Identification of Juglone in Pecan as a Possible Factor of Resistance to *Fusicladium effusum*

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Juglone (5-hydroxy-1,4-naphthoquinone) was isolated from pecan (*Carya illinoensis* Koch) and shown to be inhibitory to *Fusicladium effusum* Wint., the incitant of pecan scab. The content of juglone in pecan leaves and nuts was found to be low in June and increased through July. The level in nuts continued to increase to a maximum in August while that in leaves decreased to near zero. The content in nuts was consistently higher than that in leaves except in early season. The level of juglone in several species of the Juglandaceae family and in several cultivars of pecan was measured. There was some indication that those species and cultivars less affected by *F. effusum* in nature had higher levels of juglone.

The most serious disease affecting pecan (*Carya illinoensis* Koch) is scab, incited by the fungus *Fusicladium effusum* Wint. This disease agent, first reported in 1885 on Mockernut, *Carya tomentosa* Nutt. (Winter, 1885), is a limiting factor in pecan production.

The development of resistant cultivars of pecan has been difficult. Susceptibility to *F. effusum* has spread gradually to various commercial cultivars, including cultivars previously considered resistant (Cole, 1956; Cole and Gossard 1956; Cole and Large, 1936; Converse, 1960; Demaree, 1924; and Waite, 1911). Research has indicated that this fungus possesses a high degree of natural variability and evidently has a fairly efficient mechanism for genetic recombination (Alford, 1970; McNeill and Graves, 1970a; Soonthornpoct, 1973; Street, 1972).

The development of trees resistant to disease is always costly in time and funds. Thus, it is imperative that a source of resistance that transcends the parameters of genetic variation of *F. effusum* be obtained in pecan at the outset of any breeding program.

The quest for a technique that can be used to screen for resistance to pecan scab has proven to be complicated (McNeill, 1970; McNeill and Graves 1970a,b; Street, 1972). It is conceivable that knowledge of mechanisms of resistance could be useful in developing convenient progeny screening procedures.

The existence of a chemical mechanism for disease resistance has not been defined for any member of the Juglandaceae. Walnut, and to some extent butternut, have allelopathic effects on certain other plants (Brooks, 1951). The toxic principle present in the leaves, hulls, and inner root bark of these species was identified as juglone (5-hydroxy-1,4-naphthoquinone) (Bode, 1958). However, neither juglone nor any other compound has been associated with an observed resistance of walnut (or pecans) to disease. Juglone has not been previously reported in pecan.

The purpose of this study was to determine whether host plant constituents could be a factor in the observed differences in susceptibility to *F. effusum* among pecan and related species and to study the feasibility of using such information as a tool in screening for resistance. A concomitant study by Langhans et al. (1978) has demonstrated the efficacy of juglone (shown in this study to occur in pecan) and other extracts from pecan on *F. effusum*.

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MATERIALS AND METHODS

Biological Evaluation Techniques. Inoculum of *F. effusum* was prepared from an isolate from a native pecan at Brownwood, Texas. This isolate was previously studied by Street (1972) and designated as isolate B-10. Bioassays were conducted by the use of liquid culture techniques. Fifty-milliliter portions of potato-dextrose broth were autoclaved for 15 min and allowed to cool. The test solutions were then added individually to the sterilized broth and the flasks placed in an oven at 55 °C for 24 h to allow the solvents to evaporate. After the broth had cooled to room temperature, the flasks were seeded with 5 mL of the inoculum. The cultures were then incubated on rotary shaker tables at room temperature (approximately 24 °C). After 14 days incubation, the mycelia were collected on preweighed Whatman No. 1 filter paper using vacuum filtration, dried at 65 °C for 48 h, and weighed. Cultures which could not be filtered and dried immediately were stored at near 0 °C to prevent additional growth. In all experiments, solvent checks as well as an untreated check were included. Four replications or more were used for each data point.

Collection, Extraction, and Fractionation of Plant Material. Leaves and nuts from Stuart, Schley, and Success cultivars were collected from an orchard on the Mississippi State University campus. Those from the Van Deman cultivar were collected from an orchard located at the Blackbelt Experiment Station at Brooksville, Miss. Leaves and nuts from each cultivar were collected from four trees, combined into a single sample, chilled immediately, and stored at −10 °C until they were processed. Tissues from black walnut (*Juglans nigra* L.), white hickory (*Carya tomentosa* Nutt., synonymous to *Carya alba* Britt.), water hickory (*Carya aquatica* Nutt.), and hican (an interspecific cross between pecan and hickory) were obtained from single trees in the vicinity of Starkville, Miss.

Initially, for isolation work 0.5 kg of fresh, wet plant material was placed in a Soxhlet apparatus and extracted sequentially with pentane, chloroform, acetone, and methanol. All solvents were of reagent ACS grade or distilled from technical grade. Preliminary biological assays indicated that the bulk of the fungal inhibitory components was in the pentane and chloroform extracts. Subsequently, these extracts were combined, concentrated, and chromatographed on a 2 × 25 cm Florisil column. The contents were eluted from the column sequentially with 200-mL volumes each of methylene chloride, acetone, and methanol. Twelve fractions were collected, concentrated

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so that 1 mL was equivalent to 2 g of fresh tissue, and evaluated for antifungal activity (Langhans et al., 1978).

Analytical Techniques. Biologically active fractions and compounds were investigated by thin-layer chromatography (TLC), gas–liquid chromatography (GLC), and mass spectrometry (MS). For TLC, silica gel G plates were irrigated primarily with methylene chloride–pentane (1:3) and visualized as required by heating at 100 °C after spraying with 3% vanillin in 0.5% methanolic H_2SO_4. By GLC, juglone could be eluted from a 0.3 × 50 cm stainless steel column packed with 10% UCW-98 on Chromosorb Q in about 3.5 min at a column temperature of 105 °C and N_2 carrier gas flow of 50 mL/min. However, since pecan nut extracts contained several interfering substances, TLC cleanup was required before GLC analysis could be used. For MS, samples were introduced into a Hewlett Packard 5930 quadrupole instrument via solid probe at 100 °C or via the previously described GLC column.

Quantitative Analysis of Juglone from Plants. Extraction was made from ca. 10 g of tissue from leaves or whole nuts by grinding in a solvent mixture containing chloroform–methanol (2:1). The filtrate from three successive extractions of the tissue was combined and concentrated to 25 mL by evaporation under vacuum at 50 °C. A 1-mL aliquot was banded on a silica gel G TLC plate and chromatographed with a solvent mixture of methylene chloride–pentane (1:3). Juglone could be observed as a clearly visible yellow-orange band at about R_f 0.40. The juglone band was scraped into a test tube, eluted from the silica gel with methylene chloride, filtered, and diluted to 10 mL for spectrophotometric analysis at 420 nm. For comparison, a standard curve was prepared with dilutions of 0.05–0.50 mg/10 mL of authentic sample of juglone (Aldrich Chemical Co., Milwaukee, Wis.). MS analysis showed the fragmentation pattern of the commercial sample was identical with that reported for juglone by Aynehchi et al. (1973).

RESULTS AND DISCUSSION

Pentane and chloroform extracts of pecan leaves retarded the growth of mycelia of *F. effusum* while subsequent acetone and methanol extracts of the leaf pulp did not (Langhans et al., 1978). Twelve fractions were obtained by sequential elution of the combined pentane and chloroform extracts with a Florisil column using methylene chloride, acetone, and methanol. Of these, fractions 2 and 9, which were eluted with methylene chloride, and fraction 12, which was eluted with methanol, showed the greatest antifungal activity (Langhans et al., 1978). The fractions from the methylene chloride elution (ca. 200 mL) consisted chiefly of a yellow-orange pigment which, after standing in the solvent yielded a large quantity of crystals. The crystals (250 mg from 1 kg of pecan leaves) were filtered off and redissolved in the same solvent. From TLC, GLC, and MS analysis it was determined that this pigment possessed a R_f, a retention volume, and a fragmentation pattern identical with that of the standard juglone.

Fraction 12 was subjected to 1 N HCl hydrolysis with subsequent extraction of the hydrolysate with ethyl acetate. Juglone was found in the ethyl acetate layer. When the aqueous layer was evaporated to dryness, redissolved in pyridine, trimethylsilyated, and analyzed by GLC, evidence for glucose was obtained. This fraction therefore contained a juglone glucoside. Additional work to characterize this compound and a second also found to be present in fraction 12 is continuing.

The seasonal levels of juglone in leaves and whole nuts of several pecan cultivars are given in Figure 1. The mean values for all cultivars are given in Figure 2. There were

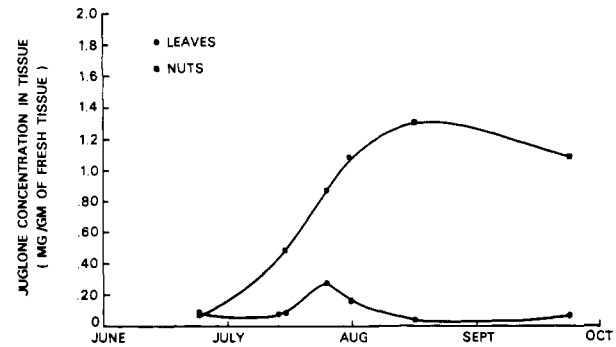

Figure 1. Juglone levels (milligram/gram of fresh tissue) in tissue samples from pecan. Each point represents four samples (one from each of four trees).

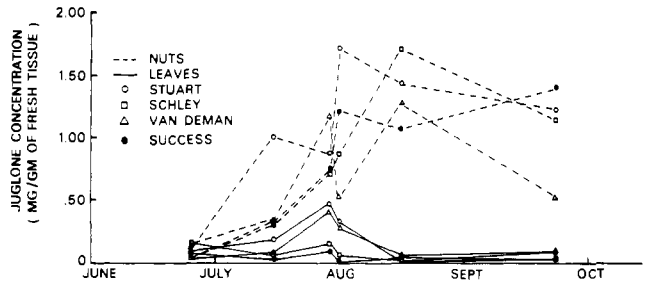

Figure 2. Seasonal fluctuations in juglone levels in leaf and nut samples of pecan. Each point represents an average of all readings from four pecan cultivars.

Table I. Comparison of Juglone Levels in Leaf and Nut Samples from Three Species of the Juglandaceae Family in Early (June–July) and Late (August–September) Season

	leaves		whole nuts	
species	early season (June–July)	late season (Aug.–Sept.)	early season (June–July)	late season (Aug.–Sept.)
pecan	0.150[a,b]	0.112	0.479	1.160
black walnut	1.850	0.472	0.790	1.928
white hickory	0.760	0.045		1.409

[a] All values given in milligram/gram of fresh tissue. [b] Each reading from pecan represents 12 samples; from walnut, one sample in early season, three in late season; from hickory, one early, two late season.

at least four replications for each data point. The juglone content was low in both the leaf and nut samples taken in June, but increased through July. In August and September, the juglone content of nuts apparently reached a maximum, while that of leaves decreased to near zero. The rapid seasonal buildup of juglone in nuts and leaves could affect the ability of a pecan culture to withstand scab. Even though these preliminary data are too variable to permit conclusions concerning relative levels among cultivars, the apparent early buildup of juglone in leaves and high levels in fruit of a cultivar such as Stuart could be one factor in the resistance phenomonen. This cultivar was considered resistant for many years, but today is susceptible to scab in some locales. Schley, Van Deman, and Success have for many years been considered quite susceptible.

The juglone level in black walnut leaves and nuts was high throughout the year (Table I). White hickory contained an appreciable amount of juglone, particularly in the early season when compared with pecan. *F. effusum* has not been reported on walnut and has been reported only rarely on hickory.

Analyses of single, late-season samples of butternut, water hickory, and hican showed juglone levels close to those of pecan. Establishment of juglone levels among representatives of the Juglandaceae family will require further investigations since only one or a few genotypes of each were studied.

Immature leaves of black walnut sampled on September 24 were found to have a higher level of juglone (1.15 mg/g) than the older, near senescent leaves sampled in August (0.045 mg/g). Most of the mature leaves on the tree had abscissed at the time of the September sampling and new regrowth was appearing.

Juglone is highly toxic to many higher plants (Brooks, 1951) and probably exists in living tissue in a nontoxic form. According to Daglish (1950), the precursor of juglone in walnut is the 5-glucoside of 1,4,5-trihydroxynaphthalene. This is hydrolyzed to α-hydrojuglone and then oxidized either in the air, the soil, or the plant to the active principle, juglone. With careful exclusion of moisture and air, Daglish was able to isolate the glucoside. Since the UV spectrum of α-hydrojuglone and the glucoside differ, he was able to demonstrate a lack of free α-hydrojuglone in walnut.

Thus, we may conclude that the juglone analyses obtained in this work do not necessarily represent free juglone levels in living tissue, but perhaps represent juglone plus any precursors that may have been oxidized to form juglone in the extraction process. Even so, it seems logical to assume that the values obtained would be proportional to available juglone in parasitized tissue. On the other hand, if precursors of free juglone are not readily converted to free juglone during attack by the fungus, this may explain why *F. effusum* can grow on tissue with apparent juglone levels that are consistently higher than those levels (0.05 mg/g) necessary to retard mycelial growth in the laboratory (Langhans et al., 1978). It also seems logical that injuries, whether pathological or mechanical, could influence the availability of the toxin in living tissue as implied by Daglish (1950). It is also possible that there are other antifungal compounds in pecan unrelated to juglone. However extraction of leaf tissue with several solvents failed to reveal any other than that containing juglone, or a glucoside of juglone.

In summary, juglone has antifungal activity and is a major chemical constituent of pecan. Even in susceptible cultivars, the concentration of juglone or its precursor in the tree is, for most of the season, high enough to inhibit the growth of mycelia in the laboratory. The concentration in the nut increases as the season progresses to levels that could account for the observed resistance of some cultivars. If juglone levels can be correlated with resistance to *F. effusum*, it may then be possible to utilize juglone analyses in the selection of scab resistant cultivars of pecan.

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