DISPARATE MODES OF ACTION OF JUGLONE IN THE RESPIRATORY SYSTEMS OF SENSITIVE AND RESISTANT PLANTS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Plants in the family Juglandaceae, especially Juglans nigra L., have long been known to have detrimental effects on certain other plants. This effect is supposedly caused by juglone (5-hydroxy-1,4-naphthoquinone), but reasons why some plants are sensitive while others appear resistant are not known. The experiments described herein were designed to determine if there are disparate modes of action of juglone in the respiratory systems of sensitive and resistant plants.

LITERATURE REVIEW

Allelopathy

The term commonly used to refer to biochemical interactions between plants is allelopathy. The effects of allelopathy are caused by chemical compounds (allelochemicals) being released into the environment by a plant (Rice, 1979). Allelopathy influences man-made as well as natural ecosystems, and is an increasingly important concept since the use of minimum tillage and stubble crop production techniques are becoming more widely employed. The influence of weeds and crop plants on other crop plants, the possibility of using allelopathy in biological weed control, and other aspects of allelopathy in agronomy and forestry have been reviewed extensively (Fisher, 1977; Horsley, 1977; Rice, 1979).

Juglone and other allelochemicals are not involved in the primary reactions of plant metabolism, and therefore are con-

sidered to be secondary plant products. Secondary plant products aregenerally believed to contribute to the survival of the producing organism (Luckner, 1972). The color andaroma of flowers attract insects to help insure reproduction, while irritants, toxins, and unpalatable characteristics help defend plants against competing species and predators (Bell, 1980). The release of juglone from walnut trees insures the survival of the trees by regulating surrounding vegetation (Brooks, 1951).

Toxicity of Juglone to Other Plants

Juglone is one of the oldest and best established phytotoxins. Pliny the Elder is accredited with the first recorded observation of the toxicity of walnut trees in 37 A.D. (Sonderquist, 1973). From his observations, Pliny concluded that all plants touched by the shadow of the walnut tree would be poisoned. Daglish (1950a) reported that juglone was first isolated from the pericarp of walnut fruit in 1856 by Vogel and Reischauer, who named the yellow crystalline compound nucin. According to Fisher (1978), the first phytotoxic effects of juglone were reported by Stickney and Hoy in 1881. Cook (1921) observed that tomato and potato plants growing under a walnut tree wilted and died within a few weeks. He concluded that there must be a toxic material exuded from the roots of the tree. Massey (1925) noted the wilted and stunted growth of alfalfa and potato plants under walnut trees, and reasoned that direct contact between the roots of the walnut and other plants

caused toxicity. Davis (1928) determined that the toxic nature of walnuts was caused by juglone. He isolated the toxin from Juglans nigra L. and found it chemically identical to synthesized juglone. He then sprayed plants with the synthetic juglone and obtained results similar to what Cook (1921) and Massey (1925) observed with naturally occurring juglone.

Many people began to refute the claims that walnut trees were toxic to other plants by presenting evidence to the contrary. Green (1929) reviewed claims supporting and denying the toxicity of black walnut, and concluded that the symptoms of toxicity were due to competition between the plants and not because of a poison released by the walnut. MacDaniels and Muenscher (1941) explained the contradictory evidence of the toxicity of black walnut by suggesting that juglone is rapidly broken down in the soil and a synergic reaction with another substance may be necessary to produce the antagonism. Brown (1943) also reviewed the controversy and concluded that the roots of walnut trees are toxic when in contact with the roots of other plants. This concept became widely accepted, and the need to understand the physiological actions of juglone became apparent (Gries, 1943).

Strong (1944) reported a list of plants he observed to be resistant or susceptible to injury under black walnut trees. He also found that after cutting down a black walnut tree, the roots in the soil were still capable of injuring susceptible plants. Brooks (1951) made an extensive twelve-year study of all plants growing within a sixty-foot radius of 300 black

walnut trees. He listed 218 species apparently susceptible to juglone, and he found a few species that actually grew better within the root zone of the walnut than outside it. A compilation of plants sensitive and resistant to juglone appears in Table 1.

MacDaniels and Pinnow (1976) reported that research at the Dow Chemical Company determined that juglone is not produced in greenhouse studies because glass filters out ultra-violet light, which is apparently necessary for the synthesis of juglone. They also suggested that juglone affects plants by reducing the absorbing function of roots, or by injuring root hairs and other root absorbing surfaces. Further work by MacDaniels (1981), and MacDaniels and Pratt (1983) showed that tomato plants growing near walnut trees developed tyloses in the xylem. This may be an explanation of the wilting behavior of tomato plants in the presence of juglone.

Work done by Fisher (1978) revealed that the antagonistic effect between black walnut trees and pine trees was dependent upon the soil moisture conditions. On a fine sandy-loam soil, the inhibitory activity of juglone disappeared under a dry moisture regime, but persisted under a wet moisture regime. This effect was also observed when black alder (Alnus glutinosa (L.) Gaertn.) trees were planted as nurse species in a black walnut plantation (Reitveld et al., 1983). Black alder and other nitrogen-fixing trees and shrubs are used to promote walnut growth, but juglone has been shown to inhibit the growth of some symbiotic, nitrogen-fixing soil microorganisms (Dawson

Table 1-A. Partial list of plants susceptible to juglone.

Family Species	Common Name	Reference
Compositae		
Chrysanthemum sp. L.	Chrysanthemum	Strong, 1944
Cyperaceae		
Carex sp. L.	Sedge	Brooks, 1951; Strong, 1944
Ericaceae		
Gaylussacia sp. HBK	Huckleberries	Brooks, 1951
Kalmia latfolia L.	Mountain Laurel	Brooks, 1951
Rhododendron sp. L.	Rhododendron, Azalea	Brooks, 1951; Pirone, 1938
Vaccinium sp. L.	Blueberry	Brooks, 1951
Gramineae		
Danthonia spicata (L.)Beauv.	Poverty Grass	Brooks, 1951; Smith, 1942
Leguminosae		
Medicago sativa L.	Alfalfa	Brooks, 1951; Massey, 1925
Liliaceae		
Asparagus officinalis L.	Asparagus	Strong, 1944
Oleaceae		
Syringa sp. L.	Lilac	Strong, 1944
Paeoniaceae		
Paeonia sp. L.	Peony	MacDaniels & Pinnow, 1976
Pinaceae		
Picea sp. A.Dietr.	Spruce	Fisher, 1977
Pinus resinosa Ait.	Red Pine	Fisher, 1977; Brooks, 1951
Pinus strobus L.	White Pine	Fisher, 1977; Perry, 1932

Table 1-A. (cont.)

Family Species	Common Name	Reference
Rosaceae		
Malus sp. Mill.	Apple	Brooks, 1951; Schneiderhan, 1927
Potentilla fruticosa L.	Shrubby Cinquefoil	James and Morse, 1902
Prunus avium (L.)L.	Sweet Cherry	Strong, 1944
Rubus sp. L.	Blackberry	Brooks, 1951
Saxifragaceae		
Hydrangea sp. L.	Hydrangea	Strong,1944
Solanaceae		
Lycopersicon esculentum L.	Tomato	MacDaniels & Pratt, 1983; MacDaniels, 1981; Perry, 1967; Brooks, 1951; Strong, 1944; Cook, 1921
Solanum tuberosum L.	Potato*	Brooks, 1951; Massey, 1925; Cook, 1921

* Potato was more susceptible to juglone toxicity than pepper, but less susceptible than tomato.

Table 1-B. Partial list of plants resistant to juglone.

Family Species	Common Name	Reference
Amaryllidaceae		
Allium sp. L.	Onion	Strong, 1944
Asclepiadaceae		
Asclepias syriaca L.	Common Milkweed	Brooks, 1951
Caprifoliaceae		
Lonicera tatarica L.	Tatarian Honeysuckle	Strong, 1944
Chenopodiaceae		
Beta vulgaris L.	Beet	Strong, 1944
Compositae		
Achilla millefolium L.	Yarrow	Brooks, 1951
Ambrosia sp. L.	Ragweed	Brooks, 1951
Aster sp. L.	Aster	Brooks, 1951
Bidens sp. L.	Beggar-ticks	Brooks, 1951
Chrysanthemum leucanthemum L.	Ox-eye Daisy	Brooks, 1951
Cirsium sp. Mill.	Thistle	Brooks, 1951
Erigeron annus L.	Daisy Fleabane	Brooks, 1951
Senecio aureus L.	Golden Ragwort	Brooks, 1951
Solidago sp. L.	Goldenrod	Brooks, 1951
Taraxacum officinale Wiggers	Dandelion	Brooks, 1951
Veronia sp. Schreb.	Ironweed	Brooks, 1951
Zinnia elegans Jacq.	Zinnia	Strong, 1944
Cruciferae		
Brassica oleraceae L.	Cauliflower, Cabbage	Strong, 1944
Equisetaceae		
Equisetum arvense L.	Common Horsetail	Brooks, 1951
Graminae		
Poa pratensis L.	Kentucky Bluegrass	Brooks, 1951
Zea mays L.	Corn	Strong, 1944
Labiatae		
Prunella vulgaris L.	Heal-all	Brooks, 1951

Table 1-B. (cont.)

Family Species	Common Name	Reference
Leguminosae		
Cercis canadensis L.	Redbud	Strong, 1944
Phaseolus vulgaris L.	Bean	Wang, 1971; Perry, 1967; Strong, 1944
Pisum sativum L.	Pea	Strong, 1944
Trifolium repens L.	White Clover	Brooks, 1951
Liliaceae		
Tulipa sp. L.	Tulip	Strong, 1944
Lobeliaceae		
Lobelia inflata L.	Indian Tobacco	Brooks, 1951
Lycopodiaceae		
Lycopodium complanatum L.	Ground Pine	Brooks, 1951
Lythraceae		
Cuphea petiola Jacq.	Clammy Cuphea	Brooks, 1951
Ophioglossaceae		
Botrychium dissectum K.Spreng	Grape Fern	Brooks, 1951
Oxlidaceae		
Oxalis sp. L.	Wood Sorrel	Brooks, 1951
Polemoniaceae		
Phlox sp. L.	Phlox	Strong, 1944
Polypodiaceae		
Asplenium platyneuron (L.)Oakes	Ebony Spleenwort	Brooks, 1951
Polystichum acrostichoides (Michx.) Schott	Christmas Fern	Brooks, 1951
Rosaceae		
Cydonia oblonga Mill.	Quince	Brooks, 1951
Geum sp. L.	Avens	Brooks, 1951
Prunus persica (L.)Batsch	Peach	Brooks, 1951
Prunus sp. L.	Cherry, Plum	Brooks, 1951
Pyrus sp. L.	Pear	Brooks, 1951
Rubus occidentalis L.	Black Raspberry	Brooks, 1951

Table 1-B. (cont.)

Family Species	Common Name	Reference
Rubiaceae		
Galium sp. L.	Bedstraw	Brooks, 1951
Solanaceae		
Capsicum annum L.	Pepper	MacDaniels & Pratt, 1983
Solanum tuberosum L.	Potato*	MacDaniels & Pratt, 1983 MacDaniels, 1981
Umbelliferae		
Daucus carrota L.	Carrot	Brooks, 1951; Strong, 1944
Petroselinum crispum (Mill.) Nyman	Parsley	Strong, 1944
Violaceae		
Viola pedunculata Torr. & A.Gray	Pansy	Strong, 1944
Viola sp. L.	Violet	Brooks, 1951

* Potato was more susceptible to juglone toxicity than pepper, but less susceptible than tomato.

and Seymour, 1983). Concentrations of 10^{-3}M and 10^{-4}M juglone inhibited both Frankia ArI3 and Rhizobium japonicum.

While investigating causes of graft failure, Prataviera et al.(1983) found that xylem exudate from grafts of Juglans regia L. shoots on J. hindsii L. rootstocks inhibited the growth of callus tissue, which resulted in a poor take between the scion and the stock. Investigations of the xylem exudate revealed the presence of juglone, which proved to be the cause of the poor callus growth.

Chemistry of Juglone

The compound actually found in walnut trees is a glucoside of 1,4,5-trihydroxynaphthalene, commonly called hydrojuglone (Daglish, 1950a). This compound is colorless, non-toxic, and easily oxidized to juglone upon exposure to the air or an oxidizing substance from the roots of other plants (Gries, 1943). The biosynthesis of juglone and its precursors is a complex process involving a mixed shikimate-citrate pathway (Bently, 1975).

Juglone ($C_{10}H_6O_3$) is the common name of 5-hydroxy-1,4-naphthoquinone. Juglone occurs as orange-yellow needle shaped crystals with a melting point of 155°C and a maximum absorption at 420 nm in methanol. The crystals are freely soluble in chloroform and benzene, somewhat soluble in alcohol and ether, and slightly soluble in hot water (Windholz et al., 1976).

Occurrence of Juglone

The concentration of juglone in various members of the family Juglandaceae changes with cultivar and time during the growing season. Juglone in the leaves tends to be translocated to the hulls during fruit development, while the quanity of juglone in the roots remains relatively high throughout the season (Lee and Campbell, 1969; Hedin et al., 1979). Juglone levels in walnut trees are higher than in hickory and pecan trees (Graves et al., 1979; Hedin et al., 1979), and there are significant differences between the cultivars of walnut trees (Lee and Campbell, 1969). There is also considerable variation in juglone content among hickories and pecans. Borazani et al.(1983) found significant differences in juglone levels of leaves and hulls in fourteen pecan cultivars, and compared these levels to eleven hickory and two hican (interspecific cross between hickory and pecan) trees. They found four hickory and one hican with juglone levels in the leaves higher than the average level of the fourteen pecan cultivars. Only two hickories (both of the same species) had juglone levels in the hulls greater than the average juglone level in the hulls of the pecan cultivars.

Because juglone exists as hydrojuglone in the plant, and because juglone toxicity is controlled by many factors other than concentration alone (MacDaniels and Pinnow, 1976; MacDaniels, 1981), Coder (1983) has suggested using the term juglone potential when refering to walnut toxicity. Juglone-potential would be defined as the total concentration of juglone

under an oxidative environment.

Biological Activity of Juglone

Effect of juglone on respiration. Each component of the respiratory chain is capable of rapid and reversible oxidation-reduction reactions. This makes possible the transfer of electrons from reduced nicotinamide adenine dinucleotide (NADH) to molecular oxygen (O_2) through a system of mitochondrial enzymes. The flow of electrons provides the energy for converting adenosine diphosphate (ADP) to adenosine triphosphate (ATP).

The effects of quinones on the electron transport chain are various (Wolstenholm and O'Connor, 1961; Webb, 1966). Because of this variability, Perry (1967) thought juglone might have different effects on respiration in sensitive and resistant plants. He used tomato (juglone-sensitive) and bean (juglone-resistant) leaf disks in juglone solutions ranging from $5X10^{-7}M$ to $3X10^{-4}M$. From his results he concluded that juglone had equal effects on both of these plants. Therefore, he reasoned that the effect of juglone on respiration could not be the cause of the differing sensitivities of tomato and bean plants to juglone.

Wang (1970) also used tomato and bean leaf disks to determine the effect of juglone on respiration. Using a juglone concentration of 10 ug/ml, he found respiration was inhibited in tomato but not in bean. He reasoned that the increased respiratory quotient of tomato in the presence of juglone could

cause reduced growth and eventually death.

Using beef heart mitochondria, Ruzicka and Crane (1970a & b) investigated the interaction of quinones and the enzyme NADH dehydrogenase. They found that juglone could accept electrons from NADH dehydrogenase, and that this reaction takes place at a unique site in the enzyme complex.

Koeppe (1972) used isolated mitochondria from corn roots to study the effects of juglone on respiration and found that his observations corresponded closely to the results of Perry (1967). He suggested that juglone at low levels (3 uM and below causes electrons to bypass some of the early flavoprotein steps and return to the electron transport pathway at or before the cytochrome b complex. At juglone concentrations greater than 3 uM, respiratory inhibitors were less effective. Koeppe interpreted this result to mean that juglone induced an increased flow of electrons directly to O_2 via an alternate pathway.

Hassan and Fridovich (1979) used Escherichia coli to screen for compounds capable of entering cells, accepting electrons from one of the electron carriers, and diverting these electrons to the production of O_2^- and H_2O_2. The diversion of electrons would cause increased cyanide resistance in respiration. They found that juglone was one of a number of compounds that behaved in this manner.

Toxicity of juglone to fungi. Besides being toxic to certain plants, juglone is also toxic to fungi and bacteria. Juglone was found to be as toxic as Bordeaux mixture when used

as a spray for control of black spot on roses (Gries, 1943). Daglish (1950b) found high concentrations of juglone precursors in the reproductive structures of walnut and suggested these compounds were present to protect the reproductive process by preventing bacterial or fungal growth. Ahmad et al.(1973) found that Juglans regia L. bark had a selective fungicidal action against some dermatophytes, and in a few cases was more effective than currently established antimycotic agents.

While looking for a substance in pecan (Carya illinoensis Koch.) to screen for resistance to pecan scab (Fusicladium effusum Wint.), Langhans et al.(1978) found juglone present in the fungitoxic fractions. The amount of juglone in pecan trees varied greatly, and the trees with more juglone appeared to be more resistant to pecan scab (Hedin et al., 1979). In a subsequent study, Hedin et al.(1980) found that leaves infected by pecan scab did not contain significantly more amounts of juglone than uninfected leaves. Since juglone does not appear to be synthesized in response to infection, Borazjani et al. (1983) investigated the range of variation in juglone levels across several species of pecan to identify individuals with high juglone concentrations. These individuals would be useful in interspecific crosses for resistance to pecan scab.

Toxicity of juglone to other organisms. Juglone toxicity is not limited to certain species of plants and fungi. Gries (1943) reported that when green walnut hulls were thrown in a still pond, the fish were stunned and would rise to the surface where they were easily collected. Westfall et al.(1961) also

reported this phenomenon and determined that the active agent in the walnut hulls was juglone. They tested purified juglone on fish, frogs, rats and rabbits, and found it had a sedative effect on these animals when used in sub-lethal doses.

Juglone has also been found to be a deterrent to feeding by bark beetles (Scolytus multistriatus) on hickory trees (Carya ovata (Mill.) K. Koch) (Gilbert et al., 1967). When juglone was removed from hickory bark extract, the beetles fed extensively on the rest of the extract. Extensive feeding was also observed on extracts from elm bark (Ulmus americana L.), but when elm bark extracts were mixed with hickory bark extracts, feeding was deterred.

True and Lowe (1978) reported that black walnut wood chips and sawdust had toxic effects on racehorses when this material was used as bedding in the horses' stalls. Other cases of walnut toxicity to horses were reported by MacDaniels (1983), who concluded that the effects may in fact be due to juglone precursors.

The effects of juglone on humans are not fully understood. Apparently, green walnut hulls have been used to treat ringworm, eczema, psoriasis, and impetigo (Gries, 1943), and juglone in walnut pericarps was effective in the treatment of Trichophytiasis (Ikekawa, et al., 1967). Walnut bark was used to treat herpes, scrofula, ulcers and tarter of gums (Ahmad et al., 1978). However, when applied to the skin of mice, juglone is carcinogenic (VanDuuren et al., 1978). Funk and

Williams (1979) reported two cases of an apparent allergic reaction to juglone, causing rashes with extensive itching and blistering similar to poison ivy. High air temperatures and high humidity seemed to irritate the condition, which lasted about five days. The blisters caused some scaring and skin peeling, and in one case, the victim experienced a reoccurrence of the rash while cracking cured black walnuts. MacDaniels (1983) also commented on the allergic reaction of humans to black walnut. In this case the symptoms were apparently caused by the air-born pollen rather than by direct contact with a walnut tree.

MATERIALS AND METHODS

Seeds of Lycopersicon esculentum Mill. cv. Marglobe were germinated on moist cheesecloth in a Manglesdorf germinator and were grown for 10 days at 27°C in the dark. Cotyledons and any remaining seed coats were discarded before these chlorophyll-free, etiolated seedlings were used. In contrast, seeds of Phaseolus vulgaris L. cv. Oregon 1604B were germinated and grown in coarse, wet sand in a growth chamber with 91.8 uE/sec m^2 * of incident light at plant level from cool white fluorescent tubes for 16 hr/day at 27°/18° C (day/night). When the bean plants reached the three true-leaf stage, they were pulled up, and only the roots were used in the experiments. Any nodulated roots were discarded. The plants were removed from the growing conditions and held in beakers of distilled water not more than an hour before they were used. The bean roots were excised from the upper part of the plant with a razor blade immediately before use.

Respiration Analysis

Respiration rates were estimated by monitoring oxygen uptake manometrically and polarographically. The plant tissue was cut into 5 mm pieces with a razor blade and placed in buffered (50 mM potassium phosphate, pH 6.7) solutions of

* Actual measurement was in W/m^2. Conversion to uE/sec m^2 was accomplished using the flux ratio table of McCree (1972).

juglone, juglone + nordihydroguaiaretic acid (NDGA), juglone + thenolytrifluoroacetone (TTFA), or juglone + potassium cyanide (KCN).

Juglone concentrations of $3X10^{-4}M$ were determined to cause nearly 70% inhibition of respiration in tomato and bean leaf disks (Perry, 1967). To insure that respiration would be inhibited in both species in this experiment, a $10^{-3}M$ juglone solution was used. The juglone solution was prepared by first dissolving juglone crystals (97% minimum purity; Pfaltz & Bauer, Inc., Stamford, CN) in 5 ml of 95% ethanol, and then diluting this to 100 ml. NDGA and TTFA were also dissolved in ethanol, but stock solutions were not prepared since different concentrations were used in each oxygen uptake method. Control solutions contained the same amount of ethanol used to dissolve each chemical.

Manometric measurement of oxygen. Basic manometric procedures were followed (Umbriet et al., 1957) using a Warburg apparatus (Precision Scientific Co., Chicago, IL). The manometers and flasks were calibrated by the bicarbonate method described by Umbriet et al.(1957). Each respirometer flask contained a final assay volume of 2.0 ml. The assay mixture in each reaction flask contained a final concentration of 50 mM potassium phosphate buffer, pH 6.7, 0.1 mM juglone (when present), 0.2 g roots, and respiratory inhibitors when appropriate. Thermobarometer flasks held the same contents as the corresponding reaction flask except that 0.2 ml water replaced the plant tissue. In flasks containing KCN, the

centerwell held 0.2 ml of 0.5 M CaCN + 10% CaOH (v/v) suspension. This was because HCN formed in the experimental fluid is volatile, and rapidly transferred to the alkaline centerwell solution. If the HCN tension is not counterbalanced in the centerwell, the concentration of cyanide in the experimental fluid may drop rapidly (Robbie, 1948). In all flasks without KCN, the centerwell contained 0.2 ml of 10% NaOH (w/v). Following a 30 min equilibration in a 25°C agitating water bath, each manometer was read at constant volume every ten minutes for the next sixty minutes. Each treatment was repeated at least three times. Slopes were calculated from the change in height (to the nearest mm) of Brodie's fluid over time, and results were expressed as percentages of the appropriate controls.

Polarographic measurement of oxygen. Respiration rates were determined using a biological oxygen monitor (Model 53, Yellow Springs Instrument Co. Inc., Yellow Springs, OH). Each reaction vessel contained a final volume of 3.0 ml with a final concentration of 50 mM potassium phosphate buffer, pH 6.7, 0.1 mM juglone when present), 0.3 g roots, and respiratory inhibitors when appropriate. Following a 30 min equilibration at 25°C, the oxygen electrode was inserted into the reaction vessel, and the concentration of oxygen in the solution was recorded over a ten minute period. Each treatment was repeated at least three times. Slopes were calculated from the change in oxygen concentration over time, and results were expressed as percentages of the appropriate controls.

NADH Assay

Bean and tomato roots were grown as previously described, and 0.8 g of each of the plant tissues was immersed in 10^{-3}M juglone or $5X10^{-2}$M KCN for various times. Control treatments consisted of distilled water for the KCN treatment and 4.75% (v/v) ethanol for the juglone treatment. When removed from the soaking solutions, excess liquid was absorbed with paper towels and the plant tissue was added to 2.0 ml of 50 mM cold potassium phosphate buffer (pH 6.7). The plant tissue-buffer mixture was homogenized in a Pyrex pestle-type tissue homogenizer in ice. The homogenates were centrifuged in a refrigerated centrifuge at 10,000 x g for 15 min to sediment cell wall fractions and organelles (Bonner, 1967). The pellets were discarded and the supernatants were kept on ice to retard the oxidation of NADH to NAD^+, and then assayed as soon as possible (within 15 minutes).

To determine NADH concentrations in the tissues, a spectrofluorometer (Model 430, Sequoia-Turner, Mountain View, CA) was used. The spectrofluorometer was zeroed using cold buffer, and fluorescence was recorded at an excitation wavelength of 365 nm and an emission wavelength of 450 nm (Klingenberg, 1974). Each treatment was repeated three times. Standard curves were prepared for each crop by adding 1 ml of a known concentration of NADH to 1 ml of a supernatant that had been previously read. The difference in the fluoresence before and after NADH was added gave the reading for a known concentration of NADH in 2 ml (Figures 1 and 2).

Figure 1. Standard curve for NADH concentration in bean roots which have been immersed in juglone and KCN solutions for various times.

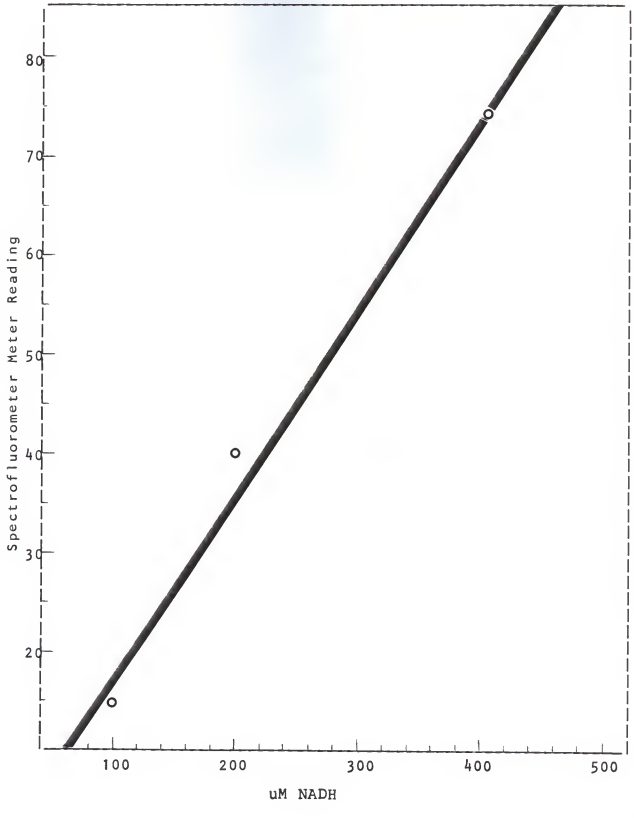
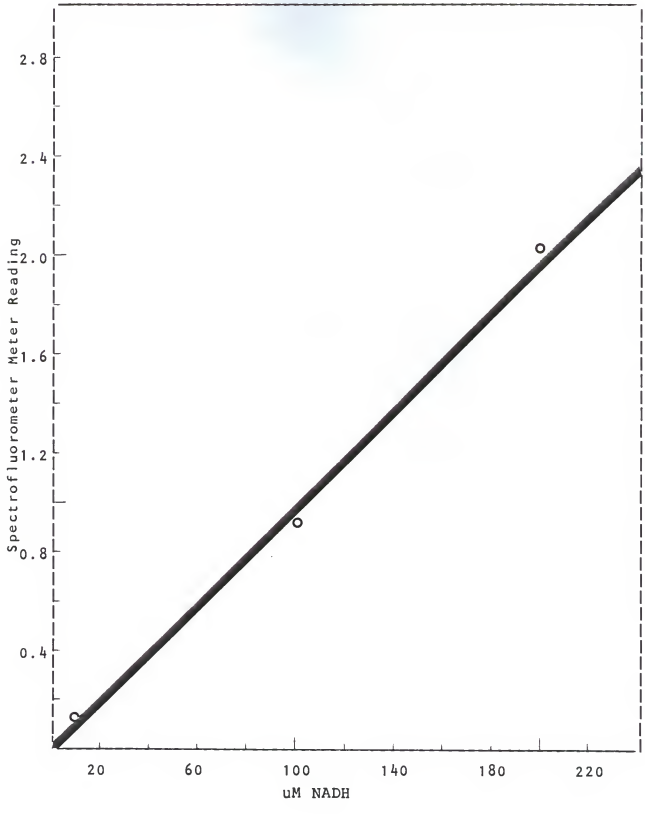


Figure 2. Standard curve for NADH concentration in tomato roots which have been immersed in juglone and KCN solutions for various times.

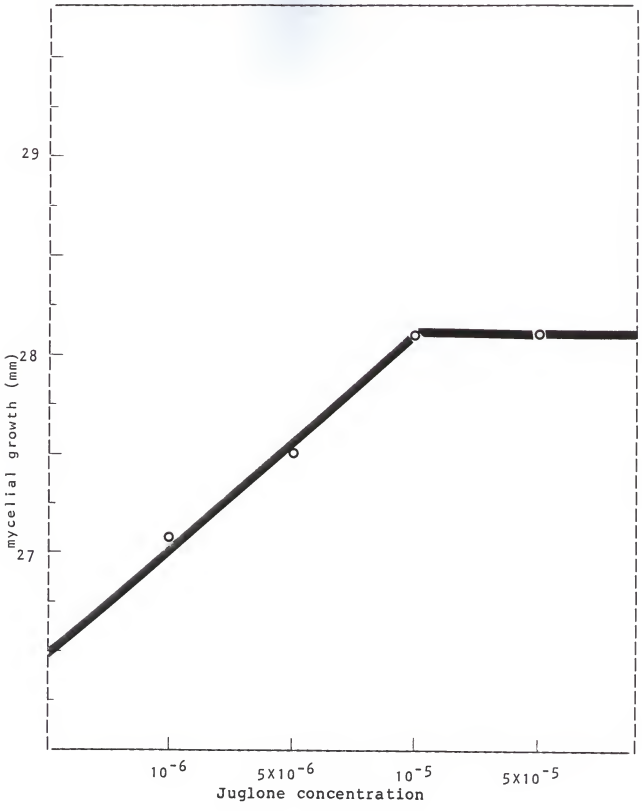


Antifungal Bioassay

Bean and tomato roots were grown as previously described, and 4.0 g of each plant tissue were immersed in juglone solutions of various concentrations for various times. Distilled water was used as a control. The plant tissue was removed from the solutions at the appropriate time, dried on paper towels, then placed in half-strength nutrient solution (Epstein, 1972) until used (1-2 hr). Since juglone has been shown to interfere with plant respiration (Perry, 1967; Koeppe, 1972), the nutrient solution was provided to insure that all elements essential for plant metabolism were avaliable. When removed from the nutrient solution, the plant tissue was ground in a Pyrex pestle-type tissue homogenizer, and the homogenate was centrifuged at 10,000 x g for 15 min to sediment cell wall fragments and organelles (Bonner, 1967). The pellets were discarded and the supernatants were collected and autoclaved for the bioassay. The bioassay was done using an agar well method (Langhans et al., 1978; Ahmad et al., 1973).

A sterile cork borer was used to cut 5 mm diameter wells in potato dextrose agar (PDA) in 100 mm diameter polyethylene Petri plates. Each plate contained 30 ml of agar. For the bioassay the wells were filled with the supernatants to be tested, and for a standard curve (Figure 3) the wells were filled with the various concentrations of autoclaved juglone. The liquid was allowed to evaporate, and the dry wells were then inoculated with 5 mm diameter plugs of mycelia cut from a PDA culture of Aspergillus niger Van Tiegh. This procedure was repeated three

Figure 3. Standard curve for Aspergillus niger mycelial growth in Petri plates containing juglone solutions.



times for each juglone concentration and time period in each species. The plates were then incubated at room temperature for 48 hr. Two measurements (to the nearest millimeter) of radial growth measured at right angles to each other gave the average mycelial growth in each Petri plate. The average mycelial growth for each juglone concentration and time was divided by the average mycelial growth in the control of each species, so results were expressed as percentages of the controls.

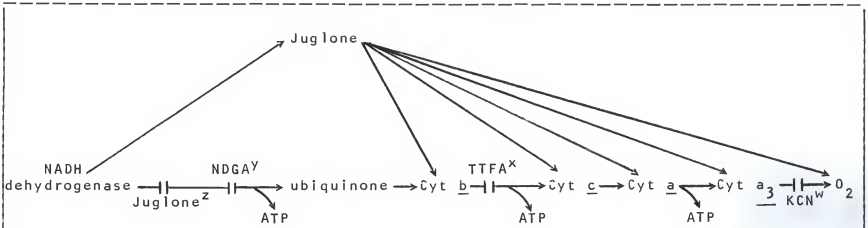
RESULTS AND DISCUSSION

Respiration Analysis

Juglone has been shown to inhibit respiration in some plant species (Perry, 1967; Wang, 1970; Koeppe, 1972), while experiments with other organisms have shown that juglone is capable of accepting electrons from the electron transport chain and diverting them to other compounds (Ruzicka and Crane, 1970 a & b; Hassan and Fridovich, 1979). Depending on redox potentials, artificial electron acceptors can also be electron donors (Klingenberg, 1968), so in this experiment, several known inhibitors of respiration (Figure 4) were used to determine if juglone is capable of passing electrons back to the respiratory chain after the site of juglone inhibition. Inhibitor concentrations causing approximately 50% inhibition were chosen to be used in the experiment (Tables 2 and 3) so that the plants would be sensitive to the presence of the inhibitors, and increases as well as decreases in respiration could be clearly seen. The concentration producing 50% inhibition was determined independently for the manometric and polarographic methods to insure that the plant tissue used in each method would be exposed to the same relative intracellular concentration of inhibitor.

An analysis of variance (Table 4) indicated that there was an overall significant difference at the 5% level between the manometric and polarographic measurement of respiration. However, when the mean method values were separated by crop and

Figure 4. The respiratory chain, showing sites of inhibition and possible electron pathways from juglone.



[z] Site of juglone inhibition as determined by Ruzicka and Crane (1970a & b).
[y] NDGA = nordihydroguaiaretic acid. Site of inhibition determined by Pardini et al.(1970)
[x] TTFA = thenoyltrifluoroacetone. Site of inhibition determined by Streichm and Avidor (1967).
[w] Site of potassium cyanide inhibition determined by Keilin and Hartree (1939).

Table 2. Manometric measurement of the effect of various concentrations of respiratory inhibitors on oxygen uptake in tomato and bean root tissue.[z]

Inhibitor Concentration (M)	Bean (%)			Tomato (%)		
	NDGA[y]	TTFA[x]	KCN	NDGA	TTFA	KCN
1	--	--	44[w]	89	58[w]	53[w]
10^{-1}	54[w]	--	--	160	--	--
10^{-2}	--	26	84	81	--	76
10^{-3}	--	42[w]	--	90[w]	--	168
10^{-4}	--	68	--	--	--	--

[z] Each value represents oxygen uptake as a percentage of the control. Each flask contained 50 mM potassium phosphate buffer, pH 6.7, 0.2 g root tissue, and various concentrations of the indicated inhibitor.
[y] NDGA = nordihydroguaiaretic acid.
[x] TTFA = thenoyltrifluoroacetone.
[w] Values indicating the concentration of each inhibitor used in the respiration analysis for each crop.

Table 3. Polarographic measurement of the effect of various concentrations of respiratory inhibitors on oxygen uptake in bean and tomato root tissues.[z]

Inhibitor Concentration (M)	Bean (%)			Tomato (%)		
	NDGA[y]	TTFA[x]	KCN	NDGA	TTFA	KCN
10^{-1}	35	--	50[w]	261	--	--
$5X10^{-2}$	48[w]	--	--	--	--	--
10^{-2}	72	47[w]	82	196	56[w]	48[w]
10^{-3}	85	106	104	102[w]	69	62
10^{-4}	122	111	122	100	86	73
10^{-5}	--	--	--	118	--	--
10^{-7}	--	--	--	102	--	--
10^{-8}	--	--	--	102	--	--
10^{-10}	--	--	--	122	--	--

[z] Each value represents oxygen uptake as a percentage of the control. Each flask contained 50 mM potassium phosphate buffer, pH 6.7, 0.3 g root tissue, and various concentrations of the indicated inhibitor.
[y] NDGA = nordihydroguaiaretic acid.
[x] TTFA = thenoyltrifluoroacetone.
[w] Values indicating the concentration of each inhibitor used in the respiration analysis for each crop.

Table 4. Analysis of variance of oxygen uptake in bean and tomato roots in the presence of various respiratory inhibitors.

Source of variation	df	SS	MS	F
Crops[z]	1	1.36	1.36	26.70**
Treatments[y]	3	3.54	1.18	23.13**
Methods[x]	1	0.24	0.24	4.64*
Crops X Treatments	3	0.71	0.24	4.62*
Crops X Methods	1	0.03	0.03	0.53
Treatments X Methods	3	0.20	0.07	1.28
Crops X Treatments X Methods	3	0.00	0.00	0.00
Errors	71	3.63	0.05	
Total	86	9.67	0.11	

[z] Crops = bean, tomato.
[y] Treatments = juglone, juglone + NDGA, juglone + TTFA, juglone + KCN.
[x] Methods = manometric, polarographic.
**, * significant at the 1% and 5% level respectively.

treatment, no differences between the methods were apparent at the 5% level (Table 5). Therefore, data from both methods were averaged. Both the manometric and polarographic methods measure oxygen-pressure changes in the reaction vessels. To avoid localized oxygen depletion in the reaction mixtures, the mixtures were constantly stirred during the time oxygen levels were monitored. The stirring process was much more vigorous in the polarographic method than in the manometric method, which may have resulted in more damage to the plant tissues. The differences in tissue damage between the methods may be responsible for the noticable difference in the actual rate of oxygen uptake between the methods (Table 6), but when the values for each treatment and crop are expressed as percentages of the controls, the values are similar to those found in Table 5.

Table 4 also indicated that there were significant overall differences between the means of the crops and between the means of the treatments. The separation of means for these values (using averaged method values) appears in Table 7.

The site of NDGA inhibition is similar to the site of juglone inhibition (Figure 4), and the results in Table 7 indicate that NDGA did not inhibit oxygen uptake in the presence of juglone in either crop. For the bean tissue, these results confirm that the site of juglone inhibition occurs before the site of NDGA inhibition since there was no additional decrease in respiration when NDGA was present. For the tomato tissue, no concentration of NDGA was found to inhibit respiration (Tables 2 and 3), which is again confirmed in Table 7. This result would

Table 5. Comparison of manometric and polarographic measurement of oxygen uptake in bean and tomato tissue.[z]

Treatment	Bean (%)			Tomato (%)		
	M[y]	P[x]	LSD (5%)	M	P	LSD (5%)
Juglone	67	59	20	119	92	42
Juglone + NDGA[w]	63	59	24	108	94	24
Juglone + TTFA[v]	57	61	22	52	44	12
Juglone + KCN	28	42	18	49	57	45

[z] Each value represents oxygen uptake as a percentage of the control. Control treatments contained water and/or ethanol solutions in the same combinations and concentrations used to dissolve the chemicals.
[y] M = manometric method.
[x] P= polarographic method.
[w] NDGA = nordihydroguaiaretic acid.
[v] TTFA = thenoyltrifluoroacetone.

Table 6. Actual rates of oxygen uptake by manometric and polarographic methods in bean and tomato root tissue.

Treatment	Bean (ul O_2/g/min)		Tomato (ul O_2/g/min)	
	M[z]	P[y]	M	P
Juglone	60.07	18.05	35.58	22.49
Control[x]	89.65	30.60	29.90	24.45
Juglone + NDGA[w]	37.55	18.05	44.28	24.16
Control	59.60	30.60	41.00	25.70
Juglone + TTFA[v]	40.50	17.90	17.76	9.68
Control	71.05	29.35	34.15	22.00
Juglone + KCN	24.21	12.85	14.65	13.94
Control	89.65	30.60	29.90	24.45

z M = manometric method.
y P = polarographic method.
x Control treatments contained water and/or ethanol solutions in the same combinations and concentrations used to dissolve the chemicals.
w NDGA = nordihydroguairetic acid.
v TTFA = thenoyltrifluoroacetone.

Table 7. Effect of various respiratory inhibitors on the oxygen uptake in bean and tomato roots.[z]

Treatment	Bean (%)	Tomato (%)	LSD (5%)
Juglone	64	107	27
Juglone + NDGA[y]	60	103	16
Juglone + TTFA[x]	59	49	11
Juglone + KCN	37	53	20
LSD (5%)	14	23	

[z] Each value represents the mean percentage of the control by manometric and polarographic methods for each treatment.
[y] NDGA = nordihydroguaiaretic acid.
[x] TTFA = thenoyltrifluoroacetone.

suggest that juglone does not inhibit respiration in tomato roots at all. Alternatively, the beginning of the respiratory chain may differ in each crop in such a way that NDGA does not inhibit electron transport in tomato roots. Douce et al.(1973) and Moreau (1978) determined that a total of three systems capable of oxidizing NADH existed in plant mitochondria. One system, common to all mitochondria and capable of oxidizing endogenous NADH, is located on the inner surface of the inner membrane. A second system capable of oxidizing exogenous NADH is located on the outer surface of the inner membrane, and a third system, also capable of oxidizing exogenous NADH is located on the outer surface of the outer membrane. Each of these systems contained a different NADH dehydrogenase and had different sensitivities to respiratory inhibitors. In the system with NADH dehydrogenase on the outer surface of the inner membrane, electron transport was not sensitive to inhibitors such as NDGA that act in the area between NADH NADH dehydrogenase and ubiquinone. The system with NADH dehydrogenase on the outer surface of the outer membrane was sensitive to these inhibitors. Therefore, if NADH in bean roots is oxidized by the outer membrane dehydrogenase, and NADH in tomato roots is oxidized by the dehydrogenase on the outer surface of the inner membrane, this would explain why respiration in bean roots is inhibited by NDGA but respiration in tomato roots is not.

When the respiratory chain is inhibited by TTFA (Figure 4), the respiration rate in bean roots is the same as in the juglone

treatment, but in tomato roots the respiration rate is significantly lower than in the juglone treatment (Table 7). The concentration of TTFA used for both crops was chosen to cause a 50% inhibition of respiration. This is apparently happening in the presence of juglone in the tomato roots, but not in the bean roots. This result suggests that juglone is capable of passing electrons back to the respiratory chain, but at different sites for each of the crops. The inhibition of respiration by TTFA in the presence of juglone in tomato roots suggests that electrons are returned to the respiratory chain before the site of TTFA inhibition, while the absence of TTFA inhibition of respiration in bean roots exposed to juglone suggests that electrons are returned to the respiratory chain after the site of TTFA inhibition. Another possible interpretation of this result is that, in bean roots, electrons are returned to the respiratory chain before the site of TTFA inhibition, but the presence of juglone supresses the inhibition caused by TTFA. Results indicating that this type of activity is possible were presented by Ernster et al.(1962). They found that the activity of a flavoprotein in rat liver mitochondria was inhibited by dicoumarol, and that this inhibition could be eliminated by menadione (2-methyl-1,4-naphthoquinone). However, they also found that juglone was not able to eliminate this inhibition, even though it is structurally similar. The work done by Douce et al.(1973) and Moreau (1978) also offers an explanation of this result. They found that when the NADH dehydrogenase on the outer surface of the outer membrane

oxidizes NADH, respiration will not be affected by inhibitors such as TTFA that act in the area between cytochrome b and cytochrome c. When NADH was oxidized by the NADH dehydrogenase on the outer surface of the inner membrane, respiration was sensitive to the inhibitors that act between cytochrome b and cytochrome c. Thus, the results from the TTFA treatment would uphold the view that the respiratory chain in bean roots differs from that in tomato roots.

The concept of juglone passing electrons back to the respiratory chain is further supported by the results of the juglone + KCN treatments. In both crops, the values in the juglone + KCN treatments were approximately 50% of the values in the respective juglone treatments (Table 7). This would be expected since the KCN concentration was chosen to cause a 50% inhibition (Tables 2 and 3), which suggests that juglone was returning electrons to the respiratory chain in both crops before the site of KCN inhibition. Bendall and Bonner (1971) presented evidence that some plant respiratory systems contain an alternate oxidase that is insensitive to cyanide, and that this oxidase seems to be associated with rapidly growing tissues such as etiolated bean hypocotyls, or skunk cabbage spadices. However, the additional inhibition of respiration in the presence of KCN (Table 7) suggests that a cyanide-insensitive pathway was not operating in either crop.

There were significant differences between the respiration rates of bean roots and tomato roots in the juglone and juglone + NDGA treatments, but not in the juglone + TTFA and juglone +

KCN treatments (Table 7). The results suggest that, in the presence of juglone, respiration is inhibited to a greater extent in bean roots than in tomato roots. If this were true, it would apparently contradict the observations that bean plants are resistant to juglone while tomato plants are susceptible (Table 1). However, work done by Bergsma et al.(1982) may offer a possible explanation.

Bergsma et al.(1982) found that membrane vesicles of the menaquinone-deficient Bacillus subtilis aroD could oxidize NADH only at a very low rate. By adding menaquinones and menaquinone analogs (including juglone) oxidation was restored, but with varying levels of efficiency for energy transfer. They concluded that the menaquinones and menaquinone analogs could be grouped into two classes based on each compound's efficiency in energy transfer. Class-1 compounds had a low rate of energy transfer and accepted electrons directly from NADH before the site of cytochrome b in the respiratory chain. Class-2 compounds had a high rate of energy transfer, nearly 10-times that of class-1 compounds, and directly oxidized NADH at a site between cytochrome c and cytochrome a in the respiratory chain.

If the system of Bergsma et al.(1982) were used to classify juglone by the results in Table 5, juglone would appear to be a class-1 compound in tomato roots and a class-2 compound in bean roots. Also, since class-1 compounds are much less efficient in energy transfer than class-2 compounds, this would explain how tomato roots could have a higher respiration rate than bean

roots and still be more susceptible to juglone toxicity than bean roots.

Another possible explanation of the difference in respiration rates for bean and tomato roots in the juglone and juglone + NDGA treatments (Table 7) would be that after juglone accepts electrons from NADH dehydrogenase, a portion of this electron flow was diverted to compounds or processes other than the respiratory chain. Since the respiration rates in tomato roots in the juglone and juglone + NDGA treatments were essentially the same as in the control treatments, juglone must not be diverting any electrons to other compounds or processes. In bean roots however, the respiration rate is about 60% of that in the control, which leaves about 40% of the available electrons that may be diverted to other compounds or processes. Hassan and Fridovich (1979) showed that juglone was capable of this type of activity in Escherichia coli. They were looking for electron-shunting compounds specifically capable of diverting electrons to the production of O_2^- (superoxide radical) and H_2O_2 (hydrogen peroxide), which are both cytotoxic. If juglone is capable of diverting electrons to these cytotoxic products in bean roots, this would again contradict the observations that juglone is not toxic to bean plants. If electrons were diverted in the bean root tissue, they may have gone to non-toxic, non-oxygen utilizing compounds or processes.

When interpreting the results of this experiment the concentrations of respiratory inhibitors used should be considered carefully (Tables 2 & 3). KCN in high concentrations

will form a strong base, and NDGA in high concentrations will form a strong acid. No attempt (other than the buffer solution at pH 6.7) was made to neutralize the pH of these inhibitors. Therefore, the variable pH of the inhibitor solutions may have influenced oxygen uptake in the plant tissues more than the presence of the inhibitors. The pH of the inhibitor solutions should have been monitored and corrected as needed.

NADH Assay

In conventional plant respiration, the respiratory chain receives reducing equivalents from TCA cycle intermediates (succinate and endogenous NADH) as well as from cytoplasmic (exogenous) NADH (Day et al., 1980). Juglone has been known to oxidize NADH dehydrogenase (Ruzicka and Crane, 1970), and may be capable of directly oxidizing NADH (Bergsma et al., 1982). Tomato roots may contain an amount of NADH significantly lower than in bean roots, which may be why tomato plants are more sensitive to juglone than bean plants. A comparison of the concentrations of the various electron carriers in the respiratory chain was done by Lance and Bonner (1968), who found that the pyridine nucleotides (NAD^+ and NADH) were the most variable component in the plants they studied. In the present experiment, the amount of cytoplasmic NADH present in bean and tomato roots was measured to see if there were significant differences between these crops.

The actual concentrations of NADH as determined from the standard curves for bean and tomato tissue (Figures 1 & 2) appear in Table 8. To standardize these values, each treatment result was expressed as a percentage of the initial (zero time) value for each crop. The KCN and juglone treatment values were then expressed as percentages of their respective controls for each crop and time. An analysis of variance of these percentage values appeares in Table 9, and Table 10 shows a separation of means by treatment and time for the percentage values.

Since KCN inhibits the flow of electrons through the

Table 8. Actual concentration of NADH found in bean and tomato roots exposed to juglone and KCN treatments for various times.

	uM NADH/mg FW							
	Bean				Tomato			
Treatment	0min	30min	60min	120min	0min	30min	60min	120min
	0.38				0.05			
KCN		0.47	0.32	0.32		0.27	0.15	0.17
Water		0.52	0.36	0.46		0.12	0.16	0.12
Juglone		0.18	0.30	0.24		0.08	0.01	0.00
Ethanol		0.34	0.38	0.35		0.02	0.04	0.08

Table 9. Analysis of variance of NADH quantity in bean and tomato roots in the presence of respiratory inhibitors for various times.

Source of variation	df	SS	MS	F
Crops[z]	1	137602.1	137602.1	270.4**
Treatments[y]	1	10227.8	10227.8	20.1**
Times[x]	2	323814.2	161907.8	318.1**
Crops X Treatments	1	0.009	0.009	0.0
Crops X Times	2	387575.7	193787.9	380.8**
Treatments X Times	2	80059.7	40029.6	78.7**
Crops X Treatments X Times	2	151040.7	75520.4	148.4**
Error	96	48856.4	508.9	
Total	107	1139176.1	10646.5	

[z] Crops = bean, tomato.
[y] Treatments = juglone, KCN.
[x] Times = 30 min, 60 min, 120 min.
** significant at the 1% level.

Table 10. Effect of respiratory inhibitors and time on the quantity of NADH in bean and tomato roots.

	Bean (%)			Tomato (%)		
Time (min)	KCN	Juglone	LSD(5%)	KCN	Juglone	LSD(5%)
30	89.8	41.9	1.7	226.2	385.0	51.9
60	88.3	78.9	6.0	91.2	17.8	13.2
120	68.9	67.8	10.6	143.7	0.0	5.7
LSD (5%)	4.9	8.5		6.3	42.3	

respiratory chain (Figure 4) and juglone can oxidize NADH either directly or through NADH dehydrogenase, the quantity of NADH would be expected to be significantly higher in the KCN treatments than in the juglone treatments. This is true in both crops for each time period except in tomato at 30 min, and in bean at 120 min. The presence of juglone appears to initially inhibit the oxidation of NADH in tomato roots, but after 30 min, NADH was rapidly oxidized until there was no detectable amount at 120 min. Evidence suggests that exogenous NADH is oxidized by a dehydrogenase system separate from that which oxidizes endogenous NADH (Palmer, 1976). Colman and Palmer (1971) determined that the operation of the exogenous NADH dehydrogenase is dependent upon the divalent cations, and unless the concentration of cations in the mitochondrial matrix is in equilibrium with the concentration of cations in the cytoplasm, exogenous NADH will not be oxidized.

Some quinones are capable of causing physical changes in proteins (Webb, 1966), which means that the presence of juglone in the tomato roots may increase the permeability of the mitochondrial membranes. This, as well as the ionic nature of KCN may be what causes an imbalance of cations between the matrix and cytoplasm. During the time necessary for the cations to reach equilibrium, NADH would accumulmlate (30 min). As the concentration of cations came into equilibrium, NADH dehydrogenase would begin to oxidize NADH, and therefore decrease the level of NADH (60 min). In the juglone treatment NADH dehydrogenase then donated electrons to juglone until the

supply of NADH was depleted (120 min). In the KCN treatment there is also a decrease in the NADH level (60 min) after the initial accumulation (30 min). This would indicate that NADH dehydrogenase is operating, but with KCN inhibiting electron flow at cytochrome oxidase, the large decrease in the NADH level (60 min) would indicate that an alternate oxidase is operating (Day et al., 1980). However, the operation of the alternate oxidase seems to be only temporary, or else is much slower at oxidizing compounds than the usual electron chain, since the NADH level eventually increases again (120 min).

In bean roots the initial decrease in NADH concentration from the control values for both the KCN and juglone treatments indicate that these chemicals increase the oxidation rate of exogenous NADH. It is known that the oxidation of exogenous NADH is stimulated by divalent cations (Palmer, 1976), and that the uptake and efflux of cations is dependent upon the pH gradient and the membrane potential of the mitochondria (Russell and Wilson, 1978). If juglone is capable of increasing membrane permeability by causing physical changes in membrane proteins (Webb, 1966), and if the ionic nature of KCN causes an attraction of cations, then the resulting concentrations of divalent cations in the mitochondria may stimulate the oxidation of NADH.

Douce et al.(1973) suggested that a third NADH dehydrogenase was present in the outer membrane of plant mitochondria. In addition to the NADH dehydrogenase on the outer surface of the inner membrane, the NADH dehydrogenase on

the outer surface of the outer membrane was also capable of oxidizing exogenous NADH. If exogenous NADH in bean plants is oxidized by the dehydrogenase on the outer membrane and exogenous NADH in tomato plants is oxidized by the dehydrogenase on the outer face of the inner membrane, differences in the activities of these dehydrogenases may explain why the initial levels of NADH in tomato and bean plants were so diverse. The outer membrane electron transport system consists of the NADH dehydrogenase and several *b*-type cytochromes. Cytochrome *c* acts as a shuttle to carry electrons across the intermembrane space, where they join the conventional respiratory chain (Moreau, 1978).

Bean roots immersed in juglone for 60 min had significantly more NADH than the bean roots immersed in juglone for 30 min (Table 10). Day and Wiskich (1977) suggested that the respiratory chain becomes saturated when exogenous NADH is oxidized in conjunction with TCA activity. The initial depletion of NADH (at 30 min) may have triggered increased activity of the TCA cycle in a process analogous to the Pasteur effect. Therefore, if the rate of electron flow through the respiratory chain is limited, this would cause a build-up of cytoplasmic NADH. With juglone present, it is possible that NADH dehydrogenase is not necessary for the oxidation of NADH (Bergsma et al., 1981), which would mean that the rate of NADH oxidized by juglone would be dependent upon the concentration of juglone (Bergsma et al., 1982).

A build-up of exogenous NADH should result in decreased

activity of the TCA cycle. With a steady rate of electron flow through the respiratory chain, the level of exogenous NADH should eventually decrease. This is apparently what happened in bean roots immersed in juglone for 120 min (Table 10).

Bean roots immersed in KCN for 30 or 60 min had the same amount of NADH (Table 10). This indicates that the initial decrease in NADH (at 30 min) did not trigger increased activity of the TCA cycle, possibly because the initial decrease in the KCN treatment was not as great as in the juglone treatment. Since some NADH was oxidized, the concentration of KCN that was used evidently was not high enough to totally inhibit electron transport. The ability of KCN to inhibit electron transport apparently deteriorated further after 120 min since the level of NADH was lower than at 60 min, indicating that more NADH was being oxidized.

The preceding results seem to demonstrate that different NADH dehydrogenase systems are operating in bean and tomato plants. The rate of electron transport through the two systems may be regulated by the presence of juglone and will influence the quantity of exogenous NADH, but this quantity may not reflect the efficiency of energy transfer of each system (Bergsma et al., 1982). Therefore, a direct comparison between the levels of NADH in bean and tomato plants would not be an indication of the resistance or susceptibility of the plants to juglone. The method of extract preparation also needs to be taken into consideration when interpreting these results. Estabrook et al.(1967) and Klingenberg (1974) determined that it

was necessary to fix tissues with an alkaline extract to rapidly terminate mitochondrial reactions so that the concentrations of NADH present *in vivo* would be preserved through the extraction process. In this experiment, the tissues were not fixed before extraction, so it is likely that due to the oxidative process during homogenization the NADH concentrations are lower than *in vivo*. Also, standard curves of juglone solutions and KCN solutions spiked with various concentrations of NADH were not prepared. Therefore, it is unknown whether or not juglone or KCN had a quenching effect on NADH present in the plant tissues.

Antifungal Bioassay

Certain plants may be susceptible to juglone because they lack a mechanism to destroy the toxic nature of juglone. Some plants have the ability to detoxify lethal substances, and the reactions involved in this process are discussed by Banz and Koster (1981). Besides certain plants, juglone is also known to be toxic to certain fungi (Langhans et al., 1978; Ahmad et al., 1973; Ikekawa et al., 1967), and Wang (1971) used juglone's toxicity to Aspergillus niger to estimate the juglone concentration in plant extracts. Based on these concepts, the antifungal bioassay experiment was devised to determine if bean plants can destroy the toxic nature of juglone, while tomato plants lack the necessary mechanisms.

Since an analysis of variance (Table 11) indicated that there were significant differences in mycelial growth between the crops and between the various times a separation of means for these variables appears in Table 12. There were no significant differences in mycelial growth between treatment values, which indicates that the concentrations of juglone that bean and tomato roots were immersed in before extraction had no influence on mycelial growth.

Table 12 indicates that there were no significant differences in mycelial growth between Petri plates containing extracts of bean tissue immersed in juglone for any of the various times. Since these values are very close to the control values, this implies that extracts of bean tissue immersed in any concentration of juglone for any amount of time have no

Table 11. Analysis of variance of mycelial growth in Petri plates containing extracts of bean and tomato tissue immersed in various concentrations of juglone for various times.

Source of variation	df	SS	MS	F
Crops[z]	1	0.50	0.50	14.62**
Treatments[y]	3	0.07	0.02	0.64
Times[x]	2	0.26	0.13	3.82*
Crops X Treatments	3	0.22	0.07	2.14
Crops X Times	2	0.03	0.02	0.42
Treatments X Times	6	0.25	0.04	1.25
Crops X Treatments X Times	6	0.10	0.02	0.51
Errors	48	1.63	0.03	
Total	71	3.06	0.04	

[z] Crops = bean, tomato.
[y] Treatments = juglone concentrations of: 1X10-6M, 5X10-6M, 1X10-5M, 5X10-5M.
[x] Times = 30 min, 60 min, 120 min.
**,* significant at the 1% and 5% level respectively.

Table 12. Effect of time and crop on mycelial growth in Petri plates containing extracts of bean and tomato which had been immersed in juglone.

Time root tissue was immersed in juglone (min)	Bean (%)	Tomato (%)	Control[z] (%)	LSD (5%)
30	101	123	100	27.5
60	90	105	100	16.2
120	97	110	100	16.0
LSD (5%)	14.2	16.6		

[z] The control values of 100% represent the mycelial growth in Petri plates containing extracts of bean and tomato roots which had been immersed in distilled water.

effect on Aspergillus niger mycelia growth. This result may imply that bean tissue does not have a mechanism to detoxify juglone, which is why mycelial growth was not inhibited by the extracts. However, Ahmad et al.(1973) found that juglone had no effect on Aspergillus niger mycelial growth, and that the fungus grew abundantly on the surface of a juglone solution. If this is true, then bean plants may not possess a mechanism to detoxify juglone, or else Aspergillus niger is a poor indicator of the juglone detoxifying abilities of bean tissue. The standard curve (Figure 3) also indicates that the juglone is not toxic to Aspergillus niger since mycelial growth seems to increase with increased concentrations of juglone. Another possibility is that the process of autoclaving may have detoxified the juglone, which would account for the lack of variation in mycelial growth between the various juglone treatments.

The analysis of variance indicated that there was a significant difference at the 1% level between mycelial growth in Petri dishes containing bean extracts and the mycelial growth in Petri plates containing tomato extracts (Table 11). However, in a mean separation of these values and the control value of 100% by time, there were no differences between the amount of mycelial growth in the Petri plates containing extracts of either crop for each time (Table 12). This result would again indicate that the juglone in the extracts of both crops has been detoxified, or else that the juglone in the extracts of both crops is not toxic to Aspergillus niger.

CONCLUSIONS

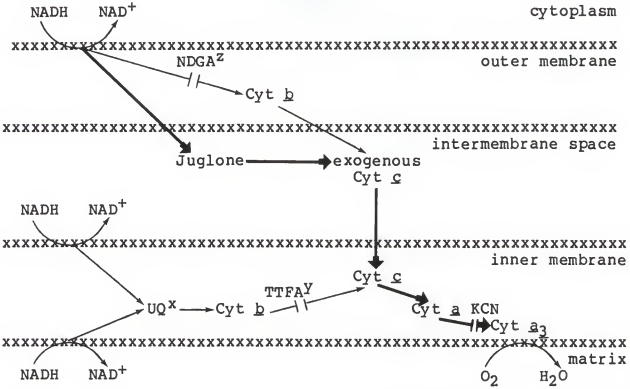
The disparate modes of action of juglone in the respiratory systems of sensitive and resistant plant species is apparently due to differences in the actual sequence of electron carriers. The results of the respiration analysis indicated that when juglone is present, the electrons it accepts are passed back to the respiratory chain before the site of KCN inhibition in both species, and that at the concentrations of KCN used, cyanide-pathways are not operating in either species. The results obtained from the work with the other respiratory inhibitors indicated that electrons in the respiratory chain of bean roots follow an NDGA-sensitive, TTFA-insensitive pathway, while electrons in the respiratory chain of tomato roots follow an NDGA-insensitive, TTFA-sensitive pathway (Figure 5A & B).

The diverse results between bean and tomato plants in the NADH assay also suggest the operation of different electron pathways in these species. With different NADH dehydrogenases oxidizing the NADH in each species, the effect of juglone may also differ depending on which dehydrogenase juglone receives electrons from.

The results of the antifungal bioassay indicated that juglone in the root tissues of bean and tomato plants is not toxic to Aspergillus niger.

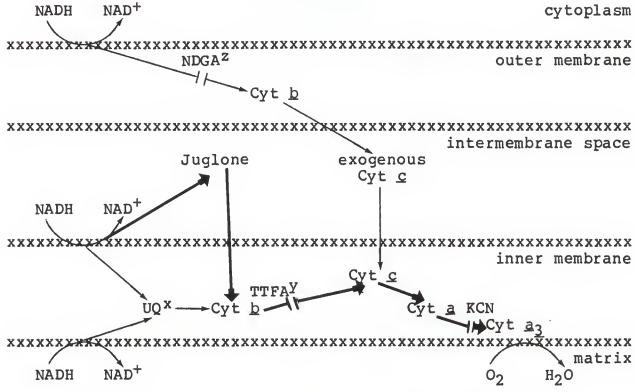
The results of these experiments indicate that electrons follow different pathways in the respiratory systems of tomato and bean plants. However, this is not sufficient evidence

Figure 5-A. Diagramatic representation of the apparent electron pathways when NADH is oxidized by juglone in bean root tissues.



z NDGA = nordihydroguaiaretic acid.
y TTFA = thenoyltrifluoroacteone.
x UQ = ubiquinone.

Figure 5-B. Diagramatic representation of the apparent electron pathways when NADH is oxidized by juglone in tomato root tissues.



z NDGA = nordihydroguaiaretic acid.
y TTFA = thenoyltrifluoroacetone.
x UQ = ubiquinone.

for the disparate modes of action of juglone in these species. More work needs to be done in this area to determine if juglone causes differences in the efficiency of energy transfer in the different pathways.

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DISPARATE MODES OF ACTION OF JUGLONE IN THE RESPIRATORY SYSTEMS OF SENSITIVE AND RESISTANT PLANTS

by

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ABSTRACT

The disparate modes of action of juglone in the respiratory systems of resistant and sensitive plants were investigated using bean (Phaseolus vulgaris L. cv. Oregon 1604B) and tomato (Lycopersicon esculentum Mill. cv. Marglobe) plants to represent resistant and sensitive species.

Respiration rates were determined by monitoring oxygen uptake in root tissues exposed to 10^{-3}M juglone alone and in combination with the known respiratory inhibitors nordihydroguaiaretic acid (NDGA), thenoyltrifluoroacetone (TTFA) and potassium cyanide (KCN). Both manometric and polarographic methods were used to measure oxygen uptake in the root tissues. Juglone mediated respiration in bean roots was sensitive to NDGA, but not TTFA, whereas juglone mediated respiration in tomato roots was not sensitive to NDGA, but was sensitive to TTFA. These results suggest that the initial electron carriers in the respiratory chain differ in bean and tomato root tissues.

The quanity of NADH present in each plant species was determined with a spectrofluorometer after roots of each species had been immersed in 10^{-3}M juglone or $5X10^{-2}$M KCN for 30, 60, or 120 minutes. Juglone and KCN in tomato root tissue caused initial increases in NADH, while in bean root tissue, juglone and KCN both caused initial decreases in NADH. After 120 minutes, tomato roots that had been immersed in juglone had no detectable level of NADH, but tomato roots that had been immersed in KCN had more NADH than the control

treatment. Bean roots immersed in juglone and KCN for 120 minutes had the same amounts of NADH. These diverse results may also indicate that the initial electron carriers in the respiratory chain of bean and tomato plants are different.

The ability of bean and tomato plants to destroy the toxicity of juglone was tested in an antifungal bioassay. Bean and tomato roots were immersed in juglone concentrations of $1X10^{-6}M$, $5X10^{-6}M$, $1X10^{-5}M$, and $5X10^{-5}M$ for 30, 60, or 120 minutes. Extracts of the root tissues were then placed in potato dextrose agar wells in Petri plates. When the extracts had evaporated, the wells were inoculated with Aspergillus niger VanTiegh mycelia. There were no differences in mycelial growth between the crops for any of the treatment times, which indicates that Aspergillus niger may be a poor indicator species.