Agrochimica, Vol. L - N. 5-6 Settembre-Dicembre 2006

Response of *Miscanthus sinensis* to prolonged applications of chromium in hydroponics

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INTRODUCTION. – Among metals that pose an environmental hazard, chromium is an abundant element on Earth, with the natural concentration averaging about 120 mg kg^{-1}. It occurs in several oxidation states, with the trivalent and hexavalent states being the most common in the environment. Whilst Cr(III) is relatively immobile and low in reactivity due to its strong adsorption capacity onto soils, Cr(VI) is highly unstable and mobile under natural conditions (MUKHERJEE, 1998). In consequence, the trivalent form is generally not regarded as toxic and it even plays an essential role for glucose, fat and protein metabolism in mammals, whereas hexavalent chromium is classified as a primary mutagenic contaminant that is extremely toxic to all forms of life (MUKHERJEE, 1998; RENGARAJ *et al.,* 2001). Nevertheless, both Cr forms should be regarded as potential environmental contaminants, since they can convert to each other (BARTLETT, 1997). According to PRUEβ (1997), in neutral conditions the mobile fraction of all Cr forms in soils is very low (approximately 15 μg kg^{-1}) but it increases more than threefold at pH values lower than 4.5. Besides, organic acids like those present in root exudates have been found to increase plant uptake of Cr(III) (SRIVASTAVA *et al.,* 1999).

Chromium salts are widely used in dyeing, tanning and plating processes and the main antropogenic sources of chromium pollution are the effluents from these industrial processes and the municipal wastes and sewages where Cr containing products are disposed (DUBE *et al.,* 2003). A substantial part of Cr wastes is produced by the leather industry, where chromium sulfate is used in the tanning process to form stable complexes with amino acids and proteins. Since the uptake of chromium into the leather is not complete, relatively large amounts of Cr escape into the effluents in which the Cr concentration can reach up to 2-5 g L^{-1} (VAJPAYEE *et al.,* 1999; KHAN, 2001). Thus, high economic efforts are needed to make the Cr concentration of waters comply with

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the limits raccomended by the World Health Organization for irrigation (0.1 mg L^{-1}) and drinking waters (0.05 mg L^{-1}) (ENDERLEIN *et al.,* 1997).

Phytoremediation is an emerging and promising technology that uses living plants for the decontamination of soils and waters. For the abatement of Cr concentration in effluents the best suited phytoremediation techniques are rhizofiltration, i.e. the use of plant roots to retain physically and adsorb chemically Cr compounds on their surfaces, and phytoextraction, that uses the ability of roots to absorb chromium and incorporate it into plant tissues (ARDUINI and MASONI, 2002). The above techniques give an advantageous alternative to conventional water remediation technologies, since growing plants is relatively inexpensive, the concentrated hazardous wastes require smaller disposal facilities and the potential exists to recover the metal from the contaminated hypogeal biomass (CHEN and CUTRIGHT, 2001). In addition, a profit can be obtained from the aerial biomass, provided that chromium is not translocated within the plant (ARDUINI and MASONI, 2002). For these reasons these techniques are attractive, especially in developing countries and considerable research is being made in order to select species able to remove chromium from waste waters and from soils contaminated with Cr wastes (RAI *et al.*, 1995; VAJPAYEE *et al.,* 1999; KHAN, 2001; MEI *et al.*, 2002; HAN *et al.*, 2004). In parallel, the effects of the most common chromium compounds on plant growth and metabolism have been investigated and, though a direct role of Cr in plant metabolism is uncertain, both stimulatory and inhibitory effects on growth have been reported (DUBE *et al.*, 2003; SHARMA *et al.*, 2003; HAN *et al.*, 2004). A higher toxicity of Cr(VI) compared to Cr(III) was reported for *Brassica juncea* and *Vigna radiata* (HAN *et al.*, 2004; SHANKER *et al.*, 2004), whereas Cr(VI) was found to be more toxic to shoots and Cr(III) to roots in *Avena sativa* (MCGRATH, 1982). In general, plants treated with hexavalent chromium show interveinal chlorosis and, later, necrosis of young leaves, loss of moisture, reduced chlorophyll concentration and reduced nitrogen uptake and assimilation (SHARMA *et al.*, 1995; VAJPAYEE *et al.*, 1999; DUBE *et al.*, 2003; SHARMA *et al.*, 2003). Due to its high oxidation power, VAZQUEZ *et al.* (1987) suggest that Cr(VI) exerts a primary toxicity on membranes causing disarrangement of cells and organelles. In contrast, the major effect of the trivalent Cr form seems to be an interference with the uptake of essential nutrients and especially with Fe (MORAL *et al.*, 1995; MEI *et al.*, 2002; PANDEY and SHARMA, 2003). Plants, however, display several detoxification mechanisms among which the most impor-

tant are the reduction of Cr(VI) to Cr(III) within the plant and the poor translocation of Cr from roots to shoots (ZAYED *et al.*, 1998; HAN *et al.*, 2004). Both characters increase the attractiveness of rhizofiltration for the abatement of chromium in waters, because of the possibility of gaining a profit from the low contaminated aerial parts of the plants.

In the present research, carried out in hydroponics, we have investigated the response of *Miscanthus sinensis* L. var. Giganteus to a long term exposure to four chromium-III concentrations between 19 and 76 mg L^{-1}. Plant growth, root morphology and Cr-uptake and distribution patterns were investigated in order to evaluate the rhizofiltration performances of this species. Miscanthus is a vigorous perennial Gramineae species originating from East Asia. It reproduces vegetatively by rhizomes and is widely cultivated as an energy crop throughout Europe. It develops a dense net of fibrous roots that could be an effective filtering system. Indeed, it was estimated that, in field conditions, roots of a three-year miscanthus crop reach a maximum depth of 250 cm and a dry weight of over 10 t ha^{-1} (NEUKIRCHEN *et al.*, 1999).

MATERIALS AND METHODS. – *Plant material and growth conditions.* Rhizomes of *Miscanthus sinensis* L. var. Giganteus were collected in early spring from a 7-year old crop and plants were grown on perlite in a nursery until culms were about 20 cm tall. They were then transferred to an open-air hydroponics installation equipped for the nutrient film technique that consisted of 10 pairs of 4 m long plastic channels (four Cr levels and one control, each replicated twice). The circulating nutrient solution had a volume of 80 L for each pair of channels (replicate) and was arranged following CLARK (1982) with slight modifications. Ion concentrations in the final solution (all the following expressed in mg L^{-1}) were: NO_3N, 351; Ca, 302.4; K, 283; S, 192; NH_4N, 128; Cl, 65; Mg, 37.8; Na, 4.56; Fe, 4; P, 2; Mn, 0.974; B, 0.536; Zn, 0.3; Cu, 0.076; Mo, 0.155. The pH and conductivity were 7.5 and 3.8 mS cm^{-1}. In order to keep the concentration of the nutrient solution constant, evaporated and transpired water was continuously replaced with tap water and, every two weeks, the nutrient solution was completely renewed. Plants were grown in the Cr-free nutrient solution for 44 days, when Cr was added as $Cr(NO_3)_3$ to final concentrations of 0 (Control), 19, 38, 57 and 76 mg L^{-1}. We chose chromium nitrate as source compound in order to avoid additional effects of the anion, and the N concentration was balanced in all treatments with ammonium nitrate. Throughout the whole experiment, secondary culms were cut just after emergence from the rhizome. Each replicate was comprised of 16 plants spaced at 50 cm intervals, which simulates a field density of miscanthus crops in Italian conditions. Four plants per replicate were randomly harvested just before chromium addition (0 days after treatment, DAT) and when control plants were flowering (104 DAT).

Growth parameters and chemical analyses. Harvested plants were rinsed with tap water and divided into shoot, rhizome and roots. For dry weight determination, all plant parts were dried at 75°C to constant weight. The Relative Growth Rates (RGR) were cal-

culated for each plant part, as:

$$RGR = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

where W is the dry biomass of each plant part at the beginning (W_1) and at the end (W_2) of the treatment period, and t_2-t_1 is the duration of the period.

A fresh root sample was taken from each plant for the estimation of root length, surface, volume and average diameter. Each root sample was thoroughly rinsed with tap water in order to remove Cr deposits from the root surface, and then it was divided into three subsamples of about 1 g fresh weight. Subsamples were weighed, cut into pieces of less than 10 cm, and distributed on a glass Petri dish of 20 cm diameter avoiding overlapping. By means of a Leica Quantimet 500 image analyser, we measured the length and the area of each root subsample, the former as the sum of the lengths of single root pieces, and the latter as the sum of the projections of single pieces on the Petri dish surface. Then, the average diameter of each root system was estimated dividing total area by total length.

The root surface was estimated, after KOKKO *et al.* (1993), as:

$$Root\ surface = \pi \infty Root\ area$$

Assuming that root systems are equivalent to cylinders whose diameter (*d*) corresponds to the root average diameter and height (*h*) corresponds to total root length, root volume was estimated as:

$$Root\ volume = \pi \times \left(\frac{d}{2}\right)^2 \times h$$

Chemical analyses and statistics. In order to verify if Cr addition and root activity affect the pH of the nutrient solution, during the whole experiment the pH was measured in each channel at a two week interval, i.e. just after (new) and just before (old) each change of the nutrient solution.

Chromium concentration of separate plant parts was determined by atomic absorption spectrometry (GBC 903 Single Beam, Australia) on ground samples (0.5 g) that were predigested overnight in 5 ml concentrated HNO_3, and then digested after adding i ml $HClO_4$ at 2200 C in an aluminium block digestor (Tecator ab, Sweden).

Data were analyzed with analysis of variance techniques (randomized block design) to test effects of Cr level.

RESULTS AND DISCUSSION. – *The pH of the nutrient solution.* The addition of chromium to the nutrient solution caused its pH to decrease immediately from 7.5 to values that ranged from 7.1 to 5.9 with increasing Cr supply (Figure 1). According to MEI *et al.* (2002), the decrease in pH was a consequence of the acidic properties of Cr(III) salts that have a tendency to hydrolyse and to form polynuclear complexes containing OH^- bridges. The precipitation of these complexes was probably responsible of the bluegreen deposits we observed on roots and that were par-

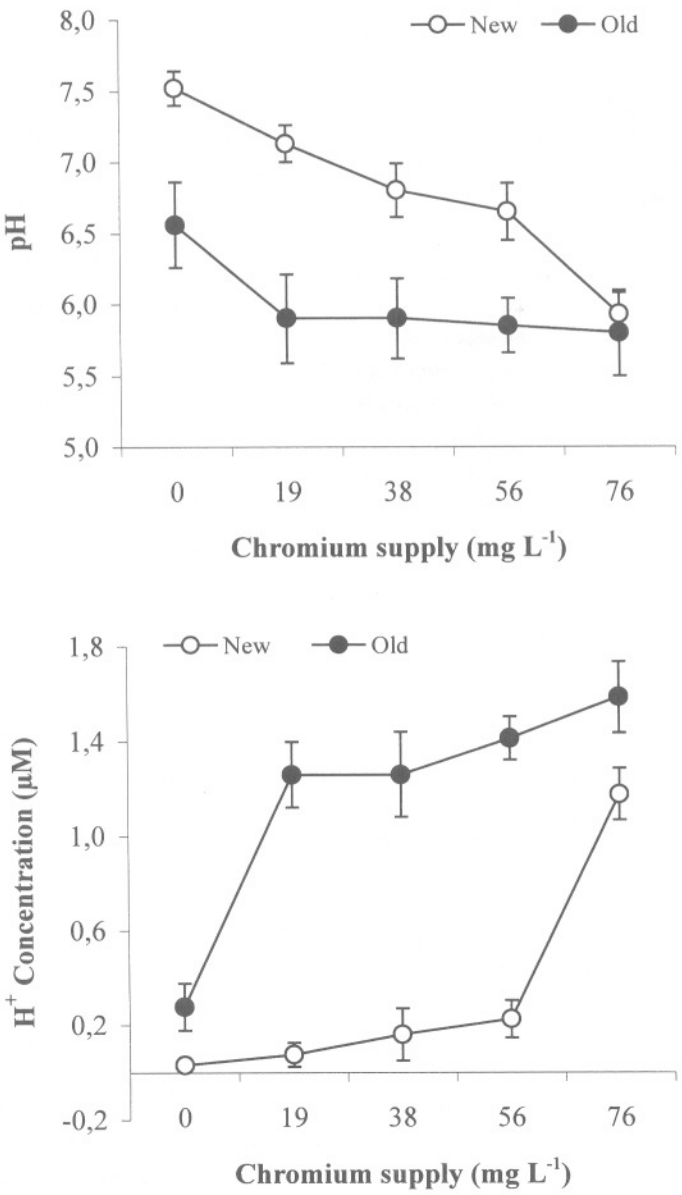

FIG. 1. – pH (top) and proton concentration (bottom) of the nutrient solution just after (new) and just before (old) each change of the nutrient solution. Data are means of 14 measurements (7 changes of solution x 2 replicates). Vertical bars denote SE.

ticularly evident with the highest Cr level. The formation of a mantle covering the root surface could be detrimental to plant growth by interfering physically and chemically with nutrient uptake (Mei *et al.*, 2002). Consistent with the decrease of pH, the concentration of protons increased in solution, and increments were especially high between 57 and 76 mg Cr L^{-1} (Figure 1). We did not measure cation concentration in miscanthus, but it is reported that Cr affected the root and shoot concentrations of Fe, K, Ca and Mg in *Brassica oleracea, Citrullus vulgaris* and *Lycopersicon esculentum* (Moral *et al.*, 1995; Dube *et al.*, 2003; Pandey and Sharma, 2003). According to Shanker *et al.* (2005), the inhibition of the activity of plasma membrane H^+ ATPase is one of the reasons for the decreased uptake of most nutrients in Cr-stressed plants. Starting from this statement, we hypothesize that the high proton concentration recorded after the addition of 76 mg Cr L^{-1} could have reduced the activity of the plasma membrane H^+ ATPase, thus reducing cation uptake. Besides, Cr(III) ions could also bind to the exchange sites of surface cells, thus competing directly with the uptake of essential cations (Zayed *et al.*, 1998; Shanker *et al.*, 2005). Finally, the precipitation of insoluble $CrPO_4$ could be responsible of the reduced P uptake by roots observed by Moral *et al.* (1995) and by Dube *et al.* (2003). Summarizing all of the above, we suggest that the addition of chromium to the nutrient solution could have affected nutrient uptake in miscanthus through the acidification of the solution, the formation of a physical obstruction at the root surface, the direct competition for uptake sites and the precipitation of nutrients into insoluble salts.

After a two-week presence of roots in solution its acidity increased further with all treatments except with the highest Cr supply, so that the pH was 6.6 in the control solution and approximately 5.9 in all Cr treatments (Figure 1). This corresponded to an increase in the proton concentration that was moderate in the control solution and with the highest Cr supply, but was very high with Cr levels between 19 and 57 mg L^{-1}. According to Hager (2003), the release of protons by the plasma membrane H^+ ATPase activates pH-sensitive enzymes and proteins within the cell wall, which initiates cell-wall loosening and extension growth. In support, Anuradha and Narayanan (1991) and Liu *et al.* (2004) found an increased extrusion of protons in the root exudates of P deficient plants, that was coupled to an enhanced root elongation.

Plant Growth. After 104 days of exposure, growth of miscanthus was differently affected by the Cr levels supplied to the nutrient solu-

TABLE 1. – *Dry weight of separate plant parts, and rhizome to root biomass ratio, of miscanthus plants as affected by chromium level.*

Chromium level (mg L^{-1})	Dry weight (g $plant^{-1}$)					
	Shoot	Root	Rhizome	Hypogeal part	Entire plant	Rz:Rt ratio
0	10.5 a	11.9 a	27.8 a	39.7 a	50.2 a	2.3 a
19	11.2 a	14.0 b	27.8 a	41.8 a	53.0 a	2.0 a
38	10.3 a	13.8 b	20.2 b	34.0 b	44.3 b	1.5 b
57	9.6 a	14.4 b	16.1 c	30.5 b	40.1 bc	1.1 bc
76	7.7 b	13.8 b	11.5 d	25.3 c	33.0 c	0.8 c

Values in each column followed by the same letter are not significantly diffferent at $P \leq 0.05$ as determined by the Duncan's test (STEEL *et al.*, 1997).

tion. A Cr concentration of 19 mg L^{-1} significantly increased root biomass and did not affect that of the shoot and the rhizome (Table 1). With Cr concentrations equal to or higher than 38 mg L^{-1}, shoot biomass was decreased, the decrease being significant only with 76 mg Cr L^{-1}. Root biomass was increased by approximately 18% with all chromium levels, whereas rhizome biomass decreased up to 59% with increasing Cr supply. As a result, the rhizome to root ratio decreased from 2.3 up to 0.8 (Table 1). Thus, compared to controls, the biomass of the entire miscanthus plant was enhanced by approximately 5% with the lowest Cr level, was decreased by approximately 16% up to 57 mg Cr L^{-1} and by 34% with the highest Cr supply. MEI *et al.* (2002) and PANDEY and SHARMA (2003) reported chlorosis and early leaf shedding as visual symptoms of Cr toxicity in *Glycine max* and in *Brassica oleracea.* None of these symptoms were observed in miscanthus leaves, suggesting that this species suffered only moderate toxicity with Cr levels up to 76 mg L^{-1}.

The positive relative growth rates indicated that, during the treatment period, biomass production occurred in all plant parts and with all Cr levels (Figure 2). However, compared to the control, the RGR of Cr treated plants decreased up to 60% in the rhizome and up to 22% in the shoot, whereas it increased by about 10% in the root. These patterns indicate that Cr reduced drastically the allocation of resources to the rhizome, which is the primary sink of reserves in miscanthus, and slightly increased their allocation to roots. ERICSSON (1995) and LIU *et al.* (2004) found that root growth of *Betula pendula* and *Zea mays* was favoured

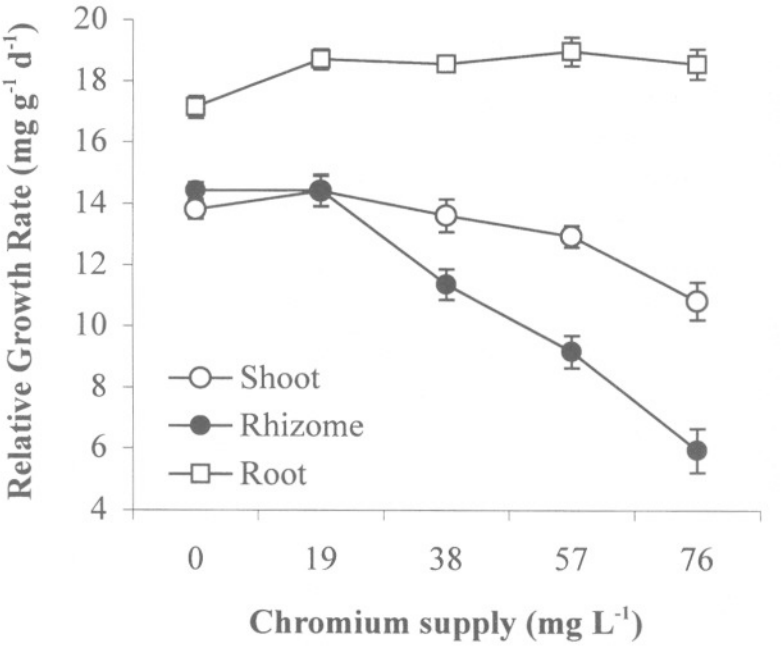

FIG. 2. – Relative growth rate of miscanthus plants as affected by chromium level. Vertical bars denote SE (n=8).

compared to shoot growth in response to N, P and S deficiency. Starting from these findings, we suggest that the higher partitioning of biomass to roots in response to Cr can be a reaction to the adverse effect of Cr on the availability of nutrients or on the overall uptake efficiency or roots.

Root morphology. Chromium supply greatly affected root morphology. At the end of the treatment period, root length was higher than in control plants with all Cr levels, and the highest increments (+94%) were recorded with 57 mg L^{-1} Cr (Figure 3). Root average diameter, in contrast, slightly decreased with increasing Cr supply. As a consequence, root surface was higher than in controls up to 57 mg Cr L^{-1} and equalled control values with the highest Cr supply. Root volume, in contrast, sligthly increased up to 38 mg Cr L^{-1}, equalled control values with 57 mg L^{-1} Cr and then decreased with the highest Cr supply. Visual observations indicated that the changes in root parameters in response to chromium were caused by the development of fine and long lateral roots, that were already evident 20 days after exposure. Increased root length in response to low Cr concentrations was reported by PANDA and PATRA (2000) and

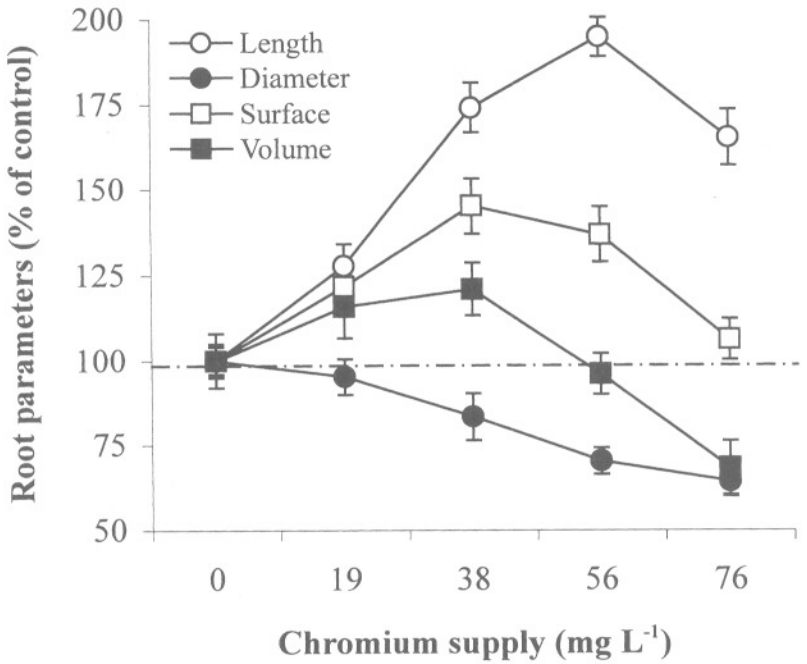

FIG. 3. – Root length, average diameter, surface and volume (% of control) of miscanthus plants as affected by chromium level. Vertical bars denote SE (n=8).

was also suggested by PANDEY and SHARMA (2003) but, generally, it was found that Cr depressed root growth (SHANKER *et al.*, 2005). Increased root length coupled to decreased average diameter was observed in response to low phospahte and cations availability and is considered a strategy to increase root surface but not root volume (BRABER and SILBERBUSH, 1984; LIU *et al.*, 2004). In this way, indeed, the redirection of resources to the root could be minimized and, actually, the root surface of miscanthus increased up to 45% but its biomass by only 18%. All above summarized, the strong stimulation of root growth we observed in miscanthus, suggests that roots of this species have a high ability to adapt to the presence of Cr in solution, at least up to 57 mg Cr L^{-1}.

Chromium concentration and content. In all plant parts of miscanthus the Cr concentration increased in response to Cr level, but marked differences were observed between organs both in the patterns and magnitude of increments (Table 2). The Cr concentration of roots increased almost linearly from 7.4 to 26 g kg^{-1} with increasing Cr level. In contrast,

TABLE 2. – *Chromium concentration and content of separate plant parts of miscanthus plants as affected by chromium level.*

Chromium level (mg L^{-1})	Shoot	Roots	Rhizome	Hypogeal part	Entire plant
			Cr concentration (mg kg^{-1})		
0	1.7 a	2 a	6 a	5 a	4 a
19	33.7 b	7412 b	4063 b	5185 b	4096 b
38	37.6 b	11228 c	6730 c	8556 c	6575 b
57	40.8 b	18594 d	7392 c	12681 d	9655 c
76	70.1 c	25964 e	12858 d	20007 e	15355 d
			Cr content (mg $plant^{-1}$)		
0	0.0 a	0.0 a	0.2 a	0.2 a	0.2 a
19	0.4 b	103.8 b	113.0 b	216.8 b	217.2 b
38	0.4 b	154.9 c	135.9 c	290.8 c	291.2 c
57	0.4 b	267.8 d	119.8 b	387.6 d	388.0 d
76	0.5 b	358.3 e	147.9 e	506.2 e	506.7 e

Values in each column followed by the same letter are not significantly diffferent at $P \leq 0.05$ as determined by the Duncan's test (STEEL et al., 1997).

the Cr concentrations of the rhizome and of the shoot showed higher increments between 57 and 76 mg Cr L^{-1}, and reached maximum values of 13 g Cr kg^{-1} and only 70 mg Cr kg^{-1}, respectively. Moreover, the increase in the Cr concentration of the shoot was insignificant between 19 and 57 mg Cr L^{-1}. Thus, the Cr concentration of roots was always approximately twice that of the rhizome, which, in turn, was 121 times that of the shoot with 19 mg Cr L^{-1}, and approximately 180 times with the higher Cr levels. Though we rinsed thoroughly roots and rhizomes, we can not exclude that part of the Cr found in hypogeal organs was only deposited on their outer surfaces or accumulated within the apoplast. Nevertheless, the great difference between the Cr concentration of the aerial and the hypogeal plant parts, so as the markedly higher Cr concentration recorded in roots compared to the rhizome, suggest the existence of internal mechanisms restricting Cr transport within the plant. The above distribution patterns seem to indicate that Cr translocation is hindered both between roots and the rhizome and between this and the shoot and, therefore, the internal barrier cannot be identified only with

the endodermis, as was suggested for cadmium (ARDUINI *et al.*, 2006). The strong retention of Cr in roots has been reported by several authors for different plant species and experimental conditions, and it is mainly considered the consequence of Cr binding on cell walls or Cr precipitation within vacuoles (ZAYED *et al.*, 1998; VAJPAYEE *et al.*, 1999; PANDEY and SHARMA, 2003; SHANKER *et al.*, 2004). In addition, in Cr treated plants of *Phaseolus vulgaris,* VAZQUEZ *et al.* (1987) observed abundant starch grains in the plastids, and precipitates in the vacuoles of parenchyma cells from the vascular cylinder of roots and from the pith of the stem. Both could be strategies for the immobilization of Cr within the non photosynthetizing parts of the plant (SHANKER *et al.*, 2004; HAN *et al.*, 2004), and they can explain the high Cr concentrations we recorded both in the roots and the rhizome of miscanthus. With the highest Cr supply, however, we observed a marked increase in the Cr concentration of the shoot coupled to a significant decrease in its biomass, which suggests that restriction mechanisms are, at least partly, inactivated with Cr levels higher than 57 mg L^{-1}.

The Cr content of the entire miscanthus plant increased from 217 to 507 mg per plant in response to increasing Cr supply from 19 to 76 mg L^{-1} (Table 2). The Cr content of the shoot, however, did not change with Cr level, and was approximately 0.4 mg per plant. This value corresponded to less then 0.2% of the total Cr content of the plant, indicating that almost all of the Cr was retained within the hypogeal organs. A low Cr accumulation in the aerial part is widely reported in consequence of both the reduced translocation of the metal and the depression of shoot growth (MCGRATH, 1982; ZAYED *et al.*, 1998; PANDEY and SHARMA, 2003). In our experiment, the accumulation of Cr in the aerial part is low also because we cut secondary culms just after their emergence from the rhizome, thus reducing the aerial sink for the metal. With the increase in Cr supply from 19 to 76 mg L^{-1}, the Cr content of roots increased markedly, whereas that of the rhizome increased only slightly and not according to Cr supply. Thus, the partitioning of Cr between the two hypogeal organs changed markedly with Cr level, in that roots retained approximately 51% of the total hypogeal Cr with the two lowest Cr levels and 70% with the two highest.

CONCLUSIONS. – A 104 day exposure of miscanthus to Cr concentrations higher than 19 mg L^{-1} markedly decreased growth of the rhizome and, consequently, of the entire plant. Shoot biomass was decreased only

with 76 mg Cr L^{-1}, whereas root biomass was increased with all Cr levels.

The presence of Cr markedly increased the length of roots and decreased their average diameter. Chromium concentrations comprised between 19 and 57 mg L^{-1} also increased root surface, whereas root volume was only slightly affected. We hypothesize that miscanthus changed its root morphology in reaction to the presence of Cr in the nutrient solution.

With increasing Cr supply, the Cr concentration increased up to 25,964 mg kg^{-1} in roots, up to 12,858 mg kg^{-1} in the rhizome, but only up to 70 mg kg^{-1} in the shoot, indicating that the translocation of Cr to the aerial part is strongly restricted in miscanthus. Nevertheless, we achieved a maximum Cr content of 507 mg per plant, which allows us to suggest that miscanthus plants can be considered for Cr rhizofiltration.

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SUMMARY. – Plants of miscanthus were exposed for 104 days to chromium concentrations ranging from 19 to 76 mg L^{-1} in hydroponics. Plant growth was slightly enhanced with 19 mg Cr L^{-1}, was decreased by approximately 16% with 38 and 57 mg Cr L^{-1}, and by 34% with 76 mg Cr L^{-1}. Root biomass and root length were increased with all Cr levels, whereas root diameter was decreased. Chromium caused the immediate acidification of the nutrient solution. After a two-week permanence of roots in solution a further decrease of pH occurred with Cr levels between 19 and 57 mg L^{-1}. In all plant parts the Cr concentration increased with increasing Cr supply, but the Cr concentrations of roots and rhizomes always exceeded by a 100 factor that of the shoot. Miscanthus plants accumulated between 217 and 507 mg Cr per plant, 99% of which was allocated in the hypogeal part.