EFFECTS OF JUGLONE ON *JUGLANS NIGRA* SEEDLING GROWTH

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ABSTRACT

Perhaps the most familiar of allelochemicals is juglone, which is a quinone produced by walnut (*Juglans*) and hickory (*Carya*) species in the family Juglandaceae. Allelopathy refers to the beneficial or harmful effects of one plant on another plant or on micro-organism by the release of chemicals from plant parts by leaching, root exudation, volatilization, residue decomposition and other processes in both natural and agricultural systems. It is often mistakenly associated only with negative effects of one plant species on other organisms. Black walnut (Juglans nigra L.) produces larger amounts of juglone than other walnut species. Juglone interferes with respiration of aerobic organisms and serves to defend walnut trees from insects and other herbivores. Because many species of plant have been observed to die near walnut trees, juglone is widely believed to be one of the walnut's primary defense mechanisms against potential plant competitors for resources (water, nutrients and sunlight). Its effects are most pronounced inside the tree's "drip line". However, plants at a seemingly great distance outside the drip line can be affected, and juglone can linger in the soil for many years even after a walnut is removed as its roots slowly decompose and release juglone into the soil. There has not been any evidence of black walnut autoallelopathy reported in the literature. The objective of this study was to test concentrations of juglone known to reduce the survival and growth of other plants on black walnut seedlings in order to determine if there were any signs of autoallelopathy. There were no significant differences in the growth of black walnut seedlings grown in sand culture with respect to controls when treated with

1mM, 0.1mM, 0.01mM, or 0.001mM of Juglone. This supports the common assumption that walnut seedlings are not harmed by juglone and that juglone could inhibit or kill seedling-associated plant competitors.

INTRODUCTION

Studies on how plants may use chemicals to inhibit the growth of surrounding organisms date back as far as 300 BC (Weston and Duke, 2003; Anaya, 1999). This relationship was termed Allelopathy by Molisch in 1937. It specifically referred to the chemical effects of one plant on the growth and distribution of another plant or microorganism, whether detrimental or beneficial (Weston and Duke, 2003; Inderjit and Nilsen, 2003; Anaya, 1999; Chou, 1999; Friedman and Waller, 1985). It has been speculated that allelopathy is an evolutionary strategy used by some plants to adapt to an unfavorable environment (Chou, 1999).

Allelopathy has become increasingly studied in recent years because it has the potential to be beneficial to agriculture and human health. The uses of allelochemicals include natural herbicides, fungicides, insecticides, and pharmaceuticals (Anaya, 1999; Singh et al., 2003). Using allelochemicals as a natural pesticide can help with the concerns of cost and potential hazards to the environment and human health associated with synthetic pesticides (Singh et. al., 2003). These current concerns in agriculture around the world are due to a dependency on pesticides to maximize crop production. Currently 37% of crops are lost to insects, pathogens and weeds, though pesticides are being used. Of the pesticides being used 60% are herbicides, 25% are insecticides, and 15% are fungicides (Anaya, 1999). It has been documented that in the U.S. 75% of crop protection is based on herbicide input, but with growing concerns the use of chemicals in agriculture has become less popular (Singh et al., 2003). Along

with easing pesticide concerns, allelochemicals also have the capability of helping to increase the reproductive fitness of plants, crop productivity, conservation of genetic diversity, and maintenance of ecosystems' stability (Anaya, 1999; Singh et al., 2003; Duroux et al., 1998).

Allelopathic interactions in agroforestry are important considerations in selecting trees to interplant with herbaceous crop plants. Agroforestry is when woody species are grown alongside agricultural crops in a certain arrangement (Rizvi et al., 1999). It can be used to increase productivity, enrich the soil with organic matter and nitrogen, transport nutrients in the soil, and reduce the infestation of pests (Rizvi et al., 1999). It has been found that there are approximately 80 taxa of tree species that exhibit allelopathy (Singh et al., 2003). The use of some allelopathic trees in agroecosystems can have positive effects on productivity of the associated crop species and economic value in crop production (Rizvi et al., 1999). Agroforestry has been considered an effective tool in combating the problems of environmental degradation, but the effects of allelochemicals on human health needs to be further investigated prior to accepting it as the perfect solution for crop yield (Rizvi et al., 1999).

One of the most studied allelopathic relationships is that between black walnut (*Juglans nigra* L.) and many other plant species. The effects that walnuts have had on surrounding species have been documented as far back as 77 AD (Coder, 1983; Weston and Duke, 2003). The symptoms of juglone toxicity include wilting, browning of vascular tissue, and necrosis.

The allelochemical responsible for these effects has been identified as 5-hydroxy-1,4-naphthoquinone, further referred to as juglone. Juglone is a derived from a chemical found in black walnut and a few other species including, butternut walnut, Persian walnut, Pecan, and shagbark hickory; though black walnut has been shown to produce the largest amount (Weston and Duke, 2003; Rietveld, 1983). The chemical found inside of black walnut is called hydrojuglone and is nontoxic. It is found in the tissue of the tree compartmentalized within cell vacuoles; specifically in the bark, fruit hulls, and roots of black walnut (Dawson et al., 1981; Weston and Duke, 2003). Hydrojuglone becomes toxic when it is oxidized to form juglone, therefore black walnut has a high potential for juglone (Coder, 1983; Rietveld, 1983). Like many other allelopathic compounds, juglone is released into the environment by exudation through the roots, leaching of the aerial parts of the plant, leaching from plant litter, and decomposition of walnut's organic matter (Anaya, 1999; Rietveld, 1983). Juglone has very low water solubility and does not tend to leach far into the soil; therefore it has been found to affect plants that are under the canopy, near the roots, leaves, and dead plant material of black walnut (Weston and Duke, 2003; Inderjit, 2001). The studies conducted by Jose and Gillespie (1998) noted that as distance increased from the black walnuts, the juglone concentration decreased rapidly.

Throughout the studies, juglone has been viewed as an effective allelopathic strategy used by black walnut to outcompete surrounding vegetation. With respect to agroforestry, black walnut has been used successfully in temperate alley- and inter-cropping systems. These include soybeans, winter

wheat, and fescue hay (Dawson and Seymour, 1983; Jose and Gillespie, 1998). Though juglone has toxic effects on many plants including tomato, pear, apple, raspberry, cucumber and black alder plants; some plants apparently tolerate juglone. These include Kentucky bluegrass, hosta, and violets (Weston and Duke, 2003; Dawson and Seymour, 1983).

When allelopathy is observed to occur among plants of the same species, various terms are used to describe the interaction including autoallelopathy, autotoxicity, and autointoxication (Singh et al., 1999; Chou, 1999). It is defined as a process in which chemicals produced by a plant or its decomposing residues in soil suppress the growth of its own, resulting in the decline of plant productivity in natural vegetation or an agroecosystem (Chou, 1999). Autotoxicity has been observed in many species of crops, orchards, and natural forests. These species include alfalfa, asparagus, rice, sugarcane, apple, citrus, and *Eucalyptus* (Singh et al., 1999). Autotoxicity can benefit a plant by increasing its fitness through maintaining seed dormancy when conditions are not conducive to growth or by increasing plant resistance to pathogens (Friedman and Waller, 1985). In previous studies concerning black walnut, the allelochemical juglone has been found to inhibit the activity of H^+-ATPase proton-pumps. This affects some of the necessary plant processes, including solute and water uptake, which in turn causes wilting, browning of vascular tissue, and necrosis (Hejl and Koster, 2004). Black walnut may pose a risk to itself due to the inhibition of H^+-ATPase proton-pumps. Though there have been studies on the allelopathic effects of juglone on many different plant species, there has yet

to be a study that has tested juglone's toxicity to black walnut, in other words autoallelopathy of black walnut.

The purpose of this study was to determine if juglone, a chemical naturally produced by black walnut trees, is toxic to black walnut seedlings. Different concentrations of Juglone known to be toxic to susceptible plants were applied to walnut seedlings to test the hypothesis that black walnut is autoallelopathic, and thus unable to tolerate substrate juglone levels toxic to other plants. This information could help to explain walnut interactions with other plants in native forest ecosystems, black walnut plantations, mixed hardwood plantings, agroforestry systems, and in horticultural cultivation.

MATERIALS AND METHODS

One hundred twenty five, 2:0, bare-root black walnut seedlings of uniform size were obtained from Carino Nurseries (Indiana, Pennsylvania, USA). The dormant seedlings were maintained at 4°C for 60 days for stratification prior to planting after being harvested from nursery beds. The seedlings had been root pruned so as to fit into our experimental pots. Initial shoot lengths were measured and recorded. Using steam-pasteurized torpedo sand as the medium, 125 of these Walnut seedlings were planted in individual 983-cc, cone-shaped pots, 6.4 cm in top diameter and 36 cm deep (Deepot™ Cells Hummert International, Missouri, USA). Drainage was provided by openings in the bottom of the containers, and the sand substrate was retained in the containers by placing multiple layers of cheese cloth next to the drainage openings in the bottom of the pots. Beneath each of these cone-shaped pots an individual saucer was used to catch and keep separate any occasional outflow of solution through drainage openings, allowing for capillary re-uptake of solution.

The potted seedlings were randomly assigned to five groups of 25. Each group of seedlings was treated with one of four different juglone concentrations: 0.001 mM, 0.01 mM, 0.1 mM, and 1 mM. Distilled water was used for the control group. Each treated seedling was placed at random on greenhouse benches in a completely randomized design. In previous studies, 1mM juglone had been found to kill or to have a pronounced adverse effect on plants studied (Rietveld, 1983).

The 1 mM stock solution of Juglone (Fisher Scientific, USA) was prepared in distilled water by stirring at 40°C for 24 hours (Rietveld, 1983). This stock solution was used as the 1 mM treatment solution then serially diluted by ten-fold with distilled water to the other concentrations of 0.1mM, 0.01mM, and 0.001mM. The 1mM treatment was 150mL of stock solution, while the other treatments consisted of 150 mL of ten-fold dilutions of stock solution or 150mL of distilled water for the control group. Solutions were also amended by adding sufficient amounts of Hoagland's nutrient solution salts (Hoagland and Arnon, 1950), including both macro- and micronutrients, so as to maintain half-strength nutrient solution concentration at pot capacity (150 ml). Pot capacity was determined by subtracting the oven-dried (40°C) weight of sand in the pots from the weight of freely drained pots 24 hours after saturation with water. It was maintained by watering with tap water daily or as needed. The seedlings were grown in a greenhouse at 25°C $\pm$ 3 °C and a 16 hour photoperiod maintained by high pressure sodium vapor lamps All plants were irrigated using a gentle spray of water applied to the tops of containers so as to avoid splashing and mixing of substrate solution in the containers. Plants were grown in the treatment sand culture solutions for 90 days. Once the experiment was complete the seedlings were harvested and the sandy potting medium was washed from the root systems. Plants were separated into root, stem, current year shoot and leaf components then dried at 40° C for 24 hours. The dry weights of each of the components were then measured using a digital balance. Along with the dry weights of seedling roots, stems, and leaves; the total shoot length, the shoot

length extension from the terminal meristems during the experiment, relative shoot extension and number of leaves were determined for analysis. The relative shoot extension was determined by dividing the current-year shoot extension by the initial shoot length.

ANOVA was performed for each of the variables in order to determine if the different treatments had a significant ($P \leq 0.05$) effect on the growth of the black walnut seedlings. In order to evaluate species sensitivity to juglone; shoot elongation, relative shoot elongation, leaf number, and dry weight accumulation were measured. Shoot elongation and dry weight accumulation are a more sensitive measure of juglone in most species than that of seed germination and radical elongation (Rietveld, 1983).

RESULTS AND DISCUSSION

There were no significant differences attributable to juglone treatments for shoot length extension from the terminal meristems (current-year shoot extension), the relative shoot extension, and number of leaves *(Table 1)*. The means and standard error for each treatment were also compared for the three variables. In similar studies, 1mM treatment had profound toxic effects on shoot elongation and dry weight accumulation of the susceptible species. In a study conducted by Rietveld (1983) thirteen species; including Ginnala maple, Autumn olive, White oak, European black alder, Eastern white pine, and scotch pine; were analyzed for shoot elongation. Of the thirteen species of seedlings analyzed, six species had no shoot elongation when treated with 1mM juglone concentration. Another five species had only 1-3 mm of shoot elongation. All species (with the exception of hairy vetch) were killed within a few days of being treated with 1mM juglone. The controls for these species had shoot elongation values ranging from 47-70 mm (with the exceptions of white oak- 21mm, Hairy vetch- 190mm, and Ginnala maple- 102mm). Fourteen species were analyzed for shoot and root dry weight changes. Seedlings of all species, if not killed outright, were found to have significant mean growth reductions with respect to controls (Rietveld, 1983). In a similar study conducted by Neave and Dawson (1989), 10^{-5}M juglone treatment caused a significant reduction in the growth of black alder seedlings. They conducted their study hydroponically as to avoid soil microbial activity, which can break down and detoxify juglone rapidly (Neave and Dawson, 1989). Black walnut did not exhibit significant growth differences

attributable to juglone in our study. Neither relative shoot extension nor number of leaves had statistically significant mean differences for overall treatment effects, nor did they have any trends or individual treatment mean differences corresponding to treatment levels (*Figures 2 and 3).*

Table 1: Single Factor ANOVA for each variable (P≤ 0.05).

VARIABLE							
Current-Year shoot extension	*Source of Variation*	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
	Between Groups	8.1248	4	2.0312	1.168659	**0.328117**	2.447237
	Within Groups	208.5672	120	1.73806			
	Total	216.692	124				
Relative Shoot Extension	*Source of Variation*	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
	Between Groups	9.472	4	2.368	1.226519	**0.30323**	2.447237
	Within Groups	231.68	120	1.930667			
	Total	241.152	124				
Leaf number	*Source of Variation*	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
	Between Groups	0.001619	1	0.001619	1.073967	**0.305244**	4.042652
	Within Groups	0.072338	48	0.001507			
	Total	0.073956	49				
Dry Root Weight	*Source of Variation*	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
	Between Groups	122.8234	4	30.70584	2.031546	**0.094261**	2.447237
	Within Groups	1813.743	120	15.11452			
	Total	1936.566	124				
Dry Weight of Current-Year Shoot Extension	*Source of Variation*	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
	Between Groups	1.190857	4	0.297714	0.835361	**0.505287**	2.447237
	Within Groups	42.76679	120	0.35639			
	Total	43.95765	124				

Figure 1. ***Means of the current-year shoot extension and the standard error for each of the treatments.***

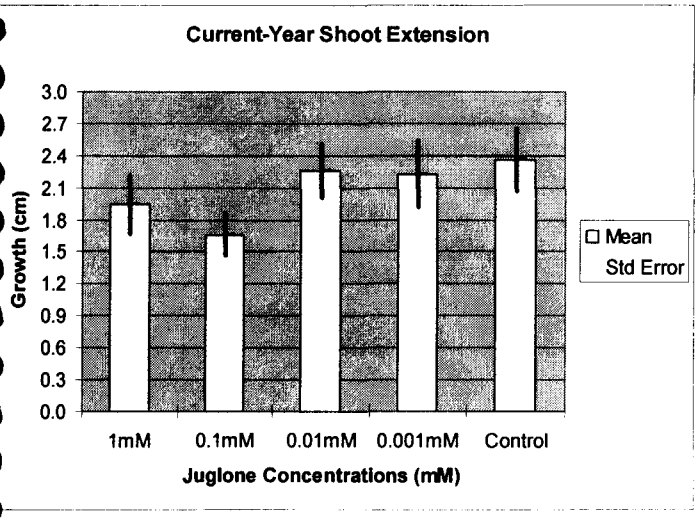


Figure 2: ***Relative shoot extension means and the standard error for each of the treatments.***

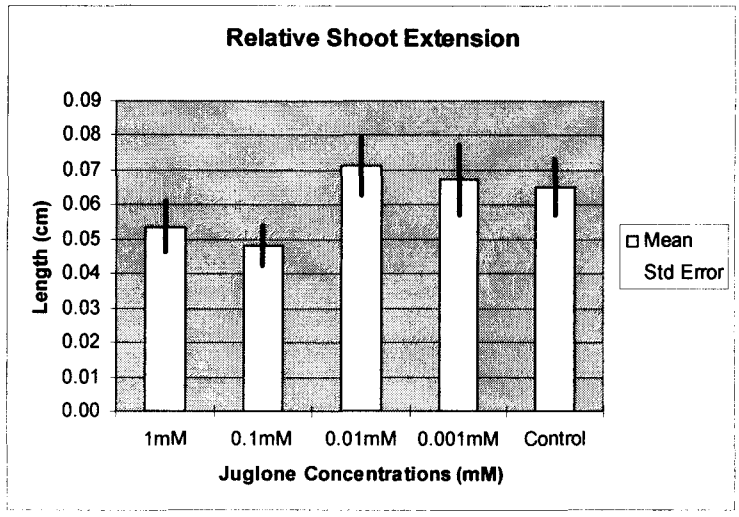


Figure 3 (right): Bar graph showing the leaf number means and standard error for each of the treatments.

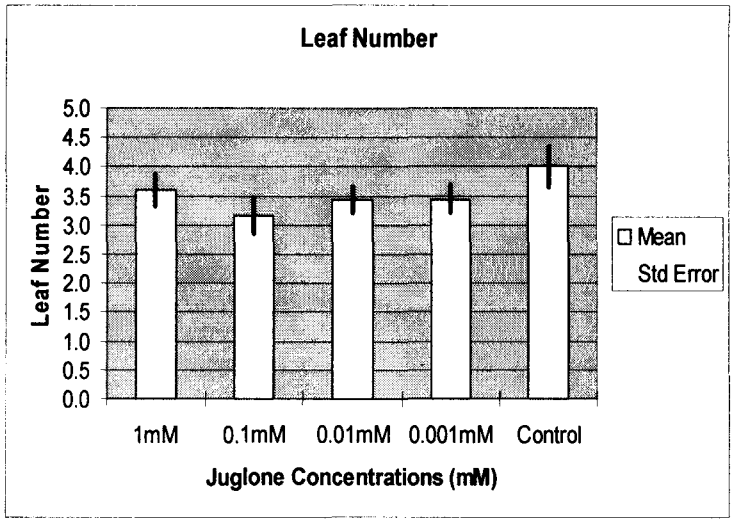


Table 2: Two-sample t-test, comparing 1mM juglone treatments to the controls for each of the variables. Each of the t-statistics were found to be less than the t-critical for each of the four variables.

VARIABLE			
Current-Year shoot extension		*1mM Juglone Treatment*	*Control*
	Mean	1.94	2.356
	Variance	1.829166667	2.072566667
	Observations	25	25
	Pooled Variance	1.950866667	
	Hypothesized Mean Difference	0	
	df	48	
	t Stat	-1.053014964	
	P(T<=t) one-tail	0.148803143	
	t Critical one-tail	1.677224191	
	P(T<=t) two-tail	0.297606286	
	t Critical two-tail	2.01063358	
Relative Shoot Extension		*1mM Juglone Treatment*	*Control*
	Mean	0.053648033	0.065026991
	Variance	0.001326945	0.001687129
	Observations	25	25
	Pooled Variance	0.001507037	
	Hypothesized Mean Difference	0	
	df	48	
	t Stat	-1.036323911	
	P(T<=t) one-tail	0.152621922	
	t Critical one-tail	1.677224191	
	P(T<=t) two-tail	0.305243844	
	t Critical two-tail	2.01063358	
Leaf number		*1mM Juglone Treatment*	*Control*
	Mean	3.6	4
	Variance	1.75	3.083333333
	Observations	25	25
	Pooled Variance	2.416666667	
	Hypothesized Mean Difference	0	
	df	48	
	t Stat	-0.909717652	
	P(T<=t) one-tail	0.183758502	
	t Critical one-tail	1.677224191	
	P(T<=t) two-tail	0.367517005	
	t Critical two-tail	2.01063358	
Dry Weight of Current-Year Shoot Extension		*1mM Juglone Treatment*	*Control*
	Mean	0.99268	1.14128
	Variance	0.169565393	0.39060746
	Observations	25	25
	Pooled Variance	0.280086427	
	Hypothesized Mean Difference	0	
	df	48	
	t Stat	-0.992722315	
	P(T<=t) one-tail	0.162911753	
	t Critical one-tail	1.677224191	
	P(T<=t) two-tail	0.325823506	
	t Critical two-tail	2.01063358	

Also the most extreme treatment of 1mM juglone was compared to the control for each of the following: current-year shoot extension, relative shoot extension, and leaf number. This was accomplished using two-tailed t-tests. Each of the t-statistics were found to be less than the t-critical, therefore no significant differences between control seedlings and the 1mM juglone treatment were found for any of these measured variables (*Table 2*).

Similarly, there were no significant differences found among the treatments for the dry weights of various tissues. These measurements included root dry weight and dry weight of the current-year shoot extension. Statistical analyses were completed by performing ANOVA statistics at the 95% probability level and comparing the means and standard errors for each of the treatments (*Table 1; Figures 4, 5).* The comparison of means agree with the findings of the ANOVA statistics. Since the current-year shoot extension was one of the more sensitive measures of growth (Rietveld, 1983), a two-tailed t-test was performed to compare the most extreme treatment of 1mM juglone, with the control. Once again, with a t-statistic less than the t-critical, no significant difference was found (*Table 2).*

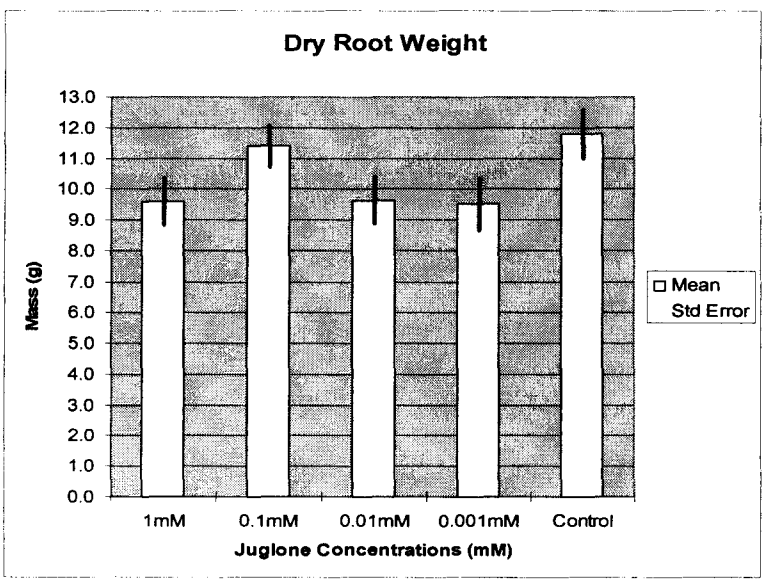


Figure 4 (right): Bar graph showing the dry root weight means and the standard error for each of the treatments.

Figure 5: Bar graph showing the dry weight means and standard error in terms of the current-year shoot extension for each of the treatments.

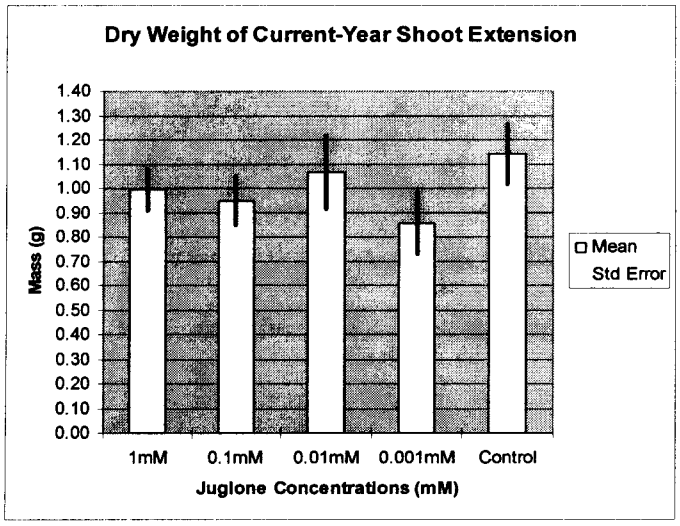
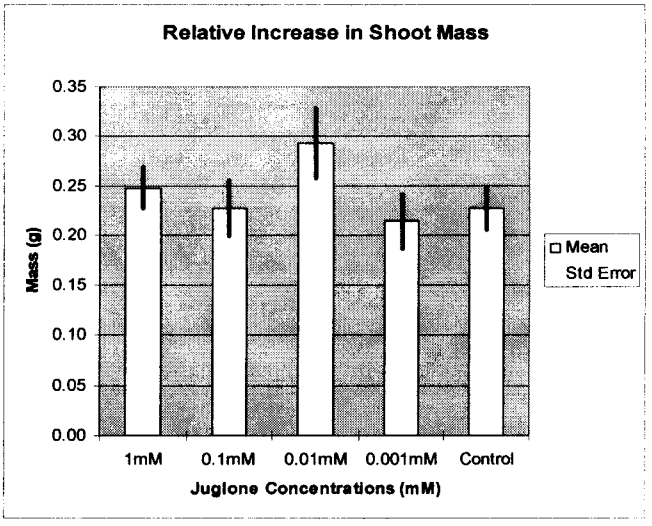


Figure 6: Bar graph showing each of the treatments means and standard error in terms of the relative increase in shoot mass.



A statistical analysis of the total dry weight of the shoots for each of the treatments was also completed using ANOVA. The P-value of 0.008 was obtained; therefore a significant difference between the treatments was noted for the total dry weight of the shoots (*Table 3)*. This shoot measurement included the current-year shoot extension. The statistical significance, however, was not associated with any biologically significant treatment effect and seemed to result from random differences in initial size and mass of experimental seedlings. The measurement of total dry shoot weight was dismissed, for the initial weights of the shoots were unknown. Also while performing a comparison of means for this variable, it was speculated that the differences were unrelated to treatment (*Figure 7)*. The observed outcome was most likely due to chance assignment of the seedlings. This observation is also supported by the fact that there were no significant differences found between the dry weights of the current-year shoot extension for each of the treatments.

Table 3: Single Factor ANOVA ($P \leq 0.05$) for total dry shoot weight.

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	40.12661	4	10.03165	3.585995	**0.00846**	2.447237
Within Groups	335.6944	120	2.797453			
Total	375.821	124				

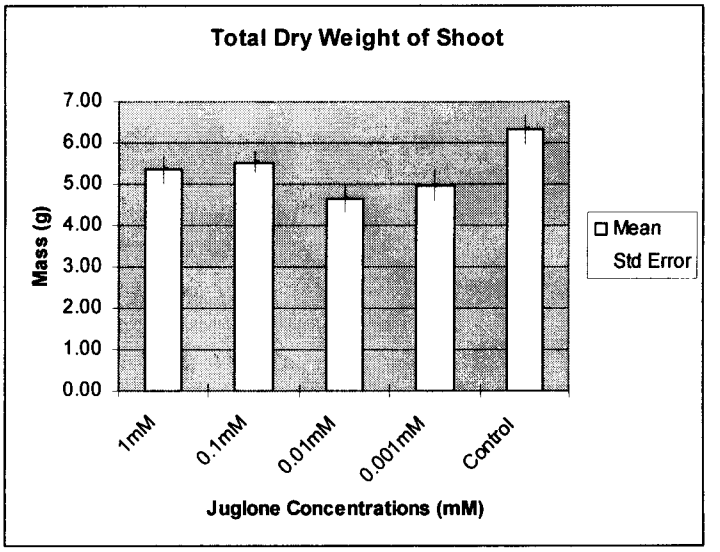


Figure 7 (right): Bar graph showing each of the treatments means and standard error in terms of the total dry shoot weight.

There were no significant differences found among juglone treatments for the variables measured. This study indicates that black walnut seedlings are apparently not strongly autoallelopathic. Rietveld's study of the allelopathic effects of juglone found that "Seedlings of all species were severely wilted and eventually killed by 10^{-3} M juglone, and most were chlorotic and severely retarded by 10^{-4} M juglone" (1983). Therefore we would have expected to see some effects at the treatment level of 1mM juglone if black walnut was autoallelopathic. Reitveld found that all species tested experienced retarded shoot elongation abruptly at the 10^{-3} M and 10^{-4} M treatment levels. This study also found that dry-weight accumulation was more sensitive than shoot elongation. In five out of fourteen species tested, 10^{-5} M and greater juglone

treatments significantly reduced both shoot and root dry weight accumulation. Some of the species strongly inhibited in this study included Amur maple, Scots pine, European alder, Amur honeysuckle, white pine, crimson clover, and Japanese silverberry (Rietveld, 1983). In a study done by Neave and Dawson (1989), black alder root and radical elongation were both significantly less in soils dosed with 10^{-3} M juglone than 10^{-4} M juglone and control treatments, although the inhibitory effects of juglone dosing on the radicle elongation of alder bioassays disappeared within a few days of juglone mixing in moist, non-sterile, loamy soils, apparently due to degradation of juglone by such common aerobic soil bacteria *as Pseudomonas fluorescens* (Schmidt, 1988).

Though black walnut has not previously been investigated for autotoxicity, other species of plants have been. In a forest ecosystem there have been many cases of autotoxicity observed. These species include Tasmanian bluegum, China fir, and Velvet mesquite (Singh et al., 1999). Autotoxicity has also been observed in alfalfa and lower plants including some ferns and algae. Plants that produce allelochemicals with the potential of being autotoxic have methods to avoid their hazards (Friedman and Waller, 1985). It has been speculated that plants that have been found to not be inhibited by their own allelochemicals may have a special mechanism to avoid autotoxicity. Some plants store the allelochemical in the outer dead layers of fruit, only being exposed to the chemical if the inner fruit shell is pierced; while others store the allelochemicals in vacuoles or between the cells (Friedman and Waller, 1985). Some plants produce roots that are well below the surface as to avoid allelochemicals that

have lower water solubility (Singh et al., 1999), which is the case for juglone. One of the main ways to avoid self-toxicity is through the presence of allelochemicals that are glycosides. These glycosides contain a variety of sugars that act as masking agents which can be removed mechanically or enzymatically to reduce the autotoxic effect (Singh et al., 1999). Juglone can accumulate as hydrojuglone β-D-glucopyranoside, which is a glycosylated form. These types of glucosides generally are found stored in vacuoles in large amounts (Duroux et al., 1998). These glucosides are converted to juglone on exposure to oxygen in the air which occurs with cell death due to insect grazing or through senescence, shedding and degradation of plant tissues. Avoidance of juglone toxicity by compartmentalization of hydrojuglone in vacuoles in living cells along with tolerance of juglone from oxidation of hydrojuglone in litter and rhizosphere soil may explain walnut's immunity to its own chemical defense against insects and plant competitors.

This study has shown that black walnut is not autoallelopathic. Additional research will be required to identify exact mechanisms by which black walnut tolerates its own phytotoxin juglone. A concentration as high as 1mM of juglone is unlikely to occur in the soil of a natural ecosystem (Rietveld, 1983; Neave and Dawson, 1989). But by determining that black walnut seedlings are not inhibited significantly in growth at a soil solution of 1mM juglone, it seems probable that black walnut is not autoalleopathic in nature. Thus it is possible that walnut can reduce competition from other plants for its seedlings through the release of toxic juglone from leaves, roots, bark and husks. This study supports the commonly

held belief that walnut favors its own regeneration through release of the phytotoxin juglone because black walnut seedlings are not harmed by levels of juglone that are extremely toxic to other potential plant competitors.

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