut (P = 0.001). Significantly different (P = 0.05) isoquercitrin concentrations among nutmeg, shagbark, and black walnut ranged from a low of 8.6 μg/μl in black walnut to a high of 10.1 μg/μl in nutmeg hickory (Fig. 2).

Condensed tannin concentrations were significantly different between trees (P = 0.028) and among nut areas (P = 0.001). Black walnut contained significantly less condensed tannin (P = 0.05) than either nutmeg or shagbark hickory (Fig. 2).

Variation between infected and uninfected tissues. Concentrations of all three phenolic compounds were significantly higher (P = 0.001) in infected compared to uninfected leaf tissues for all cultivars and species tested. Differences between infected and

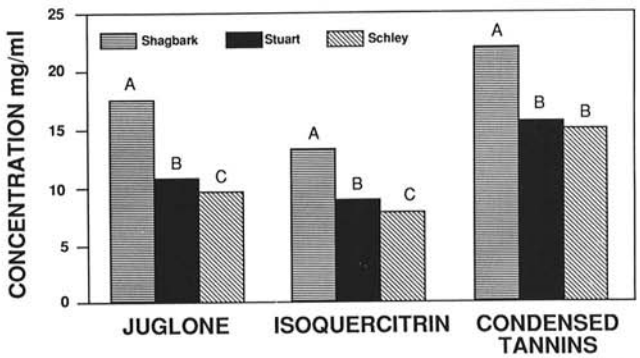

Fig. 1. Mean juglone, isoquercitrin, and condensed tannin concentrations in leaves of shagbark hickory and Stuart and Schley pecan. For each compound, different letters above bars designate significant differences (P = 0.05) among cultivar and species according to Duncan's multiple range test.

uninfected leaf tissues represented 88, 78, and 95% of the total variation in juglone, isoquercitrin, and condensed tannin concentrations, respectively.

Concentrations of all three phenolic compounds were also significantly higher ($P = 0.001$) in infected than in uninfected nut tissues. Differences between infected and uninfected tissue represented 58, 64, and 77% of the total variation in juglone, isoquercitrin, and condensed tannin concentrations, respectively, for all cultivars tested.

Variation among cultivars and species. Significant differences in all three compounds were observed among cultivars and species in leaves. In the uninfected tissue, nutmeg hickory contained a significantly greater concentration of juglone ($P = 0.05$), and Van Deman pecan contained significantly less than the other cultivars and species (Fig. 3). Only Pabst and Schley pecans contained significantly lower juglone concentrations in infected tissues (Fig. 3).

Nutmeg hickory and Pabst and Success pecans contained significantly greater concentrations of isoquercitrin ($P = 0.05$) in uninfected leaf tissue, and Van Deman pecan contained significantly less ($P = 0.05$) than the other cultivars and species (Fig. 3). Stevens, Stuart, Odom, and Success pecans contained significantly greater ($P = 0.05$) concentrations of isoquercitrin in the infected tissue (Fig. 3) than the other cultivars and species.

Nutmeg and mockernut hickories contained significantly greater concentrations of condensed tannin ($P = 0.05$) in uninfected leaf tissue, whereas Van Deman, Success, and Stevens contained significantly less than the other cultivars and species (Fig. 3). Nutmeg, Stuart, Odom, and Van Deman contained significantly greater concentrations of condensed tannin ($P = 0.05$) in infected tissues, and Schley, Pabst, and mockernut contained significantly less than the other cultivars and species.

In the nut husks, there were no significant differences among cultivars for juglone and isoquercitrin (Fig. 4) for either infected or uninfected tissue. Only Stuart pecan contained a significantly greater concentration of condensed tannin ($P = 0.05$; Fig. 4) in infected tissues, and Schley contained significantly less ($P = 0.05$) in uninfected tissue.

Comparison of TEM fixatives. Distinct differences in the appearance of phenols within vacuoles of the palisade parenchyma tissue were observed with the two fixation methods. Electron-dense phenols appeared to line the entire circumference of the inner edge of the tonoplast in cells fixed without caffeine. Cells fixed in the presence of caffeine contained phenols condensed into round masses within the vacuole. Neither fixation procedure showed darkening of the cytoplasm characteristic of phenolic

TABLE 2. Nested analysis of variance for juglone, isoquercitrin, and condensed tannin concentrations in nut husks of black walnut and nutmeg and shagbark hickories in determining variation among samples

Source of variation	Degrees of freedom	Sum of squares	F value	Pr > F
Juglone				
Among species	2	6,026.355	15.116*[a]	0.027
Between trees	3	598.001	3.632*	0.045
Among nuts	12	658.505	1.971	0.058
Among nut areas	36	1,002.199	4.435**	0.001
Error	108	677.854		
Isoquercitrin				
Among species	2	61.765	0.776	0.535
Between trees	3	119.380	5.499*	0.013
Among nuts	12	86.840	2.424*	0.020
Among nut areas	36	107.484	2.319**	0.001
Error	108	139.048		
Condensed tannin				
Among species	2	906.731	1.240	0.405
Between trees	3	1,096.534	4.291*	0.028
Among nuts	12	1,022.245	0.975	0.490
Among nut areas	36	3,146.245	5.376**	0.001
Error	108	1,755.562		

[a] * = Significance at the 0.05 level; ** = significance at the 0.01 level.

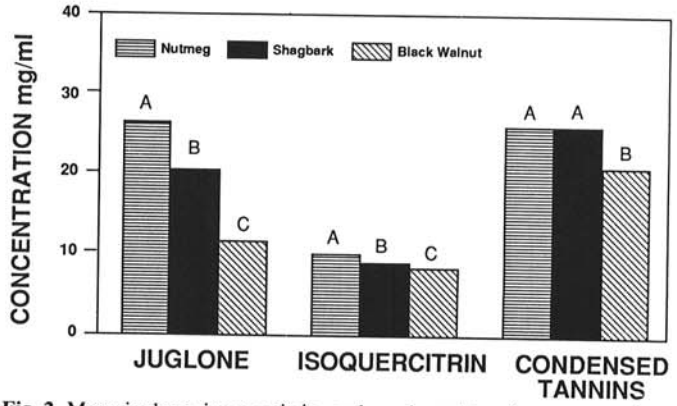

Fig. 2. Mean juglone, isoquercitrin, and condensed tannin concentrations in nut husks of nutmeg and shagbark hickories and black walnut. For each compound, different letters above bars designate significant differences ($P = 0.05$) among cultivars and species according to Duncan's multiple range test.

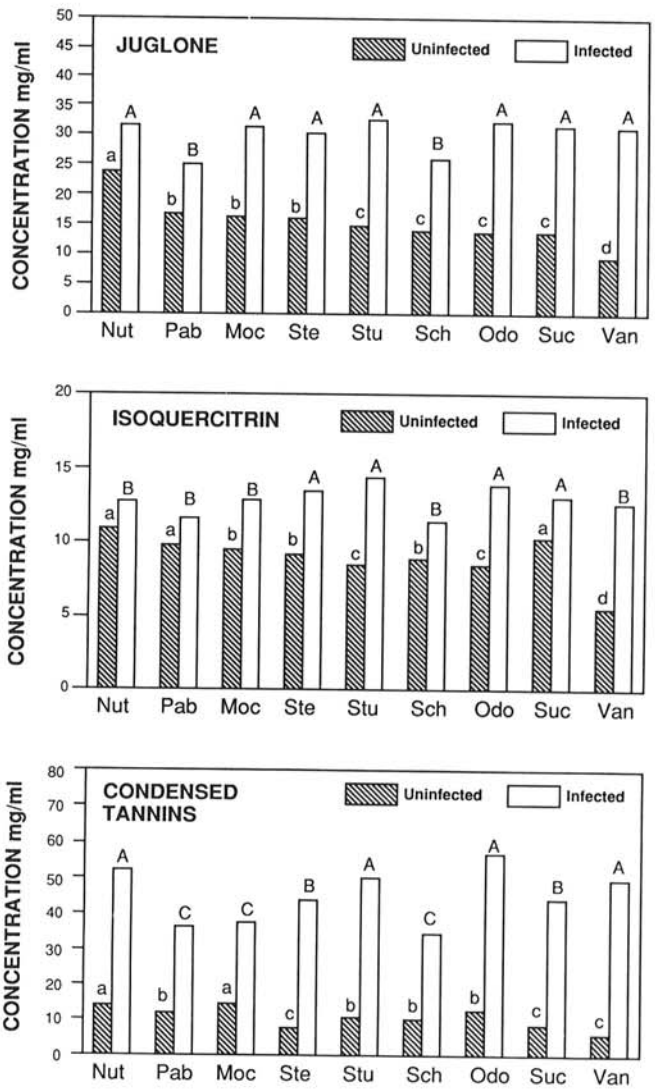

Fig. 3. Mean juglone, isoquercitrin, and condensed tannin concentrations in infected and uninfected leaf tissues of pecan cultivars and hickory species. Cultivar and species differences are based on the Scott-Knot mean separation procedure. A different letter designates significant differences ($P = 0.05$); uppercase = infected; lowercase = uninfected. Cultivar abbreviations include Nut = nutmeg; Pab = Pabst; Moc = mockernut; Ste = Stevens; Stu = Stuart; Sch = Schley; Odo = Odom; Suc = Success; Van = Van Deman.

leaching. Buffer without caffeine was selected for subsequent studies.

Comparisons of infected and uninfected tissues by TEM. Infected leaves (Fig. 5A) seemed to contain more electron-opaque phenols within vacuoles of palisade parenchyma cells than uninfected leaves (Fig. 5B), although this difference was not quantified. Most cells of the infected material were completely filled with phenols, whereas others contained phenols scattered throughout the vacuoles or lining the tonoplast. Phenolic leaching from vacuoles was observed in some cells. The upper epidermis contained little or no phenols, whereas the lower epidermis contained scattered phenols in infected tissue.

Infected nut husks (Fig. 5C,E) seemed to contain more phenols than uninfected husks (Fig. 5D). The upper layers of the infected materials were collapsed, and cells were filled with phenols, as were cells adjacent to the infection. Phenols in uninfected husks lined the wall of the tonoplast but seldom filled the vacuoles. No leaching was observed. There did not seem to be any distinguishable differences in phenolic amounts either between nuts of infected Stuart and Schley or between nuts of uninfected Stuart and Schley cultivars.

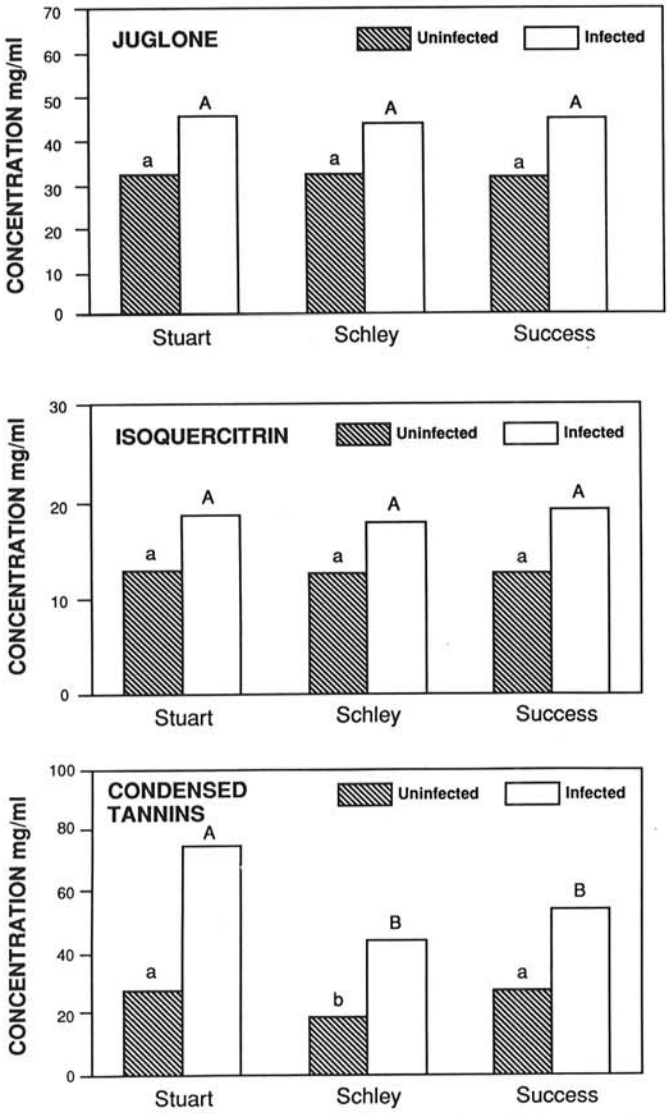

Fig. 4. Mean juglone, isoquercitrin, and condensed tannin concentrations in infected and uninfected nut tissues of Stuart, Schley, and Success pecan. Cultivar differences are based on the Scott-Knot mean separation procedure. A different letter designates significant differences ($P = 0.05$); uppercase = infected; lowercase = uninfected.

DISCUSSION

Procedures permitting quantification of juglone, isoquercitrin, and condensed tannin within tissue sections were used to determine if there were differences among selected cultivars and species and subsequently between infected and uninfected tissues. Perhaps the most important finding is that infected tissues contained significantly greater levels of juglone, isoquercitrin, and condensed tannins. This suggests a physiological response to the presence of *C. caryigenum*. In addition, hickory species other than pecan consistently contained greater concentrations of all three phenols in infected and uninfected material; nutmeg hickory contained the most. These higher phenolic concentrations may be involved in the greater resistance of hickories to scab.

There was general agreement on differences in the presence and location of phenols in resistant and susceptible cultivars and between infected and uninfected tissues with the histochemical and ultrastructural localization methods. An increase of total phenols in infected leaves and nuts was observed with both methods. With TEM, there were no visible differences in phenolic amounts between infected Stuart and Schley nut husks, or between uninfected Stuart and Schley nut husks. In the histochemical study, neither juglone nor isoquercitrin was significantly different in Stuart and Schley nut husks for either tissue; only condensed tannins showed cultivar differences.

Although there were no phenolic differences detected between the nuts of Stuart or Schley pecan cultivars, Stuart has been considered a moderately resistant cultivar to *C. caryigenum*, whereas Schley is very susceptible. It has been proposed that

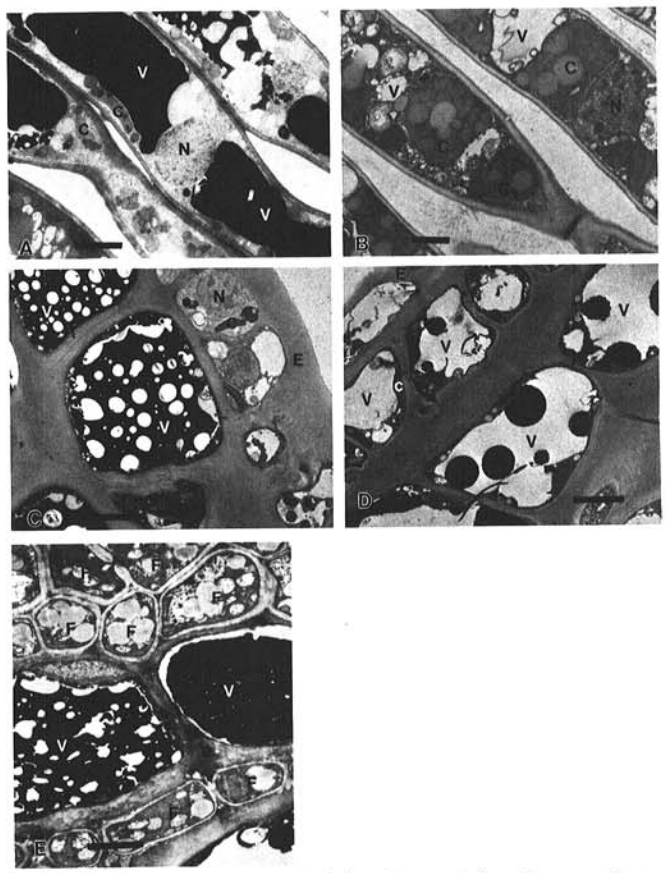

Fig. 5. *Cladosporium caryigenum* infected or uninfected pecan tissues. **A,** Palisade parenchyma cells of infected Schley pecan leaves. Phenols are electron-dense materials completely filling cell vacuoles. **B,** Palisade parenchyma cells of uninfected Stuart pecan leaves. **C,** Cells of Schley nut husk infected with *C. caryigenum* with most vacuoles filled with phenols. The smaller cells are the husk epidermis. **D,** Cells of uninfected Stuart nut husk with phenols forming globules around tonoplast but not filling entire vacuole. **E,** Infected Stuart nut husk with *C. caryigenum* mycelial cells present between the pecan cells. C, chloroplast; E, epidermis; F, fungus; N, nucleus; V, vacuole. Bars = 3, 2, 5, 5, and 3 μm, respectively.

the resistance of Stuart to the scab fungus is due to the resistance of leaves. Leaf resistance substantially decreases the amount of inoculum available for infection on the nuts. This study found phenolic concentrations were significantly greater in Stuart leaves, possibly providing leaf resistance.

Comparisons of phenolic concentrations with those of previous studies are difficult, because in previous studies concentrations were reported in milligrams of phenolic compounds per gram of fresh tissue as extracted from whole tissue. In this study, concentrations in milligrams of phenol per milliliter (or $\mu g/\mu l$) were measured only in the palisade parenchyma tissues in situ.

Graves et al (6) found that shagbark hickory contained a higher juglone concentration as compared with pecans, and Stuart pecan contained a higher concentration than Schley pecan. In this study, shagbark hickory contained greater concentrations of all three compounds in leaf tissue. Stuart pecan contained greater concentrations of juglone and isoquercitrin than did Schley pecan. No previous studies have compared isoquercitrin or condensed tannin among *Carya* spp.

Black walnut and nutmeg and shagbark hickories consistently contained lower juglone and isoquercitrin as compared with the three pecan cultivars, and the tannin contents of all cultivars were about equal. In contrast, Graves et al (6) found black walnut contained more juglone than did pecan. The low levels of phenols in the nut tissue is surprising; however, seasonal differences in juglone have been shown to be significant and could account for the present observed differences (6). Seasonal aspects were not considered in this study. Further definition of seasonal fluctuations can be done by employing the histochemical techniques now available.

Mycelia of *C. caryigenum* seem to be confined in the leaf to the intercellular areas (17) such as the middle lamella. Hyphae are found in the subcuticular region (2,3,17); they also extend down between the epidermal cells forming a mycelial mat beneath the epidermis (17). Host cells below the fungus appear to degrade (2); however, no major degenerative ultrastructural changes of host leaf tissue were observed as a result of early infection (17). The only cell destruction observed was in late stages of pathogenesis (17). If phenols play a role in pecan resistance, they must first be released from the vacuoles either by leaching of phenols from infected tissues of pecan or by cell senescence (15), before contact with the fungus can occur. For example, Treutter and Feucht (20) determined that inhibition of different pathogenic fungi on leaves of three Rosaceae species required leaching of vacuolar flavonols into the cytoplasm of stressed cells located in the lesion boundary zone. All three Rosaceae species contained appreciable amounts of flavan-3-ols in healthy tissues and exhibited a substantial increase in concentrations of these phenolic compounds with infection. Infection may alter the structural integrity and permeability of the tonoplast, making it more likely to be damaged in the TEM processing. Altered tonoplast permeability and/or phenolic leaching may be important in determining the availability of phenols for interacting with the scab pathogen. Expanded studies that include seasonal variation, broader cultivar and species comparisons, and the speed of the host's physiological response to infection will be necessary before the full role of phenolic compounds in scab resistance is clear.

Many plants exhibit an increase in phenolic content with injury, elicitation, and/or infection. Infection by any pathogen initiates a complex biological and molecular resistance response. These events occur in a very limited population of cells at or just beyond the infection sites (5). The activation of the phenylpropanoid pathways at the molecular level has been addressed in two recent review articles (5,8). The specificity of the phenolic response of pecan to the scab pathogen is unknown. We also know nothing about what chemical compounds the fungus secretes (enzymes and/or toxins) that result in host cell degradation, nor do we know if these fungal materials elicit the activation of phenolic synthesis. There have been no studies examining the presence of phytoalexins within any member of the Juglandaceae.

No one phenol correlates directly to resistance or susceptibility to scab. The total phenolic concentration seems a better indicator of resistance than that of a single phenol. The rate of the response to infection has yet to be determined. These procedures should prove extremely useful in pecan scab resistance breeding programs. They will enable researchers to determine if inheritance of phenolic enzyme gene copy numbers and activities results in increased phenolic concentrations and, thus, increased resistance in pecan-hickory progeny.

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