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Effect of Hexavalent Chromium on Photosynthetic Rates and Petiole Growth in *Nelumbo lutea* Seedlings

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ABSTRACT

Petiolar photosynthetic carbon assimilation (PCA) rates, seedling petiole length, and petiolar elongation rates were measured in *Nelumbo lutea* (Willd.) Pers. (American lotus) grown in aseptic liquid culture at pH 5.6 and 8.2 and exposed for 4, 48 and 96 hr to hexavalent chromium in initial concentrations varying from 0.5 to 100 mg/L. Within several hours considerable chromium partitioned into a phase removable from solution by filtration through 0.6-µm Nuclepore filtration. Exposure for 48 hr to soluble chromium levels as low as 0.02 mg/l in pH 5.6 media significantly stimulated PCA rates, but at pH 8.2 only plants exposed to the highest chromium concentrations for 96 hr exhibited enhanced PCA rates. The solubilizer method used for routine PCA analyses produced values that markedly overestimated net carbon fixation. Soluble hexavalent chromium concentrations of as little as 0.6 mg/l in growth media reduced mean petiole lengths in 48- and 96-hr experiments. Petiolar elongation rates over the course of 48- and 96-hr exposure experiments conducted at pH 5.6 and pH 8.2 were dose-specifically reduced by 0.1 to 4.7 mg/l of soluble chromium. In seedlings exposed for 96 hr to an initial chromium dose of 100 mg/l (3.4 mg/l as soluble chromium at T96 hr), petiole elongation was reduced to approximately 10% of zero-added-chromium control rates.

Key words: aquatic macrophyte, heavy metal, toxicity, bioassay.

INTRODUCTION

Chromium concentrations in aquatic systems receiving industrial wastewaters can reach levels of 1 to 20 mg/l, a concentration commensurate with LC50 and EC50 values in bioassay systems developed for fish, fathead minnow, aquatic invertebrate, and algal species (reviewed by Staves and Knaus 1985 and Guilizzoni 1991). Few studies have focused on the short-term (*i.e.*, a few hours to days) effects of chromium exposure on aquatic vascular plant ecophysiology.

Guilizzoni *et al.* (1984) found that chromium enhanced shoot growth in *Myriophyllum spicatum* up to a medium concentration of 50 µg/l. Higher concentrations of up to 1 mg/l caused an almost linear reduction in shoot length and weight and photosynthetic rates. Chromium concentrations exceeding 1.0 mg/l resulted in 8-day growth rate reductions in the duckweed genera *Lemna* and *Spirodela* (Staves and Knaus 1985).

Several studies examined leaf disk/shoot section bioassays as alternatives to whole-plant systems for evaluating responses of submerged macrophytes to environmental stimuli. Beer and Wetzel (1981) and Beer *et al.* (1982) developed a ^{14}C-photosynthetic carbon assimilation (PCA) rate assay to investigate carbon uptake and fixation dynamics in submerged macrophytes, including the bulrush *Scirpus subterminalis* Torr. Porter and Francko (1991) used leaf disks from *Potamogeton amplifolius* Tuckerm. to investigate the effect of chromium and copper on short-term (minutes to hours) PCA rates.

Francko (1986a,b) reported a liquid culture technique for germination and axenic cultivation of *Nelumbo lutea* (Willd.) Pers. (American lotus) seedlings and a petiole section PCA assay modeled after Beer and Wetzel (1981). Lotus petioles contained high concentrations of chlorophyll *a* (*ca.* 3% of fresh weight biomass) and exhibited PCA rates of approximately 500 to 30 µmol C mg^{-1} Chl *a* hr^{-1} over a pH range of 6.5 to 8.5. This PCA rate was comparable to that observed in leaves of bicarbonate-using submerged aquatic angiosperms (reviewed by Spence and Maberly 1985). In further work with lotus seedlings, Al-Hamdani and Francko (1992) characterized petiolar PCA and elongation rates in seedlings exposed to a variety of photon flux densities and temperatures and demonstrated that petiolar PCA may contribute to seedling growth and elongation, even under relatively cool, low- light conditions near the sediment-water interface.

The objectives of this study were: 1) to determine the effects of exposure to hexavalent chromium on PCA rates and growth of lotus seedlings and 2) to assess the potential for using lotus seedlings as a bioassay for chromium in fresh waters.

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MATERIALS AND METHODS

Lotus seeds were collected from plants growing at the margin of Sangre Isle Lake, a small eutrophic reservoir in north-central Oklahoma (Francko 1987). Seeds were surface disinfected using sequential rinses in detergent, 70% ethanol, distilled water, 5% sodium hypochlorite and distilled water, then scarified, and cultured aseptically by the method of Francko (1986a) in modified Medium II (Forsberg 1965) at pH 8.2 with 1.96 μmol l^{-1} total inorganic carbon and double-strength Hepes as a buffer. Alternatively, cultures were maintained at pH 5.6 using Mes in place of Hepes as the buffer system. Neither buffer system alters lotus growth or PCA capacity (Francko 1986b, Al-Hamdani and Francko 1992). Aseptic culture techniques were used to eliminate interference from algal, bacterial, or fungal contaminants in subsequent assays. Culture flasks containing 700 ml Medium II and 10 seeds each were incubated in an environmental chamber at 23C, using cool white fluorescent illumination to produce a photon flux density of 100 μmol m^{-2} s^{-1} and a 12-hr photoperiod.

Two petioles of unequal length differentiate upon seed germination (2 to 3 days after placement in liquid media). When the longer petioles bearing the developing floating leaf reached 4 to 5 cm in length, seedlings were withdrawn and randomly placed in fresh Medium II (N = 6 seedlings per flask) containing no added chromium or 0.5, 5, 25, 50 or 100 mg/l of hexavalent chromium (added as potassium dichromate; N = 3 replicates for control and each chromium concentration).

Individual seedlings then were labeled for later identification with a waterproof mark on the seed coat, and the length (cm) of each marked seedling's long petiole was measured. Cultures were placed randomly back into environmental chambers under the conditions described above.

A control culture and one culture at each chromium concentration were randomly withdrawn after 4, 48, and 96 hr of incubation. Seedlings were removed and blotted dry with Kim-Wipes and the length of the longer petiole of each seedling was remeasured. These petioles were then excised for use in PCA assays.

Culture media samples were withdrawn from each flask at each sampling iteration and filtered through 0.6-μm Nuclepore filters. Replicate subsamples of each filtrate were then assayed for soluble chromium via a flame AA technique using EPA reference standards. The analytical error of these measurements was approximately 16% over the range (0.5 to 50 mg/l) of reference standards. All chromium concentrations in this paper represent measured soluble chromium available at the time of assay rather than total chromium added at zero time. For consistency in data presentation, however, data are rank ordered on the basis of chromium added at time zero.

PCA rates were determined by a ^{14}C-bicarbonate assimilation/tissue solubilizer digestion method that facilitates complete digestion of plant material and incorporated radioactivity (Beer *et al.* 1982, Francko 1986b, Al-Hamdani and Francko 1992). After measurement of individual petiole lengths petioles were cut into 1-cm sections (24 ± 0.5 mg fresh wt). In order to assay petioles of relatively uniform diameter, petiole sections within 2 cm of the crown and apical meristem were discarded.

Randomly selected sections (N = 5) were placed in 50-ml glass beakers containing 20 ml of fresh sterile Medium II at either pH 5.6 or pH 8.2. Hexavalent chromium was added at concentrations of 0.5 to 100 mg/l and beakers were placed in a fume hood at the same illumination and temperature conditions noted above. At time zero, 100 μl of a ^{14}C-bicarbonate solution was added to each beaker to yield 1.5 x 10^4 Bq ml^{-1} of total radioactivity. The ^{14}C-addition did not alter significantly the initial inorganic carbon concentration of the media. PCA rates are linear for up to 45 min under these conditions (Francko 1986b).

After 15-min incubation periods, individual petiole sections were withdrawn and rinsed for 15 sec in acidified distilled water to remove unincorporated radioactivity. Petioles were then placed in glass scintillation vials and digested overnight with 0.5 ml of a quaternary ammonium tissue solubilizer (BTS-450, Beckman Co.).

Scintillation cocktail was then added and incorporated radioactivity was assayed by liquid scintillation spectroscopy using an automatic quench control program. Solubilized petiole sections that had not been incubated with radiolabel and sections incubated with radiolabel in total darkness were used to compute background-corrected mean radiolabel incorporation values (N = 5 sections for each treatment). Aliquots of solubilized petiole sections were also used for chlorophyll *a* analyses by a fluorometric method (Francko 1986b). Radiolabel uptake rates were then used to calculate carbon assimilation rates as μmol C mg^{-1} Chl *a* hr^{-1} by the ^{12}C:^{14}C ratio method of Wetzel and Likens (1979).

The PCA method we employed may overestimate net photosynthesis rates since unincorporated ^{14}-C that has exchanged with the internal dissolved inorganic carbon pool may not be removed quantitatively by a brief rinse in low-pH water. To determine the size of this error component, we conducted a replicate series of experiments in which sections were incubated at pH 5.6 or 8.2, fixed by immersion into liquid nitrogen, ground in 0.5 N perchloric acid, and then digested with solubilizer. Resultant PCA rates were then compared with those in sections treated only with tissue solubilizer as described earlier.

RESULTS AND DISCUSSION

Data on the relationship between medium chromium content and pH, exposure time period, and PCA rates are shown in Figure 1. At pH 5.6, significant increases in PCA rates ($P < 0.05$; analysis of variance (ANOVA) and Newman-Kuels multiple range test) were noted only in 48-hr exposure experiments, but no clear dose-response relationship could be determined. Plants incubated in media containing 0.02 to 2.2 mg/l of soluble chromium for 48 hr produced statistically similar effects on PCA. PCA rates were also significantly higher than those in chromium-free controls after 96 hr in media containing 0.1 mg/l of soluble chromium, and significantly reduced after 4 hr of incubation in media containing the highest titer of chromium employed.

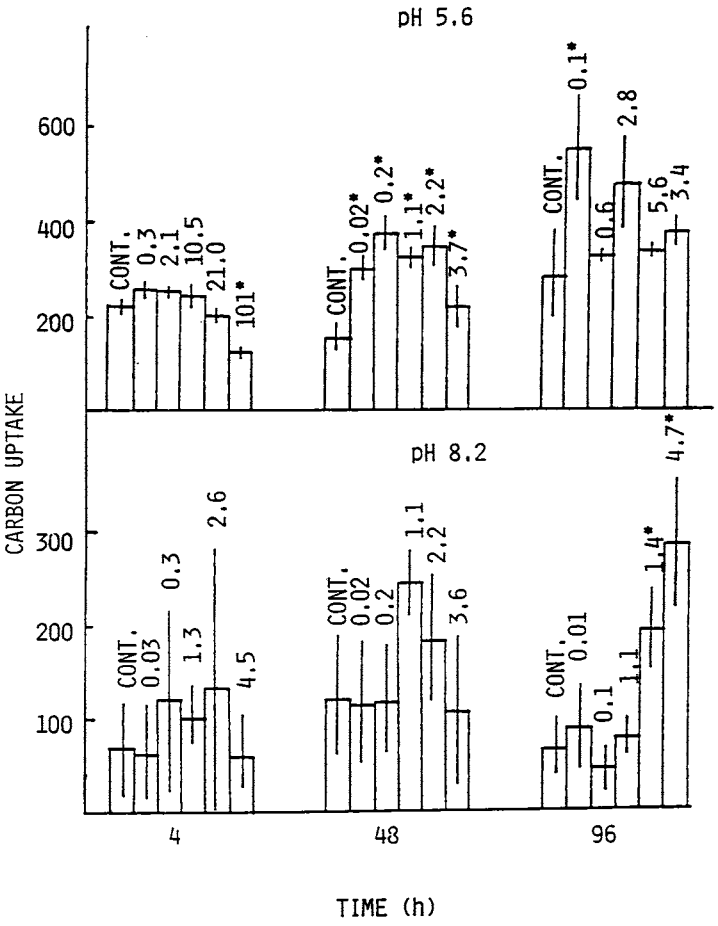

Figure 1. PCA rates in lotus petiole sections as a function of chromium concentration, exposure time, and pH. Chromium values within the bars denote soluble chromium present in culture filtrates at the time of analysis, but histograms are presented in increasing magnitude of chromium addition at time zero. Values shown as mean C-uptake rate (µmol C mg^{-1} Chl *a* hr^{-1}). Error bars denote SE (N = 6 sections per treatment variant). Asterisks denote treatments significantly different ($P < 0.05$; ANOVA and Newman-Kuels multiple range test) from control containing no added chromium (< 4 µg/l chromium present in media lacking additional chromium addition).

In pH 8.2 media, no statistically significant relationships between chromium content and PCA rates were determined in 4- and 48-hr experiments. Sections from plants incubated for 96 hr in media containing 1.4 and 4.7 mg/l of soluble chromium at the time plants were removed and assayed exhibited PCA rates 2- to 3-fold higher than the control value.

The PCA rates we measured in plants from control cultures lacking added chromium agree closely with rates previously reported for lotus seedlings assayed at the same temperature and photon flux density (approximately 220 µmol C assimilated mg^{-1} Chl *a* hr^{-1} at pH 5.6 (Al-Hamdani and Francko 1992) and 60 µmol C assimilated mg^{-1} Chl *a* hr^{-1} at pH 8.2 (Francko 1986b)). However, the data in Table 1 suggest that the method we employed more closely approximates inorganic carbon uptake into the petioles and overestimates carbon fixation rates. PCA rates in petiole sections incubated with radiolabel at pH 5.6 and treated with liquid nitrogen, acid digestion, and basic tissue solubilizer were about 7-fold smaller than PCA rates derived from petioles digested with solubilizer alone. At pH 8.2 the discrepancy between methods was 3- to 4-fold.

TABLE 1. PCA RATES IN *NELUMBO* PETIOLE SECTIONS DIGESTED WITH TISSUE SOLUBILIZER ALONE VERSUS SECTIONS FIXED IN LIQUID NITROGEN (NL_2) GROUND IN 0.5N PERCHLORIC ACID, THEN DIGESTED WITH TISSUE SOLUBILIZER. VALUES SHOWN AS MEAN µmol C fixed mg^{-1} Chl *a* hr^{-1} (SE); N=6.

	pH 5.6 Media		pH 8.2 Media	
	Trial 1	Trial 2	Trial 1	Trial 2
Solubilizer alone	239(10)	211(14)	33(3)	33(3)
LN2, Acid, Solubilizer	37(1)	36(3)	8(2)	10(1)

Accordingly, the collective data in Table 1 and Figure 1 support the view that chromium concentrations between 0.02 and 4.7 mg/l may enhance the rate of carbon uptake into lotus petioles exposed to the metal for 48 or 96 hr. Although heavy metals are typically associated with inhibition of metabolic activity, the apparent PCA stimulation we noted is not without precedent. In addition to the work on chromium and *Myriophyllum* by Guilizzoni *et al.* (1984) described in the Introduction of this paper, Jana and Choudhuri (1981) reported that copper (10 mg/l to 10 g/l) stimulated glycolate metabolism in *Potamogeton pectinatus*. Porter and Francko (1991) found that chromium and copper were capable of stimulating or repressing PCA rates in leaf disks from *Potamogeton amplifolius*, although both metals repressed PCA at or above 0.5 mg/l.

In contrast to the modest stimulatory effect of chromium on PCA, seedling growth and petiolar elongation were more markedly altered by chromium. The mean length of seedling petioles placed into various chromium treatments was statistically similar at the beginning of 48- or 96-hr incubation experiments (Table 2). Plants exposed to the two highest chromium levels for 48 hr at pH 5.6 had significantly shorter petioles than control plants, and petioles were significantly shorter in all but the smallest chromium addition after 96 hr. At pH 8.2, only the highest chromium addition employed yielded a significant decrease in mean petiole length after either 48 hr or 96 hr of exposure.

The rate of petiole elongation (change in length of individual petioles over the incubation period) was strongly and dose-specifically reduced by increasing doses of soluble chromium (Figure 2). After a 48-hr exposure period at pH 5.6, a significant decrease in petiole elongation rate was noted when as little as 0.2 mg/l of soluble chromium was present in media (5 mg/l initial dose). The magnitude of decrease increased up to 3.7 mg/l of soluble chromium (100 mg/l initial dose), at which point petiole elongation was reduced to about 20% of the control rate. A 96-hr exposure period at the same pH reduced the apparent chromium toxicity threshold to 0.1 mg/l and the maximum inhibition to approximately 10% of the control value.

In pH 8.2 media, low doses of chromium were associated with a slight increase in elongation rates after 48 hr; only the highest chromium addition yielded a decrease in elongation.

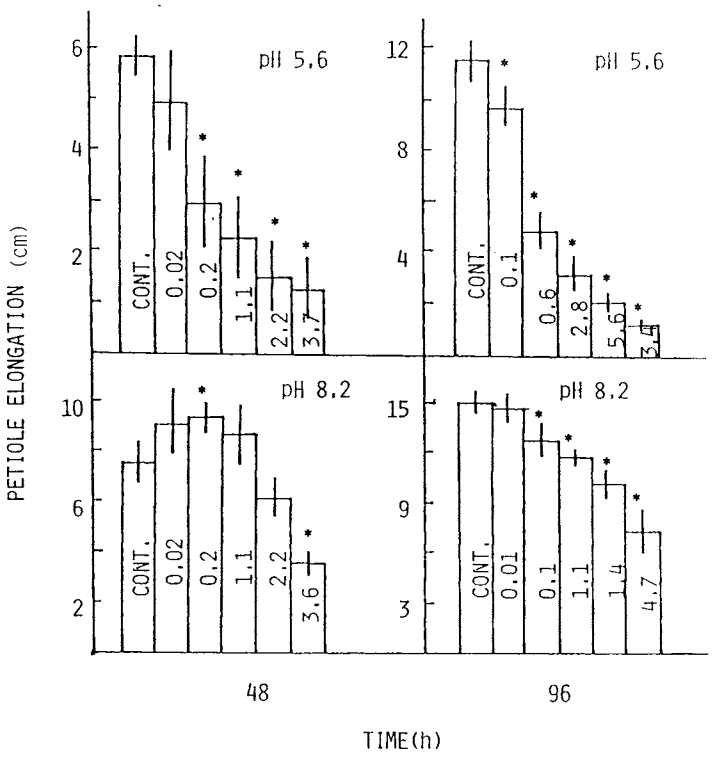

Figure 2. Petiolar elongation rates (change in cm from time zero to time 48 or 96 hr) in lotus seedlings as a function of chromium concentration, exposure time, and pH. Soluble chromium concentrations in growth media at the time of assay shown within histograms. Error bars denote SE (N = 6 seedlings per treatment). Asterisks denote treatment means significantly different from controls ($P < 0.05$; ANOVA and Newman-Kuels multiple range test).

TABLE 2. EFFECT OF 48-HR AND 96-HR CHROMIUM EXPOSURE ON PETIOLAR LENGTH IN *NELUMBO* SEEDLINGS. VALUES SHOWN AS MEAN (SD); N = 6. ASTERISKS DENOTE TREATMENTS SIGNIFICANTLY DIFFERENT FROM ZERO CHROMIUM CONTROL ($P < 0.05$; ANOVA and Newman-Kuels multiple range test).

Chromium (mg/l)	48 hr, Mean Petiole Length (cm)		Chromium (mg/l)	96 hr, Mean Petiole Length (cm)	
	Initial	Final		Initial	Final
			pH 5.6		
0	4.4(1.5)	10.2(1.3)	0	4.0(0.9)	15.4(2.0)
0.02	5.2(0.4)	10.2(2.2)	0.1	4.4(0.5)	14.0(2.5)
0.2	5.0(1.1)	7.9(1.8)	0.6	5.7(0.5)	10.1(2.2)*
1.1	5.6(0.9)	7.8(1.8)	2.8	4.4(1.1)	7.1(2.4)*
2.2	4.5(1.0)	5.9(0.6)*	5.6	5.1(1.1)	7.0(1.6)*
3.7	4.8(1.0)	5.9(1.6)*	3.4	4.7(0.8)	5.9(0.6)*
			pH 8.2		
0	11.1(2.2)	18.4(1.3)	0	11.0(0.9)	25.9(1.6)
0.02	12.2(1.3)	21.1(2.2)	0.01	10.9(2.2)	25.7(2.6)
0.2	11.3(0.9)	20.3(2.2)	0.1	11.8(3.1)	24.2(1.8)
1.1	12.3(3.2)	20.6(4.8)	1.1	10.7(2.5)	22.8(3.6)
2.2	11.7(2.5)	17.7(3.9)	1.4	11.3(2.0)	21.2(3.4)
3.6	10.9(1.3)	14.5(2.3)*	4.7	11.6(1.5)	18.9(4.6)*

After 96 hr of exposure at pH 8.2, elongation rate reductions with increasing soluble chromium resembled pH 5.6 values, although the maximum inhibitory response was considerably smaller.

The collective data suggest that 1) soluble chromium may have a slight stimulatory effect on carbon uptake rates in excised lotus petioles and 2) that the rate of petiole elongation is markedly and dose-specifically reduced by increasing concentrations of soluble chromium. Both effects were more pronounced at pH 5.6 than at pH 8.2.

Although the sensitivity and dose-responsiveness of lotus petiolar elongation support the possible utility of this technique as a bioassay system, several important points require further investigation. Our observation that the solubilizer method overestimates carbon fixation dictates the need for additional clarification on whether chromium alters carbon uptake, carbon fixation, or both processes. Petiolar elongation assays based on soluble hexavalent chromium present in media need to be coupled to tissue chromium uptake assays. Finally, petiolar elongation must be differentiated into physiological processes that reflect true growth (*i.e.*, increase in biomass) with those resulting in elongation due to cell expansion and etiolation (increase in length without an increase in biomass). Such research may shed light on the apparent contradiction between stimulation of PCA rates and reduction of petiolar elongation rates noted here. Al-Hamdani and Francko (1992) provided evidence that changing rates of elongation in lotus petioles exposed to differential environmental stimuli may result from a change in both biomass accumulation and etiolation rates.

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