Histochemical Localization of Juglone and Related Constituents of Pecan

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ABSTRACT

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Potential methods for selectively demonstrating juglone and related compounds in pecan (*Carya illinoensis*) tissue were studied and the most effective method was outlined. Fixation with formaldehyde vapor followed by the Hoepfner-Vorsatz test for phenolics, as modified by Reeve, provided the most reliable color reaction.

Additional key words: *Cladosporium caryigenum*, disease resistance, naphthoquinones, pecan scab.

Juglone, 5-hydroxy-1, 4 naphthoquinone, isolated from pecan (*Carya illinoensis* Koch) and shown to be inhibitory to *Cladosporium caryigenum* (Ell. et Lang) Gottwald, the incitant of pecan scab, is believed to contribute to disease resistance among members of the Juglandaceae (2,4,5). Juglone concentrations show wide fluctuations among species, cultivars, and periods of the growing season, diminishing in leaves and increasing in nuts as the season progresses (2,4).

Previous studies (2–5) on the location and quantity of juglone in leaves or nuts have been based on extractions from whole leaf or nut samples. As an aid in studies of host plant resistance and in breeding programs, we wished to develop a histological/histochemical procedure to determine where juglone and related materials were stored within various organs. To do this, it was necessary to use fresh or very recently frozen tissue, fix the juglone in situ so that it would not stream or wash out of the tissues when subjected to microchemical tests, and find the appropriate microchemical test(s) for selectively staining the juglone. The techniques were predicated on use of the cryostat microtome for obtaining fresh frozen sections.

MATERIALS AND METHODS

Tissue collection, preparation, and sectioning. Leaves and nuts of cultivar Stevens were collected from a pecan orchard at Mississippi State University and transported immediately to the laboratory to be frozen at −20 C in the freezing chamber of the cryostat microtome (International Equipment Co. [IEC], model CTI). Transverse rectangles of leaf tissue, approximately 0.5 × 1.0 cm, that included a midrib or lateral vein were cut from leaves, mounted on specimen holders, and quick frozen in water on the cryobar quick-freeze shelf of the cryostat for 3–5 min.

Nuts were prepared for sectioning by slicing 0.5- to 1.0-cm-thick transverse pieces out of the area to be studied and quick freezing the sections on a specimen holder on the cryobar. Prefrozen nuts were cut for mounting on the tissue holder with a warmed scalpel blade. The first few sections of nuts were discarded to assure that only previously unexposed surfaces were used for histochemical tests. After a section was cut and mounted, the next two or three sections were discarded. Cross sections of entire nuts were routinely made unless their diameters exceeded the width of the slide.

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Two wedge-shaped IEC knives were kept frozen in the cryostat and were removed for sharpening on alternate days. Frequent fine honing of the knives was essential for cutting good sections, especially when alternating between delicate leaves and coarsely textured nuts. A range of cutting temperatures (−15 to −30 C) was evaluated for both kinds of tissue. Leaves and nuts were sectioned at 20 to 30 μm and transferred to a warm (room temperature) slide, without adhesive.

Histochemical procedures. Three fixatives were evaluated for the purpose of preventing juglone from streaming from tissues when histochemical reagents were applied: 37% aqueous (aq) formaldehyde, 1 and 10% aq picric acid, and 1 and 10% aq chromic acid. Sections were mounted on slides and exposed to vapors of the fixative by inverting the slides over fixative solutions in a syracuse dish for 1–5 days. The fixation was carried out in 22.9-cm-diameter petri dishes in a fume hood.

Following preliminary testing of several procedures, three staining programs were chosen for careful study. The most promising of these, the Hoepfner-Vorsatz test as modified by Reeve (6) for phenolic compounds, was initiated by mixing equal volumes of 10% sodium nitrite, 20% urea, and 10% acetic acid. Two drops of this mixture were placed on the fixed section and 2–3 min later, two drops of 2N KOH or NaOH were added (Table 1). The color reactions that occurred are characteristic for various phenolic compounds. According to Reeve (6), the Hoepfner-Vorsatz test reagents first form a nitroso derivative of the phenol, and then a colored salt with the addition of base.

Colorimetric standards were established by adding five drops each of sodium nitrite, urea, and acetic acid to 5.0 ml of 0.01% juglone solution in distilled water, followed in 3 min with five drops of 2N KOH or NaOH. These were compared with the same test applied to solutions of other phenolics likely to be found in pecan tissues, i.e., phenol (0.2% aq), tannic acid (0.1% aq), and condensed tannins (0.1% aq). Anthocyanins extracted with 10% HCl, and anthoxanthins (flavonoids) extracted with 10% KOH from red-, purple-, and white-flowered plants of various species and neutralized with KOH or HCl, respectively, were likewise compared to standard samples. Chlorogenic acid (0.1% aq), although it has not been identified in pecan, was also tested with the Hoepfner-Vorsatz reagents since the color reaction reportedly (1) was very similar to that of juglone.

Because nickel II and copper II salts produce violet reactions with juglone (7), nickel acetate (0.02%) and cupric acetate (7.0%) were tested as histological stains. Both acetates were applied by dropper to fresh cryostat sections to the point of flooding and stained for no less than 24 hr.

TABLE 1. Histochemical test for juglone in pecan tissues

Reagents:

(a) Extractions:	pentane (or hexane)
	chloroform (or chloroform/methanol [2:1] or ethyl acetate)
	acetone
	ethanol
	methanol
	water
	sodium carbonate 1.0% and 6N HCl
(b) Color tests:	$NaNO_2$ 10%
	Urea 20%
	10% acetic acid
	KOH 2M
	nickel acetate 0.02M

Procedure for staining unbound juglone and juglone glucoside:

1. Cut 20 or 30 μm sections on cryostat at −16 to −18 C (20 μm for photos, 30 μm for gross localization).
2. Pick sections up on slide at room temperature and fix over concentrated formaldehyde vapor for 24 hr.
3. Add 1–2 small drops each of $NaNO_2$, urea, and CH_3COOH (dark forest green color evolves for a few moments; also, evolution of nitrous oxide gas is notable).
4. After 2–3 min, add a few drops of 2M KOH to complete the Hoepfner-Vorsatz test. Slide should be laid on a white sheet of filter paper or bibulous paper.

Results: Juglone turns deep red to eventually red-brown.

Procedure for membrane-bound and druse-bound juglones:

1. Cut 20 or 30 μm cryostat sections.
2. Add several sections to a slide by sweeping them off the knife with fine brush.
3. Cover with No. 1 coverslip, about 22 × 22 mm.
4. Place small pieces of absorbent paper at one edge of coverslip to draw off fluids.
5. Place slide on white filter paper under low-power dissecting microscope.
6. Begin adding, dropwise all or some of the extraction solvents to edge of coverslip opposite tissue paper. The fluids will flow rapidly under the coverslip and will clear the specimens.
7. Apply Hoepfner-Vorsatz reagents—2 or 3 drops of each to edge of coverslip: $NaNO_2$, urea, CH_3COOH and lift the tissue paper.
8. Wait 3 min and add a few drops of 2M KOH.

Results: Membranes containing juglone will turn dark red to scarlet in KOH with partial extraction, or clear with total extraction. Juglone in druses will turn dark red (small ovoid or circular druses), will retain red at edges, or will be clear (large druses). No green develops after CH_3COOH.

Procedures for controls:

Negative control

1. Extract tissue as above but also extract finally with 1.0% sodium carbonate, 5 min of continuous exchange.
2. Extract as above, *preceded* by up to 6N HCl.

Result: No reaction in cell walls or druses with complete extraction of juglone and its glucoside.

Positive control

Stain unextracted or extracted tissue for 24 hr with nickel acetate (0.5M).

Result: Stains same areas violet (difficult to detect except with a dissecting microscope).

Procedure for making semi-permanent mounts:

1. Carefully dry area around section.
2. Drop 1 or 2 drops of glycerin jelly onto a coverslip.
3. Invert slide and specimen directly onto drop, reinvert, and adjust coverslip.

Extraction procedures. Extractions were performed on sectioned, unfixed tissue to distinguish between free juglone, cell-wall-bound juglone, and druse-bound juglone. Freshly cut sections mounted on slides were held over a beaker while solvents were sequentially dripped over them. Distinctions were achieved following complete serial extractions of increasing polarity with pentane, chloroform, acetone, ethanol, methanol and water, pre-extraction hydrolysis in HCl, or postextraction treatment with 1% sodium carbonate. According to the postextraction staining properties of the tissues, pentane extraction removed vacuolar but not wall-bound juglone; the wall-bound juglone was removed by the rest of the series, i.e., cell walls would no longer give a positive reaction for juglone, leaving behind only druse-bound juglone which was intensely stained by the reagents. This was removed by pre-extraction hydrolysis in HCl or by postextraction treatment with 1% sodium carbonate after which no stain reaction could be elicited. Thus, with these extraction procedures, the location of wall- and druse-bound juglone may be observed in the absence of free juglone, which in lieu of extraction, masks the former two components.

RESULTS AND DISCUSSION

Sectioning nuts and leaves at 20–30 μm between −16 and −18 C with frequent fine honing of the knife gave satisfactory results. When cutting nuts, it was necessary to move from one area of the knife to another after a few sections, then to remove the knife for fine honing on an automatic sharpener while working with the alternate knife. Temperatures below −20 C usually caused tissue brittleness.

Fixation with concentrated formaldehyde vapor was more suitable than with picric or chromic acid in preventing streaming of juglone. The longer fixation periods produced more brilliant staining. A minimum fixation time of 24 hr was needed to prevent streaming of the juglone from leaf sections after applying the Hoepfner-Vorsatz or other microchemical reagents, although little streaming occurred after 8–16 hr of fixation. Nut tissue contained much more juglone than leaf tissue during the season this work was done; thus, longer periods (48 hr) were chosen to assure complete fixation.

Juglone could be successfully demonstrated in various tissues following fixation with chromic acid. However, even after 48 hr of fixation, some juglone was observed to stream out of the tissue and into the surrounding medium. Thus, we concluded that inadequate fixation had occurred.

Picric acid fume fixation for 24 hr produced a much less brilliant staining picture than did chromic acid fixation, and much of the juglone was unfixed, streaming out of the tissue as the Hoepfner-Vorsatz reagents were applied. Juglone staining was spotty in distribution.

The Hoepfner-Vorsatz reagents produced a bright red to wine red color with juglone. They did not give interfering colors with any of the phenolic compounds tested (including the anthocyanins and anthoxanthins) except chlorogenic acid. The visible light maxima for chlorogenic acid and juglone were 460 and 515 nm, respectively. Condensed tannins and tannic acid turned yellow to yellow-brown, and phenol turned light yellow with the same reagents. Neutralized anthocyanins and anthoxanthins failed to produce a color reaction. Anthoxanthins appeared yellow in basic solutions. Sectioned leaf trichomes and a variable portion of glandular trichomes on the nut husk also appeared yellow in basic solutions with Hoepfner-Vorsatz reagents, suggesting the presence of phenolic compounds other than juglone in these organs.

Juglone appeared in three different forms: (i) free juglone, which readily flowed out of unfixed tissue and appeared to be mainly vacuolar; (ii) wall-bound juglone, which remained bound to cell walls after extraction with pentane; and (iii) druse-bound juglone, which remained bound to druses after serial extraction in pentane, chloroform, acetone, and methanol but was removable by hydrolysis with HCl at 100 C. The alcoholic extract of (ii) was shown to be juglone glucoside, because it cochromatographed with authentic juglone glucoside and yielded juglone and glucose upon hydrolysis in 1N HCl (3). Since druses are massed crystals of calcium oxalate around a central foundation of organic material, the druse-bound material (iii) is believed to contain an unidentified juglone derivative, speculatively, juglone chelated with the salt or bound by an organic constituent.

It should be noted that quinones other than juglone would be expected to react with the Hoepfner-Vorsatz test in a fashion similar to juglone. However, extensive chemical isolation work (3,4) has failed to demonstrate quinones other than juglone and

juglone glucoside in pecan. Moreover, there evidently has not been any finding of other quinones among other members of the Juglandaceae family. However, any study involving the use of this procedure should be accompanied by periodic analyses for phenolics (or quinones) present.

Greatest concentrations of juglone in leaves were located in midribs and lateral vascular bundles. In midribs (Fig. 1 A and B), it was found in the upper and lower epidermis, bound to walls of parenchyma and phloem, and in protoplasts of the sclerified bundle sheath, but not in the bundle sheath cell walls. It was absent from

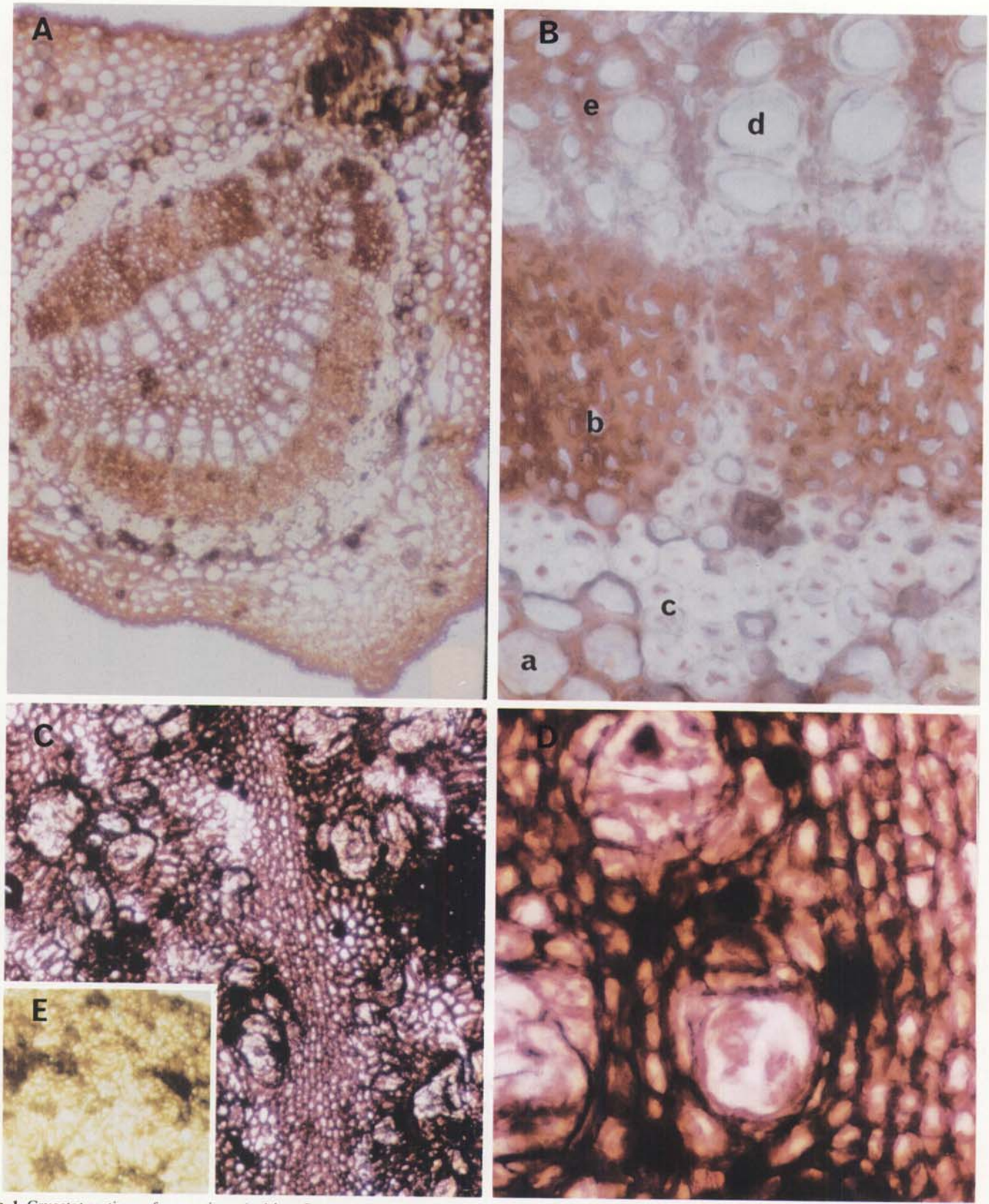

Fig. 1. Cryostat sections of pecan tissue (cultivar Stevens) fixed in formaldehyde vapors and stained with Hoepfner-Vorsatz reagents. **A,** Pecan leaf midrib (×30). **B,** Leaf tissue in close-up. Juglone is prominent in walls of parenchyma (a), phloem (b), and in vacuoles of sclerified bundle sheath, but not the bundle sheath cell walls (c), or in walls of the xylem (d). It is present in intervening fibers (e) (×120). **C,** Husk tissue with juglone in all areas except walls and vacuoles of sclerenchyma cells (×7.5). **D,** Enlarged view of husk tissues showing deeply stained (black) druses (×30). **E,** Control (juglone extracted prior to staining).

xylem walls and vacuoles, but present in intervening fibers. In the leaf blade, juglone was found mainly in the lower epidermis, vascular bundles, and in combination with other substances in the parenchyma. When located in spongy parenchyma, it gave a deeper red stain, suggesting that it was free from interfering substances. Druses of the midrib frequently gave a positive juglone reaction, but those of the blade were clear.

In nuts (Fig. 1C), free and bound juglone was abundant in all areas of the husk except in the numerous sclerenchyma cells distributed throughout the husk. Instead these had lignified cell walls and their vacuoles gave a positive starch reaction (phloroglucinol-HCl and IKI-chlor-zinc iodide tests, respectively). Embryo tissue of the nut was free from juglone. Druses stained very deep red (Fig. 1D), often appearing black because of juglone density, and could be cleared of juglone only after hydrolysis (10% HCl) following extraction with the previously specified solvents, but not by solvents alone. Tissues stained after being cleared of all juglone through extraction and hydrolysis procedures are shown in Fig. 1E.

Nickel or cupric acetates, applied as a stain for 24 hr, may serve for an initial evaluation. However, the blue-violet colors of the acetates are easily confused with natural tissue refringence in light microscopy, and are useful only for observations with incident light at low magnification with a dissecting microscope. In contrast, the Hoepfner-Vorsatz reaction is readily visualized at all magnifications. An added advantage of the Hoepfner-Vorsatz test is that it will color fixed tissue sections even after several weeks of storage under glass. The histochemical schedules for demonstrating juglone in pecan tissues are given in Table 1.

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