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EFFECTS OF JUGLONE (5'-HYDROXY-1,4-NAPHTHOQUINONE) ON THE ALGAE *ANABAENA FLOS-AQUAE*, *NOSTOC* *COMMUNE* AND *SCENEDESMUS ACUMINATUS*

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ABSTRACT

Three species of algae, *Anabaena flos-aquae*, *Nostoc commune* and *Scenedesmus acuminatus* were selected for their sensitivity to juglone and studied for the effects of juglone concentrations of 10, 1, 0.5, 0.1 and 0.01 $\mu\text{g/ml}$ upon their growth. *A. flos-aquae* was most sensitive, with significant inhibition by the 0.5 $\mu\text{g/ml}$ concentration. *N. commune* was inhibited least, with significant inhibition only in the 10 $\mu\text{g/ml}$ concentration. *S. acuminatus* was found to be moderately inhibited at the 0.5 $\mu\text{g/ml}$ concentration. All species were found to be non-viable after 14 days exposure to 10 $\mu\text{g/ml}$ juglone.

Tests with 7 and 14 day old cells of *S. acuminatus* showed significant differences in growth. Seven day old cells used as inoculum were inhibited by all concentrations while 14 day old cells showed growth in excess of controls in three concentrations (0.5, 0.1 and 0.01 $\mu\text{g/ml}$). All studies with 14 day old cells showed slight, but not significant, increases in growth in the 0.01 $\mu\text{g/ml}$ concentration. These results suggest that juglone may enhance growth of some soil micro-organisms.

INTRODUCTION

The deleterious effect of the black walnut, *Juglans nigra*, upon higher plants has been known since ancient times. Many studies have shown that juglone, 5-hydroxy-1,4-naphthoquinone, is the compound responsible for inhibiting the growth of higher plants around the walnut tree (Reitveld, 1983; Funk et al., 1979). Juglone has also been studied as a fish toxicant (Marking, 1970), and has been found to be a depressant in other animals (Westfall et al., 1961). Studies by Koeppel (1972), Harmon and Crane (1974, 1976), Cobley et al. (1973), and Grossman et al. (1974) have established a mode of action for juglone as a respiratory inhibitor, specifically of NADH dehydrogenase. Studies by Van Duuren et al. (1978) demonstrated the substance to have potent tumor promoting properties. Investigations of micro-organisms have indicated that juglone may serve as a resistance factor to plant pathogens such as *Fusicladium effusum* (Hedin et al., 1979), Solkov et al. (1972) presented evidence of juglone inhibition for a variety of plant pathogens and other microbes. Krajci and Lynch (1977) used the antibiotic disc assay method to determine inhibition by crude walnut hull extracts and pure juglone against a broad spectrum of microbes including bacteria, algae and fungi. More recently, Dawson and others have demonstrated inhibitory effects on the symbiotic nitrogen-fixing micro-organisms *Rhizobium japonicum* strain 71 and *Frankia* Ar 13 (Dawson et al., 1981; Dawson and Seymour, 1983).

Although some work has been done with members of both Cyanophyceae and Chlorophyceae, those studies have neglected to assess either extremely low-level exposure or the possibility of growth enhancement at non-inhibitory concentrations. In addition, the low solubility of juglone in water has led to the use of organic solvents to provide a carrier for the substance. The purpose of this study was to examine the effects of juglone on two free-living soil algae (Cyanophyceae), and on different aged cultures of a sensitive green alga (Chlorophyceae). Water was used as the solvent for the juglone since it is the natural carrier of the substance.

MATERIALS AND METHODS

Algae were obtained from Richard C. Starr's collection at the University of Texas. Those selected were *Anabaena flos-aquae* (Lyng.) Breb. UTEX 1444; *Nostoc commune* Vaucher UTEX 584; *Scenedesmus acuminatus* (Lagerh.) Chodat UTEX 415; and *Chlorella pyrenoidosa* Chick UTEX 252. Preliminary studies showed *C. pyrenoidosa* to be unaffected by juglone at the concentrations tested so it was abandoned.

Juglone (90% pure) was obtained from Sigma Chemical Corporation. A saturated stock solution was made by dissolving juglone in approximately 1L of distilled water with constant stirring for 24 hours. Suspended solids were removed by filtration through a 0.45 μm filter (Millipore). Concentration was calculated by deducting the weight of undissolved solid from the original amount of juglone, correcting for purity and adjusting the volume to 1L. The saturated solution was found to contain 0.1032 g/l juglone.

Test organisms were cultured in 250 ml flasks containing 100 ml of Bristol's solution in a Lab-Line Environ-Shaker at 20°C, 100 rpm on a 12 hour day/night cycle, for 14 days prior to introduction to test conditions. In studies of *S. acuminatus*, the inoculum for one series was

Conc. ($\mu\text{g/ml}$)	<i>Scenedesmus</i>	<i>Anabaena</i>	<i>Nostoc</i>
10.0	-	-	-
1.0	+	-	+
0.5	+	+	+
0.1	+	+	+
0.01	+	+	+

(+) observable growth
(-) no observable growth

Table 1. Viability study (1 ml test culture removed from flasks at end of two week exposure to juglone, cultured 7 days on Bristol's medium solidified with 15 g/l agar. Microscopic examination used to verify negative results.

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allowed to grow for only 7 days. Ten ml aliquots of inoculum were transferred aseptically to flasks containing 80 ml Bristol's solution and 10, 1, 0.5, 0.1 or 0.01 ml of filter sterilized juglone. Volumes were adjusted to 100 ml for all concentrations. Controls contained 10 ml sterile distilled water. After 14 days incubation the cells were harvested by centrifugation in tared centrifuge tubes at 15,000 rpm for 30 min. Dry weights were obtained by drying at 65°C to constant weight (approximately 7 days).

At time of harvest, 1 ml aliquots of test cultures were pipetted onto Bristol's medium solidified with agar (15 g/l) in order to test for viability (Table 1). All cultures were tested for purity by streaking on nutrient agar plates at the time on inoculation.

Data for *S. acuminatus* and *N. commune* represent the means of three series of three repetitions each. Data for *A. flos-aquae* represent the means of four series of tests. Significance was determined by both linear regression and Fisher's Least Squares Determination of analysis of variance. All tests were done at the 0.05 level of significance.

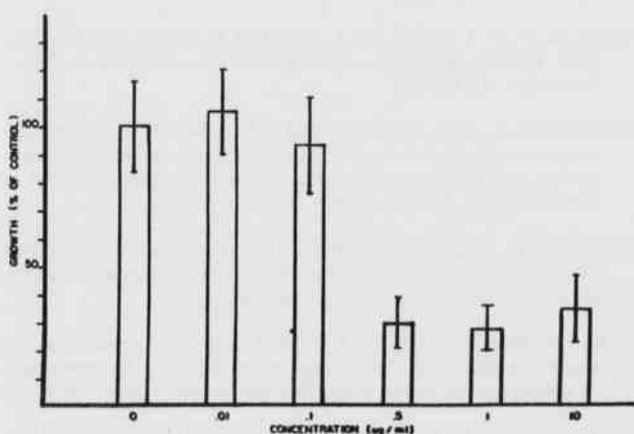


Figure 1. Effects of juglone on the growth (dry weight) of *Anabaena flos-aquae* shown as a percent of control (vertical line = standard deviation). Correlation = -0.8541 ; Significance = 0.0001 .

DATA AND RESULTS

The results of viability checks for all tests showed no growth in the 10 ug/ml concentration. *A. flos-aquae* showed no growth in the 1 ug/ml concentration. Analysis of dry weights showed a significant change between the 0.1 and 0.5 ug/ml concentrations, resulting in a bimodal distribution (Figure 1). The lower concentrations were grouped with the controls and the 10, 1 and 0.5 ug/ml tests were related. It is of interest to note the slight increase in growth associated with the 0.01 ug/ml concentration. While not significant, it nonetheless was seen in all experiments with the exception of *S. acuminatus* cultures initiated with 7 day old inocula. *N. commune* was significantly affected by only the 10 ug/ml concentration. All other data were within one standard deviation of the control. Once again there was a slight elevation in growth in the lowest concentrations of juglone (Figure 2).

The study of different inoculum ages of *S. acuminatus* displayed no significant difference between the two highest concentrations of juglone when adjusted for difference in initial inoculum weights (Figure 3). The 7 day old inoculum was inhibited in all concentrations of juglone. Significant inhibition occurred only in the 10, 1 and 0.5 ug/ml concentrations, with the 69% weight at 0.5 ug/ml being the only example of significant inhibition which differs from both the controls and the non-viable cultures. The 14 day inoculum showed growth above the level of controls in all but the 10 and 1 ug/ml concentration. There was again an increase in overall growth at 0.01 ug/ml juglone solution for the 14 day old inoculum, although the 7 day inoculum was significantly inhibited at this concentration. Results of viability checks indicated that only the 10 mg/ml solution was lethal.

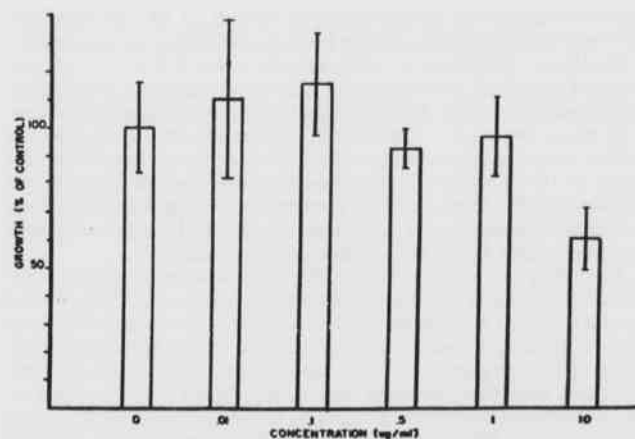


Figure 3. Effects of juglone on the growth (dry weight) of *Nostoc commune* shown as a percent of control (vertical line = standard deviation). Correlation = -0.5497 ; Significance = 0.0003 .

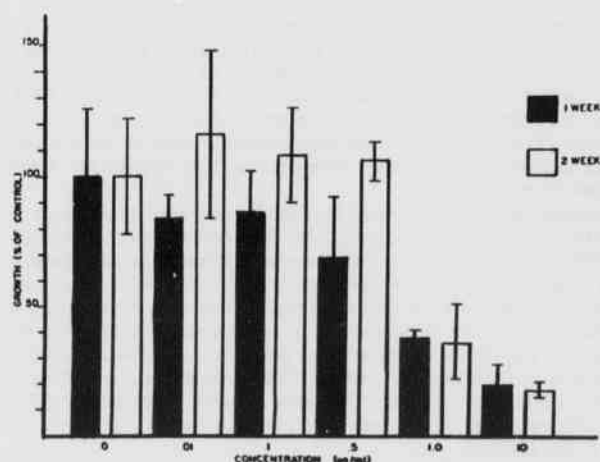


Figure 3. Effects of juglone on the growth (dry weight) of *Scenedesmus acuminatus* shown as a percent of control (vertical line = standard deviation). Comparison of 1 and 2 week old inocula adjusted for initial difference in inoculum dry weight. Correlation = -0.8585 ; Significance = 0.0001 for one week inoculum. Correlation = -0.7314 ; Significance = 0.0001 for two week inoculum.

DISCUSSION

Krajci and Lynch (1977) demonstrated the inhibition of several different bacteria, algae and fungi by juglone. The algae tested were *Calothrix flaccumbaciens*, *Anacystis* sp., *Bracteacoccus cinnabarinus*, *Coelastrum microsporum*, and *Anabaena variabilis*. Their results showed *A. variabilis* to be inhibited by juglone concentrations of 0.0625 mg/ml. Corresponding concentrations of 10 and 1 ug/ml used in this study significantly inhibited *A. flos-aquae*. Apparently, the two *Anabaena* species exhibited a similar sensitivity to juglone.

Although this is the first report of algal growth stimulation by juglone, others have reported similar results for higher plants. Funk et al. (1979) reported the stimulation of some coniferous seedlings at concentrations of juglone as low as 10^{-8} and 10^{-9} M. Reitveld (1983) also reported some increased growth in 10^{-5} and 10^{-6} M concentrations of juglone, but the increases were not significant. Data presented here indicate

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growth stimulation at concentrations of about 0.5×10^{-8} M for all test cultures except those inoculated with 7 day old cells of *S. acuminatus*.

Significant inhibition of algal growth below 0.5×10^{-5} M was observed only for *A. flos-aquae* and 7 day old inocula of *S. acuminatus*. Results of the other studies cited using approximately equivalent concentrations showed that the levels of juglone required to significantly inhibit other organisms, except *Fusicladium effusum*, were within the range of 10 - 1 ug/ml. Comparison with the work of Dawson and Seymour (1983) indicated that the symbiotic micro-organisms *Rhizobium* and *Frankia* reacted to the same concentrations of juglone as did those free-living algae found to be sensitive to the substance.

According to Langhans et al. (1976) *Fusicladium effusum* was inhibited by juglone at 0.1 mg/ml. Windham and Graves (1981) showed several different pathogenic isolates of *F. effusum* to be inhibited at 50 and 100 ug/ml concentrations. Since the species is pathogenic on mature trees, a higher level of resistance to the substance was expected.

Studies with *Candida utilis* have demonstrated juglone effects on mitochondrial activity to be affected by the growth phase of the culture (Cobley et al., 1973; Grossman et al., 1974). Their studies indicated the presence of an NADH dehydrogenase in inner mitochondrial membrane preparations which had variable sensitivity to juglone. Preparations from exponential phase cells were found to be equally sensitive to both ferricyanide and juglone inhibition; but late stationary phase cells were much more sensitive to ferricyanide than to juglone. These studies may provide an explanation for the variability noted in *Scenedesmus* cultures of different ages reported here. In general, the 0.01-0.5 ug/ml concentrations of juglone resulted in growth above the level of the control cultures when added to 14 day old cultures of *S. acuminatus*. When corresponding amounts of juglone were added to 7 day old inocula, inhibition was observed, with dry weights indicating growth of only 69-86% of controls. Growth of 69% in the 0.5 ug/ml concentration was significantly different from both controls and severely inhibited cells.

Because of the narrow range between lethal and non-inhibitory concentrations, determination of a lethal dose for 50% of the population (LD50) was not attempted. Cultures which proved to be non-viable had dry weights not significantly different from those which showed significant inhibition in both *A. flos-aquae* and *S. acuminatus*, with the above noted exception for the 7 day old *S. acuminatus*. For all other trials where no significant inhibition was noted, dry weights did not differ significantly from those of controls. Data from animal studies (Westfall et al., 1961) also noted that the range between depressant and lethal concentrations of juglone was so narrow as to make determination of an LD50 difficult.

It is possible to infer from these studies that seedlings of black walnut and related species may actually stimulate growth of some soil micro-organisms. Since both *Anabaena* and *Nostoc* are able to fix nitrogen, available soil nitrogen may be enhanced during early stages of growth. Co-cropping with leguminous plants has been shown to enhance the growth of walnut trees, although Dawson and Seymour (1983) demonstrated juglone inhibition of two symbionts responsible for nitrogen fixation in higher plants at toxin concentrations of 10^{-4} M. Their results indicated minor inhibition at concentrations of 10^{-5} and 10^{-6} M. Correlation of their study with the concentrations used in this study (0.5×10^{-4} to 0.5×10^{-7} M) showed that the blue-green algae used in this study were sensitive to the same levels of juglone. Additional study of both *Rhizobium* and *Frankia* to test for enhanced growth below 10^{-6} M concentrations would be of interest.

Reitveld (1983) stated that allelopathic effects of juglone upon other trees in mixed, even aged plantings did not become evident before 12-25 years, indicating a relatively slow accumulation of juglone in the soil. The results of this study suggest that during the period of juglone accumulation, concentrations may exist which actually stimulate the growth of soil micro-organisms. More study is needed concerning effects of low concentrations of juglone upon other beneficial soil microbes.

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