Distribution of Juglone and Related Compounds in Pecan and Their Effect on *Fusicladium effusum*

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Fractions of pecan (*Carya illinoensis* K.) leaves that contained juglone, 1,4,5-trihydroxynaphthalene (1,4,5-THN), and their glucosides inhibited mycelial growth of *Fusicladium effusum* Wint, incitant of pecan scab. Free juglone was shown to be present in intact leaf tissue by cold extraction procedures. Aqueous extracts of pecan leaves converted juglone to 1,4,5-THN and then to a partially characterized component believed to be a tetrahydrojuglone. Scab-infected leaves did not contain increased amounts of free juglone. Free juglone accounted for about two-thirds of the total "juglone" content of leaves. Two analogues of juglone showed comparable antifugal activity.

Recently, we isolated juglone from pecan (*Carya illinoensis* K.) leaves and nuts and presented preliminary evidence that it may possibly be a factor in resistance of pecan to *Fusicladium effusum* Wint (Hedin et al., 1979; Langhans et al., 1978). Scab is the most important disease of pecans in the southeastern United States.

The content of juglone in leaves and nuts was low in early season and increased through July (Hedin et al., 1979; Langhans et al., 1978). The level in nuts continued to increase to a maximum in the late season, while that in leaves decreased to near zero. The level of juglone in several species of the Juglandaceae family and several cultivars of pecan was studied. Those cultivars less affected by *F. effusum* in nature generally had a higher level of juglone (Hedin et al., 1979; Langhans, 1978). Juglone was shown to inhibit mycelial growth at 0.05 mg/g of liquid culture medium (Langhans et al., 1978), a level lower than the content of juglone in pecan leaves and nuts. The pertinent literature on pecan scab and its horticultural implications was reviewed previously (Hedin et al., 1979; Langhans et al., 1978; Langhans, 1977).

This study was undertaken to assess the total juglone synthesizing capability of the pecan and to determine whether infection by *F. effusum* elicits the release of juglone from some precursor or by another mechanism. Other objectives were to identify additional chemical resistance factors and to determine the nature of the degradation products of juglone.

MATERIALS AND METHODS

Liquid Culture Bioassay Method. A procedure, previously described (Langhans et al., 1978), was used wherein test solutions were added to potato-dextrose broth, and the broth was seeded with a native pecan isolate of the fungus and incubated on a rotary shaker at room temperature for 14 days. The mycelia were collected, dried, and weighed. In a typical bioassay, a measured quantity of the test compound was dissolved in 1 mL of acetone and added to 50 mL of broth, and the mixture was heated at 55 °C for 24 h to evaporate the acetone. The flasks were seeded with inoculum in an additional 5 mL of broth after the mixture was cooled. Solvent checks and untreated checks were included in each experiment. Replications for appropriate statistical considerations were used.

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Collection of Plant Material. Leaves of pecans and walnuts were collected from orchards in Mississippi, placed immediately in ice chests, and transported to a laboratory freezer for storage. Several cultivars were used, chiefly Delmas and Stevens. The criterion for cultivar selection was a high content of juglone as determined by an accompanying seasonal analysis and a low incidence of pecan scab on these trees.

Determination of Juglone Content in Intact Leaves. Leaves of pecan and walnut were immersed immediately upon harvesting in a solid CO_2 (−78 °C), chilled chloroform/methanol (2:1, CM) solvent mixture. Samples were flushed with nitrogen, ground in a blender, and then filtered before allowing the preparation to warm. For comparison, leaves that were iced at harvesting and oven-dried (85 :C) leaves were extracted with the CM solvent mixture at room tempature and analyzed for juglone by the previously reported procedure (Hedin et al., 1979).

Isolation, Biological Evaluation, and Identification of Juglone Glucoside and 1,4,5-Trihydroxynaphthalene Glucoside. Pecan leaves (10 g) were ground in 200 mL of the CM solvent and filtered. The filtrate was concentrated to 10 mL by vacuum distillation, and chromatographed on a 2 × 25 cm Biosil A (Biorad Laboratories, Richmond, CA) column. Eight fractions of 25 mL each were collected by elution with chloroform, then two fractions of 100 mL each were collected by elution with acetone, and finally one fraction of 200 mL was collected by elution with methanol. Aliquots of all fractions were reserved for biological evaluation.

Concentration of the methanol eluate yielded an orange pigment (45 mg) which upon hydrolysis at reflux with 1.3 N HCl for 1 h in 50% aqueous methanol yielded juglone, 1,4,5-trihydroxynaphthalene (1,4,5-THN), and glucose. The presence of these aglycons was confirmed by mass spectrometry (MS) after separation by thin-layer chromatography (TLC) utilizing procedures previously described. The glucose was identified by gas–liquid chromatrography (GLC) of the trimethylsilyl derivative on a 0.2 × 180 cm SE-52 column at 200 °C.

Synthesis of 1,4,5-Trihydroxynaphthalene (1,4,5-THN). This compound was synthesized for proof of structure by refluxing juglone (100 mg) with 2 mol of $Na_2S_2O_4$ in methanol for 1 h. Water was added to the reaction mixture which was subsequently extracted with chloroform. Residual juglone was separated from the desired product; the later migrated with a slightly lower R_f in methylene chloride/pentane (1:1) and exhibited a green fluorescence in ultraviolet (UV, 254 nm) light. 1,4,5-THN was easily reconverted to juglone by air or by potassium dichromate oxidation.

Biological Evaluation of Juglone Analogues. 1,4-Dihydroxynaphthalene, 1,5-dihydroxynaphthalene, and

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juglone (Pfaltz and Bauer, Inc., Stanford, CT) were evaluated as antifugal agents at 0.005–0.10 mg/g by the liquid culture bioassay technique.

Search for Degradation Products of Juglone. Aqueous and 0.1 M phosphate buffer (pH 7.0) extracts were made from leaves of the cultivar Stevens (10 g with 200 mL). Juglone (20 mg) was added to the filtrate, and the contents were incubated at 30 °C with stirring for 48 h. Aliquots were extracted with chloroform for analysis of juglone and other products at 2, 6, 24, 30, and 48 h.

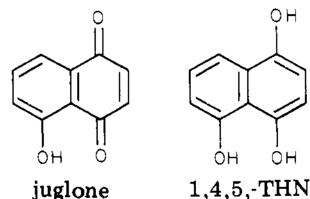
In another experiment, juglone was coated as a thin film on the inside of a round-bottom flask by evaporation of a chloroform solution during rotation. The flask was then fitted to a distilling head connected to a reflux condenser and collecting flask, and heated by an electric mantle until yellow vapors formed and sublimed in the distilling head leaving a dark residue in the distilling flask. The apparatus was disassembled, each part was washed with chloroform, and the solutions were examined by TLC for juglone or other products.

In a third experiment, juglone was suspended in water and aerated for 24 h. The suspension was extracted with chloroform as before and analyzed for juglone or other products.

Analysis of Total Juglone and 1,4,5-THN in Pecan Leaves. Leaves of the Delmas cultivar were ground in the CM solvent mixture. The filtrate was concentrated and separated by preparative TLC (dichloromethane/pentane, 1:3) to recover juglone, 1,4,5-THN, and the mixed glucosides of these two aglycons. The glucosides were hydrolyzed as previously described, and the reaction mixture was extracted with chloroform to obtain a mixture of the two aglycons. The leaf pulp remaining after filtration was extracted two times with water to remove remaining free juglone and then two times with 5% boiling aqueous sodium carbonate to remove bound juglone. Both aqueous filtrates were acidified and extracted with chloroform to isolate free juglone. The aqueous sodium carbonate filtrate was acidified to free any juglone and likewise extracted with chloroform. With each preparation, the 1,4,5-THN and juglone concentrations were determined by spectrometric analysis at 350 and 420 nm, respectively, as previously described (Hedin et al. 1979).

RESULTS AND DISCUSSION

Juglone from pecan leaves was identified by mass



juglone 1,4,5,-THN

spectral (MS), proton nuclear magnetic resonance (^{1}H NMR), and carbon-13 nuclear magnetic resonance (^{13}C NMR) analyses. Its mass spectrum (70 eV) was identical with that of authentic juglone (Aldrich Chemical Co., Milwaukee, WI) and to that reported by Aynehchi et al.(1973); characteristic fragments were *m/e* 174 (M^+), 146 (M^+ – CO), 118 (M^+ – 2CO, base peak), 90 (M^+ – 3CO), and 63 (C_5H_3).

^{1}H NMR analysis of juglone in $CDCl_3$ showed the chemical shifts: δ 6.91 [s, 2, C_2–H, C_{3-H}, (quinoid)], 7.28 (m, 1, C_7–H, (aromatic)], 7.53 (m, 2, C_6–H, C_8–H, (aromatic)], and 11.81 [s, 1, C_5–H, (phenolic)].

^{13}C NMR analysis of juglone in $CDCl_3$ showed the chemical shifts: δ 114.64 (C_{10}), 118.76 (C_6), 124.11 (C_8), 131.51 (C_9), 136.19 (C_7), 138.29 (C_2), 139.21 (C_3), 161.13 (C_5), 183.84 (C_1), and 189.88 (C_4).

Table I. Juglone Content in Leaf Tissue (Milligrams/Gram Fresh Weight)

	conditions of processing		
	−78 °C, N_2	20 °C, O_2	85 °C
walnut	0.26	0.34	0.06
pecan	0.45	0.25	0.00

MS analysis (70 eV) of 1,4,5-trihydroxynaphthalene (1,4,5-THN) showed the fragments: *m/e* 176 (M^+), 148 (M^+ – CO), 147 (M^+ – CHO), 120 (M^+ – 2CO), 119 (M^+ – CHO – CO), 92 (M^+ – 3CO), 91 (M^+ – 2CO – CHO), and 63 (C_5H_3).

^{1}H NMR analysis of 1,4,5-THN in $CDCl_3$ showed the shifts: δ 7.25 [s, 1, C_7–H, (aromatic)], 7.50 [s, 1, C_8–H, (aromatic)], 7.61 [m, 3, C_2–H, C_3–H (quinoid), C_6–H (aromatic)], and 12.10 3, C_1–H, C_4–H, C_5–H (phenolic)].

Daglish (1950) demonstrated that there was little or no free juglone in intact walnut leaves. When he rigorously excluded moisture and air during the extraction he isolated primarily 1,4,5-THN glucoside. In the present study, juglone was isolated from walnut leaves that had been immediately immersed in solid CO_2–CM solution; air was excluded by flushing with nitrogen until after the extraction and filtration processes. For comparison, leaves that were collected over ice but not otherwise specially treated, and leaves that were dried at 85 °C, were also analyzed for juglone. The free juglone content of the CO_2-chilled walnut leaves extracted under N_2 was about 75% (0.26 vs. 0.34 mg/g) of that in unprotected leaves (Table I). This is partial confirmation of the study by Daglish (1950). The juglone content of the dried leaves was severely decreased.

In contrast to walnuts, the highest juglone content in pecan was found in CO_2-chilled leaves extracted under N_2; lower amounts were extracted at room temperature and none was obtained from oven-dried leaves. These data indicate that free juglone is present in intact pecan leaves. Therefore, resistance to disease in pecan may involve free juglone as well as precursors that are converted to juglone upon plant injury. Because free juglone diminishes in harvested tissue, specimens should be frozen quickly after harvest and analyzed as quickly as possible for comparable results.

Of the 11 chromoatographic fractions from pecan leaves, only two showed fungitoxic activity. The first (actually fraction 5), which was eluted with chloroform, contained juglone and 1,4,5-THN. The second (fraction 11), eluted with methanol, contained an orange pigment. Acid hydrolysis of this pigment yielded two aglycons that were identified as juglone and 1,4,5-THN. Glucose was also isolated and identified from the acid hydrolysate. Fraction 11 which contained the glucosides was identical with the previously reported fraction 12 which was the only other major factor toxic to *F. effusum* (Langhans et al., 1978). Thus, the entire juglone system in pecan should be taken into account when considering possible relationships of naphthol derivatives with resistance of pecan to scab. The ratio of juglone to 1,4,5-THN obtained from the mixed glucosides varied from 2:1 to 1:2.5 as the summer progressed.

In a previous study, Thomson (1950) determined the structures of α- and β-hydrojuglone. Mylius (1885) had shown that α-hydrojuglone (1,4,5-THN) was readily transformed into β-hydrojuglone (5-hydroxy-1,4-diketo-2,3-tetrahydronaphthalene) by heating above its melting point. While both isomers formed the same acetates, only α-hydrojuglone was readily oxidized to juglone. β-Hy-

Table II. Metabolism of Juglone by Aqueous Leaf Extracts; Content of Juglone and 1,4,5-THN (Milligrams/Gram)

incubation period, h, 30 °C	aqueous extract (pH 6.5)		phosphate buffer ext (pH 7.0)	
	juglone	1,4,5-THN	juglone	1,4,5-THN
0	20.10[a]	0.07	20.10	0.08
2	8.60	0.81		
6	9.40	0.89		
24	7.80	1.31	11.20	1.68
31	7.00	1.22		
48	7.40	0.99	10.40	1.34

[a] Juglone content of aqueous extract equivalent to 0.1 mg/g of original tissue; 20 mg of juglone was added to the extract.

drojuglone has recently been isolated from walnut leaves (Muller and Leistner, 1978). However, the present work excludes the probability that appreciable β-hydrojuglone was present in pecan because ^{1}H NMR showed no cyclomethylene protons to be present (cyclomethylene protons α to C=O in alkanone systems show chemical shifts of 2.0–2.5 ppm). Moreover, both the isolated and synthesized 1,4,5-THN in this study were easily oxidized to juglone.

To demonstrate that analogues of juglone may possess antifungal activity for *F. effusum*, two commercially available compounds were evaluated at 0.005–0.10 mg/g (mL) by the liquid bioassay procedure. 1,4-Dihydroxynaphthalene and 1,5-dihydroxynaphthalene were found to be as effective at 0.05 mg/g as juglone. It is recognized that there is an extensive literature on the fungitoxicity of phenols and quinones in general and of juglone particularly. This suggests that there may be a number of phenols and quinones that are effective antifungal agents for *F. effusum*; however, the only phenols and quinones found in pecan leaves were juglone and its precursors and derivatives.

There was no indication that invasion of pecan leaf by *F. effusum* elicited the release or biosynthesis of additional juglone. When infected and infection-free areas of individual leaves affected by scab were sectioned and analyzed, the juglone content in free and infected tissue was 0.53 and 0.55 mg/g, respectively (mean of two analyses). Scab-free leaves from adjacent branches possessed a juglone content of 0.51 mg/g.

Over 2 h at 30 °C, aqueous and pH 7.0 phosphate buffer extracts of pecan leaves converted approximately 60% of added juglone to other products. The content of 1,4,5-THN increased through 30 h, but the increase accounted for only a small fraction of the decrease of juglone. Beyond 30 h, 1,4,5-THN also decreased (Table II). A third aglycon was isolated from the aqueous reaction mixture by chloroform extraction and subsequent chromatography. By MS analysis, the molecular weight was found to be 178 which is consistent with a structural assignment for a tetrahydrojuglone. This study suggests that 1,4,5-THN is not only a precursor of juglone, but also a derivative (but not the only one). In our previous study (Hedin et al., 1979) the juglone content of leaves was found to decrease as the summer progressed. This model study along with the decreased ratio of juglone to 1,4,5-THN in the glucosides suggests two products that may be formed.

Table III. Total Juglone and 1,4,5-THN Content in Pecan Leaves (Milligrams/Gram)

component	juglone	1,4,5-THN
aglycon	1.330	0.254
glucoside	0.130	0.360
bound	0.002	0.000
total	1.462	0.614

When juglone was heated to 190 °C as a thin film in a distilling flask, yellow vapors formed that sublimed in the distilling head, but none actually passed into the receiving flask. Some brown residue was formed in the distilling flask. The pigment in the distilling head contained juglone and 1,4,5-THN in a ratio of 4:1, whereas the distilling flask contained the same two components in the ratio of 1:9. No evidence was found for β-hydrojuglone.

Finally, the total content of juglone and juglone-related compounds were estimated in the leaf. For this purpose, the contents of free aglycons, glucosides, and bound juglone were determined. The most prominent compound was free juglone, and small additional amounts of juglone were presented as the glucoside and in bound form (Table III). On the other hand, more 1,4,5-THN was present as the glucoside than in the free state. Overall, free juglone accounted for about two-thirds of the total "juglone" content. There, of course, may still be other related compounds of significance.

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