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Response to Elevated CO_2 of Indian Mustard and Sunflower Growing on Copper Contaminated Soil

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Phytoextraction has been defined as the direct use of living green plants in order to extract pollutants from contaminated soils and concentrate them into roots and easily harvestable shoots (Cunningham and Berti 1993; Raskin *et al.* 1994; Salt *et al.* 1995; Baker *et al.* 1989; Cunningham and David 1996). When compared to physical- and chemistry-based processes such as excavation and soil washing, interest in the technique has been increasing during recent years (Black 1995) because it offers a cost-effective and environmentally sound pollution-remediation option. However, there still are many problems that need to be addressed before this technology is widely accepted and utilized. One of the key problems is how to enhance the uptake of metals by plants in order to increase absolute phytoremediation efficiency, but at the same time increase biomass production in order to increase relative phytoremediation efficiency (Tang and Wilke 1999). A review of literature showed that more than 400 plants have been identified to possess ability to uptake and absorb unusually large amounts of metals, but the majority of them have very low biomass production in their native habitats (Karenlampi *et al.* 2000). Thus, the possibility of increasing plant biomass production, enhancing the plant uptake of metals, but meanwhile creating no secondary contamination, has intrigued many scientists in recent years. Much of the previous work on phytoextraction has involved study of soil amendments (Huang *et al.* 1997a, b; Anderson *et al.* 1998; Blaylock *et al.* 1997) and transgenetic plants (Glass 1997; Black 1995; Karenlampi *et al.* 2000; Clemens *et al.* 2002). However, either technique may pose a potential risk to the environment (Steinbrecher 1996; Huang *et al.* 1997a, b; Glass, 1997).

There exists a large body of literature which reports that the enrichment of the atmosphere with carbon dioxide (CO_2) could increase the yield of crops (Luxmoore *et al.* 1986; Luxmoore 1981). This leads to the speculation that enrichment of CO_2 to plants in a growth chamber may yield higher biomass production of metal accumulators, increase the tolerance of plant species to metal toxicity, and possibly induce hyperaccumulation in plants. However, to our knowledge, little has been done so far in order to investigate the responses of metal accumulators to CO_2 enrichment, from the viewpoint of phytoremediation.

In order to test this hypothesis, we investigated the responses, to CO_2 enrichment, of Indian mustard (*Brassica juncea L. Czern.*) and sunflower (*Helianthus annuus*

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L.), both used as model plant species for phytoremediation. Our objectives of this study were (a) to investigate the possibility of using CO_2 fertilizer in order to increase biomass production of the two species, (b) to study the possible improvement of copper tolerance of these metal-accumulating plant species to higher levels of copper through CO_2 enrichment, and (c) to trigger copper hyperaccumulation in plants through the use of CO_2 fertilization.

MATERIALS AND METHODS

A greenhouse experiment was conducted using 1 kg of topsoil, collected from the experimental agricultural field at Huajiachi Campus, Zhejiang University. Table 1 shows some properties of the soil tested. Soil organic matter, pH, CEC, and soil texture were measured using the same methods as described previously (Tang *et al.* 1999).

Table 1. Physical and chemical characteristics of the soils used for this study.

Total N (%)	0.06
Total P(%)	0.24
Available P (mg kg^{-1})	58.10
Available K (mg kg^{-1})	22.74
Cu (mg kg^{-1})	7.33
Zn (mg kg^{-1})	19.81
Organic matter (%)	1.50
CEC (cmol/100g soil)	7.27
pH(H_2O)	7.14
pH($CaCl_2$)	6.89
HWCmax(%)	29.45
Soil texture	
Clay	9.7
Silt	74.7
Sand	15.6
Soil texture (USDA)	Silty loam

Fresh soils were sieved to pass a 3 mm mild steel sieve, and mixed with a basal fertilizer (100 mg N kg^{-1} soil as $Ca(NO_3)_2.4H_2O$; 400 mg P kg^{-1} soil as CaH_2PO_4; 252 mg K kg^{-1} soil as KH_2PO_4) (Brown *et al.*, 1994). After allowing four weeks for equilibration, the soil was artificially contaminated with varying volumes of copper nitrate solution (10% w/v) in order to produce a suite of soils containing different concentrations of the metal. A further sample of soil was retained uncontaminated as a control. The contaminated soils were denoted as *low copper* (LCu; 100 mg Cu kg^{-1}) soil and *high copper* (HCu; 200 mg Cu kg^{-1}). Both concentrations of copper were based upon weights of dry soils. After addition of copper, the soil was kept in the darkness for a further six weeks in order to attain equilibration.

Seeds of Indian mustard and sunflower were first sterilized with 0.1% sodium hypo-chlorite and then germinated in a mixture of perlite and vermiculite (volume ratio 1:1) for 11 days. Three viable seedlings of each species were subsequently transferred to individual plant pots. Soil moisture content and maximum water holding capacity (WHC_{max} %) were determined prior to the experiment. The soil moisture was maintained at 80% WHC_{max} using deionized water during the period

of the experiment. Each treatment was replicated four times. The experiment was carried out in a greenhouse at ambient temperature with natural illumination, but without climate control. The experiment was performed during April and June 2000.

After an initial growth period of 24 days following transplanting, treatment with elevated levels of CO_2 commenced. All pots were divided into three sets. One set received only CO_2 from the ambient atmosphere (about 350 $\mu L\ L^{-1}$) and acted as a control. The other two sets of the pots received 800 $\mu L\ L^{-1}$ and 1200 $\mu L\ L^{-1}$ CO_2, respectively. Pots treated with an enhanced supply of CO_2 were encased with vaulted polyethylene film (0.2 mm in thickness), the base of which was sealed around with water, forming a closed chamber of ca 2 m^3 volume. Two parallel 8 mm diameter perforated PVC pipes were fixed inside the chamber. Both pipes were connected to another extending to the outside for the supply of CO_2 gas. Each chamber was fitted with a blower capable of delivering approximately 3000 L min^{-1}. The blower was fixed vertically in the center of the chamber. CO_2 gas (99% purity) was added to the chamber by the blower through the external PVC pipe. The flow rate of CO_2 was controlled by a flow meter connected to the gas cylinder. Canopy CO_2 levels were maintained at approximately 800 and 1200 $\mu L\ L^{-1}$ in each treatment chamber, respectively. The CO_2 level was monitored with an infrared gas analyzer (APBA-250E, Holland). For the non-enriched control, the canopy CO_2 level averaged 350 $\mu L\ L^{-1}$. CO_2 enrichment was applied to plants from 08:00 to 11:00 h on sunny days. Total CO_2 treatment time was twelve days.

Plants were then harvested by cutting the shoots 2 cm above the soil surface, and roots were separated from the soil. Plant samples were divided into leaves, stems, and roots. Roots of the plant samples were rinsed with deionized water, and blotted dry on a paper towel. Pooled samples (the parts of plants) grown in the same pot were recorded for fresh weight and then dried to constant weight for at least 3 days at 70°C. Plant material was then ground using an agate ball mill. Subsamples of ground material (ca 0.5 g) were digested with a mixture of concentrated HNO_3-$HClO_4$ (1:1; 10 cm^3). The resultant solutions were made up to a final volume of 50 cm^3, and analysed by flame atomic absorption spectrometry (FAAS, Spectr AA 220).

All data obtained were statistically analyzed by the three-way ANOVA routine in the software package SAS (Version 6.12, SAS Institute Inc., Cary, USA) in order to allow comparison of treatment means. Significant differences between plant species, CO_2, and Cu treatments were identified at the $p<0.005$ confidence level, using Dunnett's multiple unplanned comparison of means.

RESULTS AND DISCUSSION

After ten days following transplanting, all sunflower seedlings growing in LCu and HCu treated pots and under ambient CO_2 levels were observed to show toxic symptoms in the leaves while those in control pots grew well. Chlorosis developed in the leaves of all Indian mustard growing in LCu and HCu treated pots and at ambient CO_2 levels twelve days following transplanting, showing obvious toxic symptoms compared to the corresponding controls. All Indian mustard and sunflower seedlings growing at elevated CO_2 levels exhibited better growth than the CO_2 controls, while those growing at ambient CO_2 level showed poor growth at high levels of copper, suggesting an improvement of growth following the application of CO_2.

The biomass production of shoots increased at elevated CO_2 levels, with the average dry shoot weight increasing by 5% to 200% for both species (Table 2). The highest dry shoot weights for Indian mustard and sunflower were obtained from the low copper treatment, along with exposure to 1200 $\mu L\ L^{-1}$ CO_2. At ambient CO_2 levels, all copper contamination caused highly significant decreases in shoot and root yields of Indian mustard and sunflower, which exhibited obvious toxic symptoms (Table 2). However, when grown under elevated CO_2 conditions, sunflower and Indian mustard exhibited an increase in average shoot dry weight per pot, but the magnitude of increase was different between pots treated with copper and without copper. It was apparent that species growing in enriching CO_2 exhibited a significant increase in shoot biomass from pots treated with high concentrations of copper, than from those without copper, compared to those grown in ambient CO_2 (Table 2). At high CO_2 levels of 1200 $\mu L\ L^{-1}$, both Indian mustard and sunflower showed a significant net increase in dry shoot weight. In addition to biomass increase with increasing CO_2 in the growth chamber, it was also observed that Indian mustard and sunflower, grown under elevated CO_2 levels, possessed more leaf area when compared to those grown at ambient atmospheric CO_2 levels. These results were in agreement with that documented in the literature (Morison and Gifford 1984; Idso and Idso 1994).

It is realized that with CO_2 enrichment in the air, plants generally grow larger, with more branches or tillers, more and thicker leaves, and more extensive root systems as well as an increase in flowers and fruit (Idso *et al.* 1989). Similar results from our present study have also been achieved. In consequence of CO_2 enrichment, Indian mustard and sunflower grew higher and larger, and had more and thicker leaves, and produced larger leaf areas, compared to the plants growing under ambient CO_2 levels. At toxic concentrations of copper, CO_2 application more than doubled plant biomass production and obviously alleviated the toxic symptom produced by copper contamination. This is a significant finding, since the increase

of plant biomass resulting from CO_2 application could suggest that more metal be taken up from the contaminated growth media, and that the tolerance to metal toxicity be improved. Obviously, this could help metal accumulators survive on the metal stress conditions, shorten the time needed for clean-up of contaminated sites, and, therefore, increase relative phytoremediation efficiency.

Table 2. Dry weight (g /per pot, DW) of Indian mustard and sunflower grown in pots and exposed to different levels of CO_2.

Plant specie	Cu added to soil ($mg\ kg^{-1}$)	CO_2 concentration ($\mu L\ L^{-1}$)	Root Average±SD	Shoot Average±SD
Sunflower	0	350	2.96±0.44 A, a, a'	8.84±0.53A, a, a'
		800	3.63±1.9 A, a, a'	9.01±1.19A, a, b'
		1200	3.04±0.73 A, a, a'	9.28±0.48 A, a, c'
	100	350	2.84±1.46 A, b, a'	7.66±3.4 A, b, a'
		800	2.23±0.25 A, b, a'	8.11±0.50 A, b, b'
		1200	2.51±0.52 A, b, a'	9.29±0.87 A, b, c'
	200	350	0.38±0.23 A, b, a'	2.13±0.86 A, c, a'
		800	0.36±0.08 A, b, a'	3.14±1.02 A, c, b'
		1200	0.66±0.35 A, b, a'	4.30±0.46 A, c, c'
Indian mustard	0	350	0.83±0.82 B, a, a'	2.35±0.07 B, a, a'
		800	0.81±0.22 B, a, a'	2.49±0.49 B, a, b'
		1200	0.73±0.23B, a, a'	3.61±0.71 B, a, c'
	100	350	0.89±0.96 B, b, a'	2.36±1.57 B, b, a'
		800	0.83±0.35 B, b, a'	3.24±0.44 B, b, b'
		1200	0.85±0.43 B, b, a'	4.42±0.60 B, b,c'
	200	350	0.52±0.12 B, c, a'	1.83±1.03 B, c,a'
		800	0.43±0.19 B, c, a'	1.94±0.72 B, c, b'
		1200	0.47±0.07 B, c, a'	2.81±0.83 B, c, c'

Note: Within each column, values followed by the same letter are not significantly different as determined by Dunnett's test ($p<0.005$) for all values. A, a, and a' represent plant species, copper treatments, and CO_2 treatments, respectively.

Table 3 illustrates the accumulation of copper by Indian mustard and sunflower growing under elevated CO_2 levels and at ambient atmospheric CO_2 levels. All plants growing in pots treated with copper, but under enhanced CO_2 levels, exhibited hyperaccumulation of copper, with concentrations of the metal being more than 1000 $mg\ kg^{-1}$ in the plant tissues on a dry weight basis. Copper concentrations in plant tissues were significantly higher in plants exposed to 800 $\mu L\ L^{-1}$ CO_2 than in plants growing under either 350 or 1200 $\mu L\ L^{-1}$ CO_2. In plants exposed to 800 $\mu L\ L^{-1}$ CO_2, copper concentrations in the roots, stems, and leaves were more than two times higher than that of controls, while enhanced CO_2 treatment had less influence on copper concentration in the roots, stems, and leaves

Table 3. Influence of CO_2 application on copper concentration in roots, stems, and leaves of Indian mustard and sunflower grown in pots treated with different levels of copper.

Species	Cu added to soil ($mg\ kg^{-1}$)	CO_2 ($\mu L\ L^{-1}$)	Copper concentration in plant tissues (mean±SD, $mg\ kg^{-1}$, dry weight)			Average leaf/root ratio	BF*
			Leaf	Stem	Root		
Indian mustard	0	350	154±44 A, a, a'	245±31 A, a, a'	199±23 A, a, a'	0.49	7.0
		800	527±125 A, a, b'	621±129 A, a, b'	287±52 A, a, b'	2.56	101.3
		1200	186±3 A, a, c'	121±46 A, a, c'	197±60 A, a, c'	0.83	37.8
	100	350	423±123 A, b, a'	361±171 A, b, a'	765±152 A, b, a'	0.11	0.6
		800	4586±263 A, b, b'	13696±1853 A, b, b'	2301±1751 A, b, b'	1.79	23.7
		1200	1587±173 A, b, c'	831±175 A, b, c'	672±245 A, b, c'	2.03	10.7
	200	350	538±87 A, c, a'	443±187 A, c, a'	977±114 A, c, a'	1.27	4.1
		800	2277±325 A, c, b'	1091±282 A, c, b'	2270±166 A, c, b'	2.24	10.3
		1200	1382±236 A, c, c'	957±342 A, c, c'	1362±503 A, c, c'	1.49	5.0
Sunflower	0	350	51±10B, a, a'	42±11 B, a, a'	106±25 B, a, a'	0.78	21.1
		800	664±214 B, a, b'	506±159 B, a, b'	290±73 B, a, b'	1.84	71.9
		1200	277±27 B, a, c'	192±96B, a, c'	333±73 B, a, c'	0.94	25.4
	100	350	60±7 B,b, a'	69±16 B, b, a'	557±28 B, b, a'	0.55	3.9
		800	2539±1110B, b, b'	1401±402 B, b, b'	1418±507 B, b, b'	1.99	42.7
		1200	1567±106 B, b, c'	1031±371 B, b, c'	564±117 B, b, c'	2.36	14.8
	200	350	857±297 B, c, a'	558±108 B, c, a'	674±51 B, c, a'	0.55	2.6
		800	2143±507 B, c, b'	1433±442 B, c, b'	958±12 B, c, b'	1.00	11.0
		1200	1037±149 B, c, c'	957±342 B, c, c'	696±183 B, c, c'	1.01	6.7

Within each column, values followed by the same letter are not significantly different as determined by Dunnett's test ($p<0.005$) for all values. As for A, a, and a' represent plant species, copper treatments, and CO_2 treatments, respectively.; BF*=average copper concentration in leaf/average copper concentration in soil. For the copper control pots, average copper concentration in soil = average copper concentration determined in soil. For the copper treated pots, average copper concentration in soil =average copper concentration in soil+ copper added.

of both species (Table 3). It was obvious that Indian mustard accumulated copper to a greater degree than the sunflower tested. For pots treated with same
of both species (Table 3). It was obvious that Indian mustard accumulated copper to a greater degree than the sunflower tested. For pots treated with same concentration of copper and exposed to the same CO_2 levels, copper concentrations in the tissues of Indian mustard were, in general, higher than in shoots of sunflower (Table 3).

The bioaccumulation factor (BF), calculated as the ratio of average copper concentration in leaf to copper concentration in soil, increased with increasing CO_2. This value was significantly higher in plants exposed to 800 $\mu L\ L^{-1}$ CO_2 than in plants growing under either 350 or 1200 $\mu L\ L^{-1}$ CO_2, suggesting that CO_2 supply substantially altered this factor (Table 3). The application of CO_2 also altered the leaf/root ratios of copper in both plants (Table 3). With increasing CO_2 levels in the growth chambers, plants exhibited a significant increase in the ratios, but the highest leaf/root ratios for copper were obtained in plants grown under 800 $\mu L\ L^{-1}$ CO_2 levels. The changes of leaf/root ratios in plants treated in atmospheres of enriched CO_2 may suggest the alteration of the plant-soil relationship (Baker 1981), possibly from excluder to accumulator, or even hyperaccumulator. This finding is of great significance, since that the leaf/root ratios of Indian mustard and sunflower increased with CO_2 enrichment, which may suggest enhancement in the translocation of copper from root to shoot when exposed to higher CO_2 levels up to 800 $\mu L/L$.

The large increase in uptake of copper by Indian mustard and sunflower, and the alleviation of chlorosis in their leaves with elevated CO_2, suggest that both species may be able to increase the internal recycling of deficient nutrients resulting from copper stress, as such mechanisms could be favorable to plant growth. It is apparent that greater translocation of copper from root to shoot requires some additional energy, and the enhanced application of CO_2 may act to compensate this energy consumption, but meanwhile provide extra energy to maintain rapid growth. This aspect of energy budget requires further investigation. Another possibility could be that atmospheric CO_2 enrichment could help plants cope with those limitations to growth imposed by insufficient water and soil nutritional deficiencies resulting from metal stress (Idso and Idso 1994). The third possibility may be related to changes in the characteristics of the rhizosphere. It is already known that CO_2 enrichment is generally associated with rhizospheric acidification. Our experimental measurements indicate that the application of CO_2 decreased the rhizospheric soil pH values down by 0.2 to 0.5 pH units. Such acidification could improve the bioavailability of copper in solution. CO_2-triggered hyperaccumulation may be associated with this process. It is more likely that the enriching CO_2 is involved both in the mechanism of copper tolerance and in high

rates of copper transport into the leaves. The latter process is suggested since it is known that atmospheric CO_2 enrichment acts to enhance the activity of soil rhizosphere organisms, which may then secrete more organic acids and thus extend the area of root surface available for nutrient uptake (Boyle and Voigt 1973; Fogel 1983; Lamborg 1983; Tinker 1984; Clarkson 1985). The enhancement of organisms in the rhizosphere is also favorable to the uptake of metals and other nutrients by plants. Further research in this area is needed in the future.

Carbon dioxide-triggered phytoextraction technology has more advantages than chelate-mediated phytoextraction. For the latter, there exists the worry that unbridled use of chelates may lead to groundwater contamination, if their application to the soil is not performed in a reasoned, controllable manner. It is clear that CO_2-triggered hyperaccumulation has advantages in not only reducing the risk of contaminant movement from soil to groundwater but also by enhancing the accumulation of contaminants by plants, and increasing the biomass production. Application of this CO_2-assisted phytoremediation in the field will speed up the removal of contaminants from contaminated soils and provide a cost-effective and environmentally friendly means for the clean-up of polluted sites.

When compared to chelate-assisted hyperaccumulation (Anderson *et al.* 1998), the costs involved in this technology are slightly higher, but still within an acceptable range. It is estimated that to build, in China, one hectare of simple greenhouses suitable for CO_2 fertilization would cost about $50,000. Such additional increase of cost may be recouped in several ways. First, covering contaminated sites with simple polyethylene film greenhouses can prevent the contaminants from blowing away and leaching down to underground water by rainfall. Second, use of simple greenhouses may provide better growth conditions for plants and would thus affect both yield and economy.

Last, a simple greenhouse may provide the optimum conditions for CO_2 fertilization, which could increase the number of crops. As a result, plants would be induced to accumulate more metals and produce more biomass, and thus shorten the time required for phytoextraction. Despite this, there is a strong need for more research before the advantages of this particular effect of CO_2 on biomass production and metal accumulation can be fully utilized in practice. We believe that the use of CO_2 fertilizer for triggering hyperaccumulation in plants, and increasing biomass production could open up the way for enhanced phytoremediation and for phytomining.

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