Relationship Between Juvenile-Leaf Resistance to Anthracnose and the Presence of Juglone and Hydrojuglone Glucoside in Black Walnut

Steven Cline and Dan Neely

Research assistant, Department of Plant Pathology, University of Illinois, Urbana 61801; and plant pathologist, Illinois Natural History Survey, 172 Natural Resources Building, 607 East Peabody, Champaign 61820.

The authors express appreciation to Paul D. Shaw and Jack D. Paxton, Department of Plant Pathology, University of Illinois, and Claus Grunwald, Section of Botany and Plant Pathology, Illinois Natural History Survey, for granting the use of facilities and for consultation during the course of this work. We also thank Ken Kessler, U.S. Forest Service, Carbondale, IL 62901, for supplying *Cylindrosporium juglandis*.

Accepted for publication 9 August 1983.

ABSTRACT

Cline, S., and Neely, D. 1984. Relationship between juvenile-leaf resistance to anthracnose and the presence of juglone and hydrojuglone glucoside in black walnut. Phytopathology 74:185-188.

The effects of juglone (5-hydroxy-1,4-naphthoquinone) and hydrojuglone glucoside (HJG) on anthracnose (*Gnomonia leptostyla*) development in black walnut (*Juglans nigra*) seedlings were correlated with resistance expressed in juvenile leaves. Anthracnose lesions on ontogenetically immature leaves were smaller and had fewer acervuli in contrast to fully expanded leaves. In shake culture, the effect of juglone on the growth of three leaf spot pathogens of black walnut and three nonpathogens was monitored. Two of three pathogens were more tolerant of juglone than were the nonpathogens. Hydrojuglone glucoside, isolated from walnut husks, stimulated the growth of the anthracnose fungus in shake culture at concentrations up to 750 μg/ml with a reduction in growth observed at 1,000 μg/ml. A high-pressure liquid chromatographic method was developed to determine the juglone and HJG content in walnut leaves. Higher levels of both compounds were observed in juvenile seedling leaves than in mature leaves. The concentration of juglone decreased while the HJG level increased in anthracnose-infected leaves. Seasonal levels of juglone and HJG varied considerably between trees and over time in field-grown trees. Immature leaves had the highest juglone levels over the growing season. We conclude that the pathogenic relationship between the anthracnose fungus and black walnut may be modified by juglone and its precursors in juvenile leaves.

The most destructive foliar disease (anthracnose) of black walnut (*Juglans nigra* L.) in the central United States is caused by *Gnomonia leptostyla* L. (imperfect stage *Marssonina juglandis* (Lib.) Magn.).

At present, resistant cultivars are not available to commercial growers and foresters. Attempts have been made to monitor disease resistance in the field (2) and to select for resistant hybrids of *Juglans* species (3); however, further development has not been pursued. Preliminary investigations on black walnut seedlings have determined that smaller lesions (13) and reduced numbers of fruiting bodies (acervuli) are associated with juvenile leaves. Similar observations have been made on field-grown trees. Reduced lesion development and acervulus formation would be significant to the epidemic spread of this disease. The mechanism of juvenile-leaf resistance is not understood, but it may be important to consider such characteristics in breeding for disease resistance. The search for disease-resistant cultivars would be advanced by techniques of rapid screening for progeny that express resistance to this disease.

A chemical basis for resistance to the pecan scab fungus has been implicated in pecan (8). The toxic principal was identified as juglone (5-hydroxy-1,4-naphthoquinone), a compound endogenous to members of the Juglandaceae. Higher levels of juglone in certain cultivars of pecan were correlated with reduced scab disease in nature (8). Juglone was shown to inhibit fungal growth of the pecan pathogen in vitro at concentrations less than those found in pecan leaves and nuts (11). Walnut is reported to contain free juglone in its leaves and nut hulls (7,8). Significant quantities of juglone have been extracted from walnut leaves and nut hulls in the form of a glucoside (6,16,17). Hydrojuglone glucoside is reportedly present in relatively high concentrations in juvenile and reproductive tissues, and it has been speculated that juglone and hydrojuglone glucoside are involved in disease resistance (5).

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The purpose of this study was to examine the possible role of juglone and hydrojuglone glucoside in juvenile-leaf resistance to the walnut anthracnose fungus in black walnut.

MATERIALS AND METHODS

Plant material and inoculation. Black walnut seedlings were grown in 3.8-L containers under greenhouse conditions. Leaf age was monitored by counting the number of leaves on the main stem upward to the apical tip where a new leaf was emerging from the bud. A juvenile leaf at the tip was considered to be 1 day old. Leaf age was expressed in days from emergence at the apical growing point. The ontogenetic maturity of individual compound leaves was estimated by measuring leaflet expansion directly.

Leaves of six trees with leaf ages of 10–12, 13–16, 17–20, 21–24, and >24 days were inoculated by atomizing conidia (1.5×10^5/ml) of *G. leptostyla* grown on oatmeal agar (14) onto the abaxial leaf surface. Individual leaves were bagged with plastic fastened at the petiole for 48 hr and incubated at 21 C under a 14-hr light period in a growth chamber. Mean lesion diameters for each leaf age were calculated on three leaflet pairs in positions of closest (P_1) to farthest (P_3) from the main stem 15 days after inoculation.

In a separate experiment, acervulus production was evaluated on five trees with leaves of 10–12, 17–20, and >24 days of age that had been inoculated with a conidial suspension, bagged, and incubated at 21 C for 72 hr in a growth chamber. The number of acervuli per lesion on both leaf surfaces of leaflet pairs P_1, P_2, and P_3 on leaves of each age was determined microscopically 15 days after inoculation.

Preparation of hydrojuglone glucoside. Hydrojuglone glucoside (4,8-dihydroxy-1-naphthalenyl-β-D-glucopyranoside) (HJG) was isolated from nut hulls as described by Daglish (6) with minor modifications. Two hundred grams of sliced, green nut hulls were placed in 470 ml of 1% HCl in 95% ethanol; homogenized in a Waring blender; and filtered successively through cheesecloth, cotton, Seitz filters, and Whatman No. 2 filters. To the filtrate was added 142 ml of distilled water, and this solution was adjusted to pH 4.0 with 1 N sodium hydroxide. The extract was applied to a 4×

25-cm column of aluminum oxide (Woelm acid washed). Two to 4 ml of 1 N sulfuric acid were added to the receiver flask, and the column was placed under partial vacuum. HJG was eluted with four 500-ml portions of 60% ethanol. The effluent was flash-evaporated at 40 C to 400 ml, and HJG was extracted from the resultant aqueous phase with four 100-ml portions of ethyl acetate. The ethyl acetate fractions were combined, washed 3× with 100 ml of 4% NaCl, dried over anhydrous sodium sulfate, and the volume was reduced to 50 ml. Upon the addition of 200 ml of hexane to the ethyl acetate extract, a precipitate formed. The supernatant was decanted, and the precipitate was washed with hexane and dried under N_2 gas. About 50 ml of 0.25% stannous chloride, containing two to three drops of concentrated HCl, was added to the precipitate. The mixture was partially dissolved by heating it to 70 C on a hot plate, mixed with activated charcoal with gentle stirring, filtered, and crystallized by cooling at 4 C. HJG was identified by its characteristic UV-absorption spectrum (6). The melting point with decomposition was determined microscopically.

Liquid culture bioassay. Three black walnut leaf spot pathogens (*G. leptostyla, Cylindrosporium juglandis*, and *Cristulariella moricola*) plus three fungi nonpathogenic to black walnut (*Gnomonia quercina*, *G. platani*, and *Sclerotinia sclerotiorum*) were grown in shake culture for 3–5 days at 21 C. Stock cultures were made by seeding potato-dextrose broth (PDB) (200 g peeled potatoes boiled in 1 L distilled water, strained; 20 g dextrose, filtered) with 5-mm-diameter agar plugs of each fungus. *Cylindrosporium juglandis* was grown on Czapek's-Dox media. Juglone (Sigma Chemical Co., St. Louis, MO 63178), 20 mg/ml in chloroform, was placed in sterile 125-ml flasks, and evaporated to dryness under N_2 gas. Sterile distilled water was added to each flask, the flasks were covered and heated for 5 min to solubilize the juglone. Aliquots of juglone and 5 ml of a mycelial suspension of each organism were added to 25 ml of PDB in 50-ml flasks, and the cultures were incubated for 3–5 days at 21 C on a rotary shaker. Fungal growth was determined by weighing the mycelial mats dried at 70 C for 5 days on preweighed filter paper. The pH was recorded before and after growth. The effective dosage of juglone, which resulted in a 50% decrease in dry weight relative to the controls (ED_{50}), was calculated for four or five replications per organism.

TABLE 1. Lesion size and acervulus density of anthracnose infections on different-aged leaves of black walnut seedlings

Leaf age at inoculation (days)	Lesion diameter (mm)[x]	Acervuli per lesion[y]
10–12	1.1 a[z]	17 a
13–16	1.5 ab	
17–20	2.1 bc	26 a
21–24	2.6 cd	
>24	3.3 d	42 b

[x] Mean lesion diameter of the five largest lesions at each of three leaflet positions replicated six times per leaf age 15 days after inoculation.
[y] Values represent the mean of 60 lesions evaluated per leaf age.
[z] Values in columns followed by the same letter do not differ significantly ($P = 0.05$) according to Fisher's least significant difference test.

The effect of HJG on the growth of *G. leptostyla* was examined in shake culture. Shake stock cultures of the anthracnose fungus were grown in PDB as previously described. HJG was added directly to 20 ml of PDB in 50-ml flasks at concentrations of 0, 100, 250, 500, 750, and 1,000 μg/ml. Five milliliters of a mycelial suspension was pipetted into each test flask. Mycelial dry weights were determined following incubation at 21 C for 5 days on a rotary shaker.

Analysis of HJG and juglone in seedling leaves. The concentration of HJG and juglone in seedling black walnut leaves was determined by high-pressure liquid chromatography. Three 1.1-cm-diameter leaf disks were cut from leaflets with a cork borer, placed immediately in 3 ml of redistilled ethanol in a tissue grinder in ice, and homogenized. The extract was filtered through a 0.45-μm Millipore filter and injected directly into an ODS reverse phase column (250 × 5.0 mm). The mobile phase for HJG analysis was 40% methanol in distilled water, while 2% acetic acid in 70% methanol was used for juglone elution. The flow rate was 1 ml/min, and the absorption was monitored at 308 nm for HJG and 420 nm for juglone.

Determinations of HJG and juglone concentrations in anthracnose-infected and uninfected seedling leaves of black walnut were made. The lower surface of leaflets on leaves 12, 21, and 30 days old on 1.5-mo-old seedlings were inoculated by atomizing a conidial suspension (1.8×10^6/ml) of *G. leptostyla*. One leaflet of each pair was inoculated; opposite leaflets were atomized with sterile distilled water. Treated leaves were bagged in plastic for 72 hr and placed in a growth chamber at 21 C with a 14-hr light period. At day 7 following inoculation, single 1.1-cm-diameter leaf disks were collected from each leaflet in positions P_1, P_2, and P_3 and analyzed for HJG and juglone content. Dry weights were determined for leaf disks taken concurrently and dried for 5 days at 70 C.

Seasonal variations in HJG and juglone in field-grown trees. Fluctuations in HJG and juglone concentrations in leaves of field-grown black walnut trees were monitored in 1982 at Urbana, IL. Five trees 8–10 years of age were randomly selected from approximately 80 trees. In April, apical buds on terminal branches were selected at random and marked. Starting on May 12, and at intervals of approximately 2 wk, the third, fourth, and fifth compound leaf in succession was collected from one branch per tree and placed in a sealed plastic bag. From leaflets P_2, P_4, and P_6, single 1.1-cm leaf disks were sampled, pooled, and analyzed by HPLC for the HJG and juglone content.

RESULTS

The compound leaves of black walnuts grown in the greenhouse averaged 32 days of age between bud break and ontogenetic maturity. The leaflet pair closest to the main stem was the first to reach full expansion, followed by the next leaflet pair and sequentially ending with the terminal leaflet.

The smallest lesions were found on the most juvenile leaves, and lesion size increased proportionally with leaf maturity (Table 1). Acervulus production followed a similar trend, with fewer and smaller acervuli observed on progressively more juvenile leaves

TABLE 2. Effect of juglone on the growth of pathogens and nonpathogens of black walnut in shake culture

	Replications	ED_{50} (μg/ml juglone)[a]	pH	
			Initial	Final[b]
Nonpathogen				
Gnomonia quercina	4	0.20–0.40	5.7	5.3
Gnomonia platani	4	0.25–0.50	5.8	5.4
Sclerotinia sclerotiorum	5	0.20–0.30	6.1	2.8
Pathogen				
Cristulariella moricola	4	0.25–0.50	5.5	3.2
Cylindrosporium juglandis	4	1.00–2.00	7.5	7.0
Gnomonia leptostyla	5	4.00–5.00	5.9	5.6

[a] Effective dosage (ED) of juglone where the test fungus grew to 50% of the weight of the control as derived from a growth curve.
[b] Determinations of pH were made 5 days following incubation except for *S. sclerotiorum* and *C. moricola* which were made at 3 days.

(Table 1). Roughly 2.5× more acervuli per lesion were observed on leaves of >24 days of age than were found on the most juvenile leaves.

In general, juglone was more toxic to nonpathogens than to pathogens of black walnut (Table 2). Of the pathogens, *G. leptostyla* was the most tolerant, with an ED_{50} range of 4.0–5.0 μg/ml, while *C. juglandis* followed with a range of 1.0–2.0 μg/ml. *Cristulariella moricola* was as sensitive to juglone as were the nonpathogens whose combined ED_{50} range was between 0.2 and 0.5 μg/ml.

Approximately 132 mg of HJG was isolated from 200 g of green nut hulls. Shake culture bioassays of the effect of HJG on the growth of *G. leptostyla* showed that between concentrations of 0 and 750 μg/ml, fungal growth progressively increased. At 750 μg/ml, the fungal growth exceeded by 233% the dry weight of the control. A slight reduction in dry weight (10%) was observed at 1,000 μg/ml. When fungal growth was abundant, the solutions turned from a light yellow to a dark green during the 5 days of incubation. At that time, no HJG could be detected in ethyl acetate extracts of the culture filtrates.

Tissue samples extracted for HJG and juglone in 1% ethanolic HCl, whether stored at room temperature or at −10 C, showed a significant loss of HJG over a 48- to 72-hr period. While storage at low temperature reduced this loss, HJG was relatively stable only in nonacidified ethanolic extracts. Juglone was affected less by acidification and storage in ethanolic extracts, and only small changes in juglone concentration occurred over time.

Levels of HJG and juglone extracted from walnut leaves fluctuated with leaf age (Table 3). In uninfected leaves, the highest concentrations of both compounds occurred in the most juvenile leaves (12 days). Progressively lower concentrations were found in increasingly older leaves. These concentration differences in uninfected leaves were more pronounced for juglone than for HJG. Infected leaves contained 1.4–1.6% juglone and 8.1–11.6% HJG in contrast to 1.8–3.1% and 6.4–7.3%, respectively, in uninfected leaves. The concentration of HJG was consistently higher in infected leaves, while juglone levels were always lower.

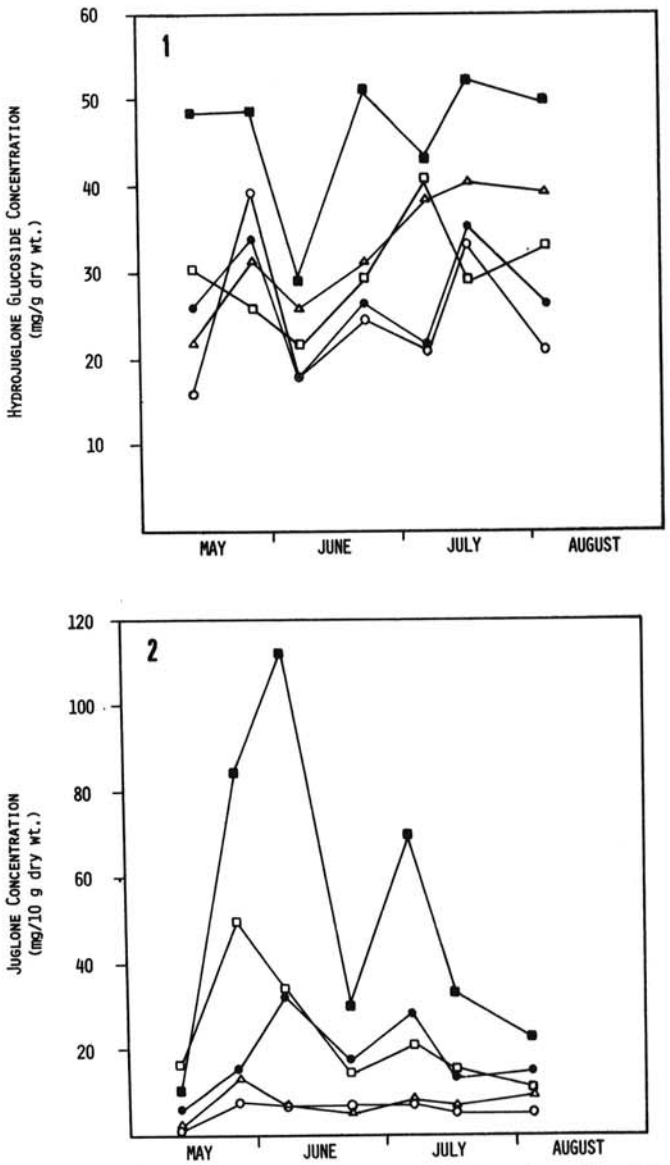
The seasonal fluctuations in the concentrations of HJG and juglone in walnut leaves varied greatly between trees (Figs. 1–2). Juglone levels were lowest at the first evaluation (12 May), 1–2 wk after bud break. When leaves were considered ontogenetically mature, juglone levels had peaked (7 June). A slight rise again occurred at 7 July before declining through the end of the evaluation period. Of the five trees monitored, one (B) consistently produced high juglone levels, two (C and E) produced very low levels, and the remaining two trees (A and D) fell between these values. Levels of juglone averaged between 0.75–3.8 mg/g dry weight from May through August.

HJG concentrations fluctuated similarly throughout the evaluation period. Average peak levels occurred in late May (35.2 mg/g dry weight) just before full leaf expansion and again in late July (38.0 mg/g dry weight). One tree (B) consistently produced high levels of HJG; however, the variation in HJG levels among the other trees was not as consistent as with juglone.

DISCUSSION

In estimating resistant reactions to fungal plant pathogens, it is common to consider the number of reproductive structures and measure lesion size. Reduced lesion growth and acervulus formation of walnut anthracnose associated with more juvenile leaves has previously been observed (13); however, leaf age was not quantitated. The mechanism of resistance to lesion growth and fruiting body production in juvenile leaves of black walnut is unknown. Histopathological studies (4) did not reveal anatomical limitations to explain these effects.

Juglone, which is produced by many, if not all, members of the



Figs. 1–2. Seasonal fluctuations in levels of **1,** hydrojuglone glucoside and **2,** juglone in five field-grown black walnut trees. Each point represents an average of three analyses per tree (trees: A = □, B = ■, C = o, D = •, and E = △).

TABLE 3. Comparison of hydrojuglone glucoside (HJG) and juglone levels relative to leaf age in anthracnose-infected and uninfected leaves of black walnut seedlings

Leaf age at inoculation (days)	Uninfected		Infected[z]	
	HJG (mg/g dry wt)	Juglone (mg/g dry wt)	HJG (mg/g dry wt)	Juglone (mg/g dry wt)
12	73.4 a	31.4 a	116.1 a	14.7 a
21	70.2 a	28.6 ab	106.0 b	16. 0a
30	63.9 s	18.4 b	80.9 c	14.3 a
Mean	69.2	26.1	101.0	15.0

[z] Each value represents an average of four replications. Values in columns followed by the same letter do not differ significantly ($P = 0.05$) according to Fisher's least significant difference test.

walnut family, has been suggested to be involved in disease and decay resistance (5,7,8,11). Nonpathogens used in this study were generally more sensitive to juglone than the pathogens of black walnut, indicating a possible adaptative immunity to potentially harmful levels in leaves. From in vitro juglone toxicity tests in shake culture, the pecan scab fungus appears to tolerate higher levels of juglone than the walnut pathogens used in the present study. Juglone levels in pecan were found to be higher than in walnut (7). Pathogens may be less sensitive than nonpathogens to endogenous toxic compounds produced by their hosts (1). In this study, a major exception to the trend of juglone tolerance was found with *C. moricola* which, although it is reported to be a pathogen of black walnut, exhibited juglone sensitivity equal to that of the nonpathogens. *Cristularielle moricola* and *S. sclerotiorum* are thought to be closely related (M. N. Cline, J. L. Crane, and S. D. Cline, *unpublished*) and are known producers of oxalic acid (10,15). Acid production was evident by the extreme drop of pH in shake culture. The role of oxalic acid formation by *S. sclerotiorum* and its relationship in the infection process has been postulated (15). The tolerance to juglone exhibited by *C. moricola* may be related to alterations of the host tissue through oxalic acid production during growth. While *S. sclerotiorum* is not a reported pathogen of black walnut, mycelial and ascospore inoculations of greenhouse-grown seedlings produced infection during preliminary tests. Thus, for these two fungi the predisposition of host tissue by acid production may be more important in establishing infection of black walnut than a tolerance to juglone.

Problems in repeating the reported HJG isolation procedure from walnut hulls (6) warranted using additional cleanup steps. Alternative procedures (16) have since proven more consistent. While some in vitro toxicity of HJG against selected pathogens has been indicated in other studies (7,18), the fungicidal activity of this compound against the branch wilt pathogen of Persian walnut (18) was relatively low. Most preformed inhibitors are present in plant tissues as glycosides, and the low biological activity of such compounds is not unusual (20). The antibiotic potential of secondary products, however, is difficult to assess in vitro. A pathogen growth response to certain substances may change in the presence of other growth-regulating compounds in the media (9,21).

Daglish (5) first hypothesized that HJG, upon oxidation due to wounding or invasion by an organism, would generate juglone that could serve as a protective agent against infection. Although the presence of free juglone in walnut tissues has been questioned, recent evidence (7,16) supports its occurrence. Elevated juglone levels in juvenile leaves of seedlings as determined in this study may influence fungal development and contribute to the observed reductions in growth and acervulus formation by the anthracnose fungus. High levels of juglone in juvenile leaves formed late in the season of field-grown black walnut have been reported (8).

The mechanism influencing concentration differences of HJG and juglone between infected and uninfected leaves is unknown. The increased concentration of HJG and reduced levels of juglone in infected leaves indicates a host-pathogen interaction resulting in a disruption of the biosynthesis of juglone from its precursor. This is consistent with the proposed metabolic pathway described for the synthesis of juglone and HJG (17). Reduced juglone levels may enable more rapid fungal development and ensure pathogenic success. Hypothetically, the major obstacle to growth would, therefore, be the amount of preformed juglone initially present in the leaf tissue, and it has been shown here that these levels change with leaf age. In other studies (7), differences in juglone concentrations between infected and uninfected leaves were not found. Detection methodology, infection severity, and leaf age may alter the interpretation of the effect of juglone upon the infection process and development of a pathogen.

The highest concentrations of juglone found in field-grown black walnut on an individual tree basis were observed while leaves were still immature. These levels are in reasonable agreement with those found in other studies (12,19), but are much lower than levels observed in greenhouse-grown seedlings. The same trend resulted for HJG. Soil fertility and growth conditions can influence juglone and HJG production in seedlings (S. Cline, *unpublished*) and may also contribute to the oscillation of juglone and HJG levels in field-grown trees.

Factors other than concentration may be involved in roles for juglone and HJG in pathogenesis. Enzymatic activity, host or pathogen inhibitors, and anatomical restrictions may complicate the relationship between endogenous inhibitors and the pathogenic process.

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