Linking phytotechnologies to bioeconomy; varietal screening of high biomass and energy crops for phytoremediation of Cr and Cu contaminated soils

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Abstract

Enerbiochem was a project devoted to study new strategies of industrial valorisation of high biomass crops grown on brownfields or contaminated soils not suitable for food production. Chromium and copper accumulation and toxicity were examined in different species of agronomic interest. Cultivars of *Brassica carinata* A. Braun (7), *Brassica juncea* (L.) Czern. (4), *Brassica napus* L. (4), *Raphanus sativus* L. (4), inbred lines of *Helianthus annuus* L. (6) and cultivars of *Nicotiana tabacum* L. (3) were screened for the best genetic materials to be used with the aims: i) to produce the highest biomass in contaminated soils; and ii) possibly to phytoremediate them. Cr and Cu accumulation in shoots were evaluated on 16 days old plants grown for additional 5 days in the presence of either Cr (60 μM) or Cu (2 μM) in hydroponic. They were characterised for Cr and Cu concentrations in roots and shoots, shoot biomass, and total chlorophyll as well.

Shoot biomass was significantly lower in *Brassica* species than in *R. sativus*, *H. annuus* and *N. tabacum* under Cr treatments. On the contrary, under Cu treatments, *N. tabacum* produced the lowest biomass in respect to other species. Potentially toxic element concentrations varied among genetic material and some genetic material resulted less affected (higher chlorophyll content and shoot biomass) even under higher Cu or Cr concentrations in shoot. Potential candidates within each species, to be used for coupling phytoremediation and biomass production on slightly Cr-Cu potentially contaminated soils are listed.

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Introduction

The greatest challenge of this century will be the transition from a fossil-based economy to bioeconomy. That is needed in response to several global mega-trends such as: i) fast-growing global population and higher life expectancy; ii) rise of food production and water demand; iii) high dependence on fossil-based resources; iv) needing of a diversified energy supply options; v) increasing greenhouse gases emission; vi) increasing land use competition; vii) land and water resource pollution (Nita *et al*., 2013).

Driven by environmental concerns, an increasing focus is paid worldwide to sustainable technologies which are part of the concept of a bio-based economy or bioeconomy (EC, 2012). Bioeconomy is an economy in which bio-based materials instead of fossil-based ones are used for the production of energy, transport fuels, chemicals and many other materials (Prasad, 2016). According to European Community (2012) *significant growth is expected to arise from sustainable primary production, food processing and industrial biotechnology and biorefineries, which lead to new bio-based industries, transform existing ones, and open new markets for bio-based products*. With regard to land pollution, several remediation technologies are available to clean up or securing the contaminated sites. Extensive studies over the last three decades demonstrated the potential of plant based technologies (phytotechnologies) as biological remediation techniques (Fagnano, 2016). Being more energy-efficient and less disruptive to contaminated sites they emerged as viable alternatives to conventional remediation techniques. The traditional term *phytoremediation* has been recently associated with the term *phytotechnologies*. In addition to the degradation and/or removal of contaminants, phytotechnology also includes techniques such as stabilisation and volatilisation of pollutants, terminate the exposure pathways of pollutants, thus securing the sites (Fagnano, 2017).

The expected take-off of the bioeconomy will lead to

increased demand for biomass. The use of brownfields and marginal lands or contaminated sites to produce non-food biomasses for energy valorisation or chemical industry through plants, which are able to grow and to develop healthy in such environments is a feasible option. While remediating contaminated substrates or wastewaters, phytotechnologies provide plant biomass to be reused that can be used as renewable energy sources, green fine chemistry, bioplastics, *etc*. (Prasad, 2014). That implies that phytotechnology, beyond its primary role, is attractive also as an additional source of biomass and it can be considered as an integral part of sustainable development and bioeconomy (Prasad, 2016). However, development of economically sound valorisation pathways for complete chain of phytoproducts of value addition and value chain from phytotechnologies would go a long way (Grison 2015; van der Ent *et al*. 2015).

The multidisciplinary project *PON01_01966 ENERBIOCHEM - Agricultural and industrial supply chain with energetic high efficiency for the setup of bio-compatible production processes of energy and bio-chemicals from renewable sources and for the improvement of the local areas* was aimed at developing integrated agro-industrial chain processes to produce bio-fuel (*i.e*. bio-ethanol and bio-diesel) from renewable sources in respectively marginal lands subjected to erosion (Fagnano *et al*., 2015) and polluted soils (Fiorentino *et al*., 2013). Our group was involved in studies regarding two agro-industrial biomass chains, respectively from oleaginous crops (*Brassica* species, *Helianthus annuus* L., *Nicotiana tabacum* L) and ligno-cellulosic field crops (*Sorghum bicolor* L., *Arundo donax* L., and *Cynara cardunculus* L.), subjected to Cr and Cu contamination.

Chromium (Cr) and copper (Cu) are among the most widely used heavy metals in industries which effluents contain ionic Cr and Cu in high concentrations which can accumulate in soils and cause serious environmental pollution (Costa, 2003; WHO, 2003a, 2003b).

Chromium is a potentially toxic element (PTE) non-essential in plant physiology and present in environment mostly in hexavalent or trivalent form (Marschner, 2012; Shanker *et al*., 2005). Both hexavalent (CrO_4^{2-}, $HCrO^{4-}$ and $Cr_2O_7^{2}$) and trivalent (Cr^{3+} and $CrOH^{2+}$) chromium species are present in industrial waste solutions (Shanker *et al*., 2005; Miretzky and Cirelli, 2010). However, Cr toxicity in plants depends on its valence state and hexavalent chromium is much more toxic than trivalent chromium (Shanker *et al*., 2005). Moreover, Cr(VI) is considered hazardous to public health due to its mutagenic and carcinogenic properties (Shanker *et al*., 2005).

Copper is a PTE essential in small amounts for plant metabolism and can be present as Cu^{+} or Cu^{2+} under physiological conditions (Marschner, 2012). While Cu is an essential micronutrient, prolonged exposure to the metal causes adverse health effects in both plants and animals (WHO, 2003b). Large acute doses can potentially produce fatal effects in humans (WHO, 2003b).

The aim of this research was to test a number of genetic materials from six different species of agronomical interest (*Brassica carinata*, *Brassica juncea*, *Brassica napus*, *Helianthus annuus*, *Nicotiana tabacum* and *Raphanus sativus*) for their biomass and Cr-Cu accumulation capacities under plant exposition to slightly toxic Cr or Cu concentrations in hydroponic system. By determining parameters such as chlorophyll content, shoot dry weight, along with Cr and Cu accumulation in the shoots, the phytotoxic effects of these two heavy metals were quantified with the aim to determine if these species display sufficient tolerance and metal accumulating ability to be used for phytoremediation and biomass purposes on slightly Cr-Cu polluted soils.

Materials and methods

Seeds of different genetic material from *Brassica carinata* A. Braun, *Brassica juncea* (L.) Czern., *Brassica napus* L., *Raphanus sativus* L., *Helianthus annuus* L. and *Nicotiana tabacum* L. (Table 1) were sown in a garden peat soil and left for ten days in a growth chamber. Seedlings were then transferred to aerated hydroponic culture in 1-L polyethylene pots (one plant per pot) according to Mei *et al*. (2002) with slight modification. In particular, our culture contained a modified half-strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 20 µM Fe(Na)-EDTA, 1 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.1 µM $CuSO_4$ and 0.1 µM $(NH_4)_6Mo_7O_{24}$ in demineralised water buffered with 2 mM 2-N-morpholino-ethanesulfonic acid (MES), pH 5.5, adjusted with KOH. Plants were grown in a growth chamber (22/16°C day/night; light intensity 220 µE m^{-2} s^{-1}, 14 h day^{-1}; relative humidity 70-80%).

After six days of pre-culture, plants were transferred to the test solution, which was of the same background composition as the pre-culture solution, but with Fe-EDDHA instead of Fe(Na)-EDTA, to avoid Cu-EDTA complex formation, owing to displacement of $Fe^{(III)}$ (Lucena and Chaney, 2007). Plants were exposed to either 2 µM Cu (0.127 mg L^{-1}) or 60 µM Cr (3.12 mg L^{-1}) obtained from $CuSO_4$ and $K_2Cr_2O_7$ respectively. These concentrations were chosen after literature screening (Marchiol *et al*., 2006; Shahbaz *et al*., 2010; Terzi and Yıldız, 2015). While contaminated real soils are expected to have a range of pH we used a slightly acid pH - typically applied in hydroponic toxicity experiments in order to keep all the nutrients available and to avoid precipitation (Marchiol *et al*., 2006). We also choose to test Cr(VI) despite the most frequent Cr(III) because Cr(VI) is more toxic and with carcinogenic properties (Mei *et al*., 2002). Plants were grown in treatment solution for additional six days (one plant per pot, three plants per treatment per genetic material in a randomised design). The treatment solutions were refreshed after three days in order to keep concentrations of elements in solution constant.

Prior harvesting, small disk (5 mm in diameter) from one adult leaf of each plant was collected in 2 mL Eppendorf, grinded under liquid nitrogen and re-suspended in 2 mL of 80% acetone (Sudhakar *et al*., 2016). After ultracentrifugation, total chlorophyll content was determined using a spectrophotometer (Pharmacia Biotech, Novaspec II, 80-2088-54) (Sudhakar *et al*., 2016).

ICP-OES analysis of chromium and copper in soil and plant fractions

Immediately after harvesting plant specimens were divided into shoots and roots and the latter were carefully rinsed with ice-cold $Pb(NO_3)_2$ for 30 min to desorb metals from the root free space and then blotted with paper tissue (Cestone *et al*., 2010). Shoots and roots were oven-dried for 24 h at 105°C and total shoot dry biomass was measured through technical-balance. Subsequently, roots and shoots were acid-digested in a microwave oven (CEM, MARS Xpress) according to the USEPA 3052 method (USEPA, 1995). After mineralisation, extracts were filtered (0.45 mm PTFE), diluted and analysed. Elemental analysis was performed through ICP-AES (Varian Inc., Vista MPX) and the accuracy of the analytical procedure was checked running standards every 20 samples. Quality control was conducted using Y as the internal standard, reagent blank samples, and triplicates reading for each sample. Detection limits were: 2 µg L^{-1} and 4 µg L^{-1} for Cr and Cu respectively. Total shoot accumulation of Cr and Cu was estimated as the product of shoot concentration times biomass.

Data analysis

Statistical analysis used the nested ANOVA (genetic material nested within species) according to Sokal and Rohlf (2010). A posteriori comparison of individual means was based on the minimum significant difference (MSD) method obtained from the T statistic (Sokal and Rohlf, 2010). Calculations were made through PC spread sheet utilities.

Results

Shoot biomass and development

Different genetic material within *Raphanus sativus*, *Helianthus annuus* and *Nicotiana tabacum* developed higher shoot biomass while all the varieties of *Brassica* spp. except the *B. carinata* 79444 have shown a remarkably and significantly lowest shoot biomass development under Cr exposition (Table 2). *B. carinata* 79444 biomass was not significantly different from *H. annuus* 28 R Mt and *R. sativus* Hazera Red but it would be expected a larger difference if the experiment would stay longer. *R. sativus* varieties Nabo Amazela, Pegletta and Cavalrondo, and the *H. annuus* inbred line R 1954/1 developed the significantly highest biomass among genetic material (Table 2).

In the Cu experiment, all the tobacco cultivars showed the lowest biomass among analysed genetic material. On the other side, no other plants show a clear species specific response. Rather shoot biomass was very variable among different genetic material (Table 3). In the Cu experiment, the biggest shoot biomass was developed by *H. annuus* 3620 MT, on average nine times bigger then the tobacco biomass which was the lowest (Table 2). However, the tobacco inbred lines were not significantly different from three of *H. annuus* inbred lines (R 1954/1, R 569 and RT2) and R 569 was not significantly different from the inbred line 3620 MT biomass. The great variability in shoot biomass between individual plants and the genetic material is probably due to a complicated relationship between species and the genetic material.

Leaf chlorophyll content in all Cr treated plants except *N. tabacum* cultivars was little affected relatively to the corresponding control leaves with an average of 96% of chlorophyll content relatively to that present in control plants. Only in *N. tabacum* cultivars the relative chlorophyll content was lower, although not always to a significant extent, being only 47% of that present in control plants. This is giving insights that the Cr test concentration chosen (60 µM) was still under partial plant homeostasis control in all samples except tobacco. Tobacco plants are thus resulting to be very sensitive to Cr. Moreover, some genetic material as *B. carinata* varieties B.car 0.99, BRK35 and SVP nr.12, *B. juncea* variety Newton, *H. annuus* inbred lines 28 R MT, R 1954/1, R 569 and R T2, and *R. sativus* variety Hazera Red, although not significantly, shown an increased leaf chlorophyll content relatively to corresponding control leaves (Table 2).

Leaf chlorophyll content of Cu treated plants relatively to the corresponding control leaves, was generally lower with an average of 92% of chlorophyll content relatively to that present in control plants. Moreover, the relative leaf chlorophyll content was not significantly different among genetic materials in Cu experiment.

Table 1. Genetic material description: species name, accession name of different genetic material and their source are reported.

Species	Accession name	Source
Brassica carinata	79444	DI4A, University of Udine, Udine (I)
	B.car 0.99 (CGN04028)	CGN, Wageningen (NL)
	BRK35	DI4A, University of Udine, Udine (I)
	Gommenzer 1 (CGN03966)	CGN, Wageningen (NL)
	Gommenzer 2 (CGN04019)	CGN, Wageningen (NL)
	L194252	CGN, Wageningen (NL)
	SVP nr.12 (CGN04025)	CGN, Wageningen (NL)
Brassica juncea	Newton (CGN19972)	CGN, Wageningen (NL)
	Primus (CGN06615)	CGN, Wageningen (NL)
	Trowse (CGN19973)	CGN, Wageningen (NL)
	Vitasso (CGN19974)	CGN, Wageningen (NL)
Brassica napus	Buko (CGN18958)	CGN, Wageningen (NL)
	Cobra (CGN18960)	CGN, Wageningen (NL)
	Pulsar	CGN, Wageningen (NL)
	Ramses (CGN17382)	CGN, Wageningen (NL)
Helianthus annuus	Inbred line 28 R MT	DI4A, University of Udine, Udine (I)
	Inbred line 3620 MT	DI4A, University of Udine, Udine (I)
	Inbred line 458 MT	DI4A, University of Udine, Udine (I)
	Inbred line R 1954/1	DI4A, University of Udine, Udine (I)
	Inbred line R 569	DI4A, University of Udine, Udine (I)
	Inbred line R T2	DI4A, University of Udine, Udine (I)
Nicotiana tabacum	G165 (Bright type)	CRA-CAT, Scafati (I)
	G19 (Bright type)	CRA-CAT, Scafati (I)
	P2B (Oriental type)	CRA-CAT, Scafati (I)
Raphanus sativus	Cavalrondo (CGN20741)	CGN, Wageningen (NL)
	Hazera Red (CGN20758)	CGN, Wageningen (NL)
	Nabo Amazela (CGN06912)	CGN, Wageningen (NL)
	Pegletta (CGN06952)	CGN, Wageningen (NL)

CGN accession codes are given between brackets.

This can give an indication of similar stress response among the different analysed species. However, in some plants such as *B. carinata* variety BRK35, *B. juncea* varieties Primus, Trowse and Vitasso, *H. annuus* inbred line R 1954/1, *N. tabacum* cultivar P2B, and *R. sativus* variety Cavalrondo, leaf chlorophyll content, although not significantly, was slightly higher than the corresponding control leaves (data not shown).

Roots and shoots potentially toxic element concentrations

Chromium concentrations in the roots were similar and not significantly different among all genetic material with an overall mean of 2269 μg g^{-1} (Table 2). Similarly, Cr concentrations in the shoots varied little among genetic material. Although not significantly different from the majority of genetic materials, the lowest Cr concentrations in the shoots were found in *H. annuus* (on average 6.82 μg g^{-1}). The no significance can be, however due to high variation within replicates and further experiments should assess this. The highest Cr concentration in shoots relatively to the other genetic material was found in *R. sativus* variety Hazera Red with a mean value of 35.4 μg g^{-1} which was significantly different from all the other means except from the *B. juncea* variety Primus which showed also notable Cr concentration in the shoots of 21.9 μg g^{-1} on average (Table 2).

However, there was a clear restriction to Cr translocation into the shoots for all the genetic material and the translocation coefficients (shoot concentration to root concentration ratio) was not significantly different among genetic materials and approximately 0.0056 - that is only 0.56% of Cr in roots was present in shoots.

Copper concentration in the roots was highest in *N. tabacum* cultivars while the lowest in *B. carinata* variety 79444. There was no clear species-specific response in root Cu concentrations with great variability among genetic material within species (Table 3). Given the great variability among replicates, significant differences among genetic material and species was hard to be detected. *N. tabacum* cultivars G19 and P2B had significantly higher root Cu concentrations respect to all the other genetic materials reaching mean values of 1525.1 and 1105.3 μg g^{-1} of Cu in roots respectively. Also in the shoots the higher Cu concentration were found in *N. tabacum* cultivars which were significantly higher from the other genetic materials with an average of 87.4 μg g^{-1} of Cu in shoots. However, there was also for Cu a clear restriction to its translocation into the shoots for all the species and the translocation coefficients was not significantly different among genetic materials and approximately 0.13 - that is 13% of Cu in roots was present in shoots.

Shoot heavy metal accumulation

To assess the effectiveness of the different genetic material within species for phytoremediation of Cr and Cu polluted soils taking into account also their shoot biomass for energy valorisation or chemical industry, total shoot accumulation (plant aboveground biomass multiplied by the PTE concentration) was calculated for Cr and Cu (Table 2 and 3 respectively).

Table 2. Cr concentration in roots and shoots (μg g^{-1} d.w.), shoot d.w. (mg), Cr accumulation in the shoots (μg d.w.) and relative chlorophyll content (% of control plants) of 28 analysed accessions from six different species (mean±SE, n=3) after exposure to 60 μM Cr for six days.

Species	Accession name	Root Cr	Shoot Cr	Shoot d.w.	Shoot Cr accumulation	Relative chlorophyll content
Brassica carinata	79444	2331^{a}	12.4^{bcd}	21.7^{ef}	0.273^{de}	83.9^{abc}
	B.car 0.99 (CGN04028)	2125^{a}	13.6^{bcd}	12.1^{f}	0.163^{e}	110.8^{abc}
	BRK35	2224^{a}	11.6^{bcd}	14.9^{f}	0.178^{e}	132.7^{ab}
	Gommenzer 1	2205^{a}	11.2^{bcd}	15.2^{f}	0.167^{e}	80^{abc}
	Gommenzer 2	2300^{a}	9.2^{bcd}	15.3^{f}	0.151^{e}	74.6^{abc}
	L194252	2666^{a}	13.9^{bcd}	20.0^{f}	0.258^{de}	90.6^{abc}
	SVP nr.12	2086^{a}	9.9^{bcd}	20.7^{f}	0.201^{de}	143.9^{a}
Brassica juncea	Newton	2201^{a}	12.0^{bcd}	15.7^{f}	0.186^{de}	107.2^{abc}
	Primus	2199^{a}	21.9^{ab}	19.3^{f}	0.404^{de}	89.3^{abc}
	Trowse	2406^{a}	14.3^{bcd}	15.5^{f}	0.216^{de}	79.1^{abc}
	Vitasso	2733^{a}	12.^{8bcd}	18.2^{f}	0.206^{de}	56.8^{bc}
Brassica napus	Buko	2322^{a}	13.5^{bcd}	18.4^{f}	0.243^{de}	88.3^{abc}
	Cobra	2120^{a}	21.^{3bc}	15.0^{f}	0.328^{de}	69.6^{abc}
	Pulsar	1831^{a}	14.3^{bcd}	16.8^{f}	0.235^{de}	98.5^{abc}
	Ramses	2485^{a}	9.9^{bcd}	16.9^{f}	0.159^{e}	89.1^{abc}
Helianthus annuus	28 R MT	1862^{a}	7.9^{cd}	88.6^{def}	0.742^{cde}	116.3^{abc}
	3620 MT	2464^{a}	8.0^{cd}	146.2^{bcd}	1.168^{cde}	88.5^{abc}
	458 MT	2025^{a}	4.8^{d}	150.7^{bcd}	0.757^{cde}	90.5^{abc}
	R 1954/1	1624^{a}	2.8^{d}	210.8^{ab}	0.589^{cde}	107.6^{abc}
	R 569	2172^{a}	8.2^{bcd}	123.1^{cd}	0.938^{cde}	119.4^{abc}
	R T2	2403^{a}	9.3^{bcd}	102.4^{cde}	0.823^{cde}	114.8^{abc}
Nicotiana tabacum	G165	2396^{a}	9.5^{bcd}	120.4^{cd}	1.236^{cd}	48.6^{c}
	G19	2180^{a}	8.9^{bcd}	172.7^{bc}	1.490^{c}	46.6^{c}
	P2B	1833^{a}	8.8^{bcd}	110.4^{cd}	0.972^{cde}	45.9^{c}
Raphanus sativus	Cavalrondo	2750^{a}	14.5^{bcd}	210.3^{ab}	3.026^{b}	85.3^{abc}
	Hazera Red	2595^{a}	35.4^{a}	102.7^{cde}	3.287^{b}	108.0^{abc}
	Nabo Amazela	2660^{a}	12.3^{bcd}	279.6^{a}	3.412^{ab}	84.7^{abc}
	Pegletta	2324^{a}	19.5^{bc}	217.8^{ab}	4.342^{a}	78.4^{abc}

$^{a-f}$Different letters indicate significant differences between accessions separately for each parameter ($P<0.05$, T test).

The variation of Cr accumulation in shoots varied from 0.15 ng of Cr per plant in *B. carinata* variety Commencer 2, to 4.34 ng of Cr per plant in *R. sativus* variety Pegletta. Variation of Cr accumulation among genetic material within species was very low and similar among all species varying from 1.4 in *R. sativus* till 2.2 in *B. juncea*. It can be clearly seen a significantly higher accumulation of Cr in all varieties of *R. sativus* compared to all the other genetic material. On the contrary the other three species of genus *Brassica* shown the lowest level of Cr accumulation with mean value of 0.22 ng of Cr per plant. *H. annus* and *N. tabacum* showed a middle accumulation capacity of about 0.97 ng of Cr per plant. However, Cr accumulations in *H. annuus* and *N. tabacum* were not significantly different from most of the other *Brassica* spp.

Cu accumulation in shoots ranged from 0.02 ng of Cu per plant in *H. annuus* inbred line R T2, to 2.29 ng of Cu per plant in *B. carinata* variety Gommenzer 1. Cu accumulation did not show species specific responses and it was very variable even among genetic material within species. However, varieties of *B. juncea* and *B. napus*, and *N. tabacum* cultivars showed a lower degree of differences in Cu accumulation among genetic material. On the contrary Cu accumulation differed up to 28 and 36 folds among *R. sativus* and *B. carinata* varieties. In *H. annuus* an exceptionally high variation was found of up to 118 fold due to a very low accumulation in the inbred line R T2. If to not take in account the above inbred line, the variation of Cu accumulation in among inbred lines of *H. annuus* is reduced only to 2 fold (Table 3).

Discussion and conclusions

Root concentrations were considerably higher than those in the shoot for both Cr and Cu. Thus, all the plants showed a typical excluder strategy (Hanikenne and Nouet, 2011). In fact, a very small Cr translocation factor of 0.56% on average was found (Table 2). However, Cr concentrations reached considerable levels in shoots (12.6 μg g^{-1} d.w. on overall species mean) taken into account that normal range of Cr concentrations found in plants from uncontaminated soils are 0.2-1 μg g^{-1} d.w. (Nagajyoti *et al*., 2010). On the contrary, plants from contaminated soils had shown variable Cr accumulation and up to 490 μg g^{-1} d.w. but this can be attributed to the soil dust deposited on the leaves as the plants were not properly washed (Jaison and Muthukumar, 2017). These results indicate that the analysed species could be useful for phytoextraction of, but mainly for phytostabilisation, thanks to the progressive removal of the bioavailable form from the substrate (Visconti *et al*., 2017). For the phytoremediation and biomass purposes it is desirable also that plants survive in the contaminated land and a good indicator of plant stress is its total chlorophyll content. Plants with Cr in nutrient solutions reduced only a little the total chlorophyll content relatively to control plants indicating little stress under Cr experiments. Moreover, the main feature of chromium intoxication, which is chlorosis was not observed (Shanker *et al*., 2005). Although also root Cu concentrations were considerably higher than those in the shoot, the Cu translocation

Table 3. Cu concentration in roots and shoots (μg g^{-1} d.w.), shoot d.w. (mg), Cu accumulation in the shoots (μg d.w.) and relative chlorophyll content (% of control plants) of 28 analysed accessions from six different species (mean ± SE, n=3) after exposure to 2 μM Cu for six days.

Species	Accession name	Root Cu	Shoot Cu	Shoot d.w.	Shoot Cu accumulation	Relative chlorophyll content
Brassica carinata	79444	24.9^{j}	1.4^{ghi}	62.6^{bcde}	0.081^{f}	88.5^{a}
	B.car 0.99 (CGN04028)	175.5^{hij}	18.1^{cd}	45.2^{bcde}	0.81^{bcdef}	98.4^{a}
	BRK35	32.6^{ij}	1.9^{fghi}	50.4^{bcde}	0.095^{f}	108.6^{a}
	Gommenzer 1	172.7^{hij}	17.3^{cde}	132.6^{abc}	2.285^{a}	82.5^{a}
	Gommenzer 2	126.1^{hij}	13.4^{cdefgh}	135.4^{ab}	1.771^{ab}	75.2^{a}
	L194252	309.2^{efghij}	0.7^{hi}	76.1^{abcde}	0.064^{f}	84.4^{a}
	SVP nr.12	32.5^{ij}	14.6^{cdef}	123.9^{abc}	1.878^{ab}	100.8^{a}
Brassica juncea	Newton	586.9^{def}	7.0^{cdefghi}	38.4^{cde}	0.264^{de}f	65.5^{a}
	Primus	712.7^{cd}	4.3^{fghi}	69.2^{bcde}	0.287^{cdef}	117.2^{a}
	Trowse	317.7^{efghij}	5.6^{defghi}	61.7^{bcde}	0.340^{cdef}	106.5^{a}
	Vitasso	353.8^{efghij}	4.1^{fghi}	56.2^{bcde}	0.286^{cdef}	109.6^{a}
Brassica napus	Buko	214.6^{ghij}	3.3^{fghi}	90.4^{abcde}	0.252^{def}	94.8^{a}
	Cobra	462.8^{defgh}	5.4^{defghi}	63.4^{bcde}	0.321^{cdef}	76.2^{a}
	Pulsar	50.1^{ij}	1.5^{ghi}	93.4^{abcde}	0.164^{f}	85.2^{a}
	Ramses	531.8^{defg}	4.5^{efghi}	53.1^{bcde}	0.240^{ef}	79.7^{a}
Helianthus annuus	28 R MT	149.7^{hij}	13.8^{cdefg}	135^{ab}	1.935^{ab}	88.8^{a}
	3620 MT	247.5^{fghij}	9.9^{cdefghi}	164.7^{a}	1.558^{abc}	63.4^{a}
	458 MT	119.7^{hij}	13.1^{cdefghi}	116.6^{abc}	1.538^{abcd}	75.1^{a}
	R 1954/1	175.7^{hij}	18.6^{c}	69.8^{bcde}	1.221^{abcdef}	126.9^{a}
	R 569	172.9^{hij}	7.4^{cdefghi}	111.2^{abcd}	0.873^{bcdef}	76.8^{a}
	R T2	79.2^{ij}	0.3^{i}	54.8^{bcde}	0.016^{f}	77.3^{a}
Nicotiana tabacum	G165	984.4^{bc}	80.0^{b}	20.0^{de}	1.574^{abc}	73.6^{a}
	G19	1525.1^{a}	95.0^{a}	15.8^{e}	1.491^{abcde}	96.6^{a}
	P2B	1105.3^{b}	87.4^{ab}	17.0^{de}	1.485^{abcde}	137.0^{a}
Raphanus sativus	Cavalrondo	274.4^{fghij}	1.0^{ghi}	124^{abc}	0.137^{f}	96.5^{a}
	Hazera Red	719.6^{cd}	1.0^{ghi}	54.6^{bcde}	0.054^{f}	104.0^{a}
	Nabo Amazela	657.9^{cde}	11.5^{cdefghi}	125.3^{abc}	1.539^{abcd}	97.7^{a}
	Pegletta	416.8^{defghi}	4.5^{efghi}	76.6^{abcde}	0.371^{cdef}	90.5^{a}

$^{a-j}$Different letters indicate significant differences between accessions separately for each parameter ($P<0.05$, T test).

from roots to shoots was considerably higher compared to that of Cr experiment with overall mean translocation factor of 13% (Table 3). This is not surprising as Cu is also a micronutrient and it has a dedicated homeostasis pathway, while Cr is a toxic heavy metal with no rule in plant development (Marschner, 2012) and it is possibly actively excluded from the shoots even if it was applied at higher concentrations than Cu. The overall species mean value of Cu concentration in shoots excluded *N. tabacum* plants was 7.4 μg g^{-1} d.w. ranging from 0.3-18.6 which is in the range of 4-15 μg g^{-1} d.w. typical for plants grown in uncontaminated lands (Nagajyoti *et al*., 2010). On the contrary *N. tabacum* has great potential for Cu phytoremediation as it reached high Cu concentrations in shoots (on average 87.4 μg g^{-1} d.w.) in all three analysed cultivars. This is also demonstrated by its high level of Cu accumulation even if it was not significantly different from many other genetic materials. However, this is due to a lower biomass value in *N. tabacum* in respect to the other plants which had a faster rate of development. When we collect plants *N. tabacum* was still very small while the other plants developed at a much faster rate and it is believed if to prolong the testing period of Cu stress, *N. tabacum* would developed much more biomass thus accumulating more Cu. However, Cu can be also toxic over a certain critical point, that is believed not to have been achieved by plants in our experiment because our Cu doses were lower than typical critical concentrations encountered in literature (Shahbaz *et al*., 2010). Moreover, the total chlorophyll content in the Cu treated plants was only little reduced in comparison to control indicating that plants were still active. To evaluate the ability of a species to extract heavy metals for phytoremediation purposes, the tissue concentrations of shoots alone it is not enough because it does not take plant biomass into consideration (Shahbaz *et al*., 2010). Species which develop more biomass in stressful condition such as heavy metal contamination are more adapted for non-food biomass production as a strategy for risk management of polluted soils, by avoiding cultivation of food crops. On the same time, species which have higher heavy metal concentrations in their shoot are more adapted for phytoextraction purposes, since they could be able to progressively remove the bioavailable fraction of metals from the soils, thus reducing risks for human health due to PTE entrance into the food chain. Having an intention to reach both purposes, the total shoot accumulation (the product of shoot concentration times biomass) was chosen as a composite index to select the best performing genetic material within each species. All the species appeared to be low accumulators of both Cr and Cu thus low phytoextraction capacities are expected for all the genetic material, which is in accordance with the available literature (Han *et al*., 2004; Shahbaz *et al*., 2010). On the other hand, a negligible root-to-shoot metal translocation could be very useful in the case of industrial valorisation of aerial crop biomass. Our experiment showed undoubtedly that at low level of available Cr and Cu - a typical condition in slightly anthropogenic polluted soils - the majority of the analysed genetic material (except *N. tabacum* cultivars under Cr stress), can grow with little or no stress as shown by good quantity of leaf chlorophyll content relatively to the corresponding leaf chlorophyll content in control leaves. Thus, among the genetic material within each species, we can choose the best performers according to the accumulation coefficient. There is a good correlation between Cr and Cu accumulation performance. Therefore, the best performers in Cr accumulation are also the best performers in Cu accumulation except in *B. carinata* and in *H. annuus* for one inbred line. This is not surprising as the species were tested in solution under Cr or Cu concentrations which were inhibiting the root growth at the same degree of 50%, and Cu and Cr have similar atomic and covalent radius. Our observations are in accordance with the references as it was already noted that Cu and Cr accumulation in shoots are highly correlated in different varieties of water spinach (*Ipomoea aquatic*) (He *et al*., 2015) and within each analysed seaweed species the accumulation of Cr and Cu were similar (Murphy *et al*., 2009). Therefore, it can be supposed, that these two PTEs are following similar stress response pathways in plants.

Taken into account that Cu is more toxic than Cr (WHO, 2003a, 2003b; Hemachandra and Pathiratne, 2015), the choice of *B. carinata* and *H. annuus* genetic material to test further under Cr and Cu stress in nutrient solution and in Cr and Cu contaminated soils should be chosen among best Cu performers. Moreover, the best performers in Cr accumulation in *B. carinata* and *H. annuus* are not significantly different in Cr accumulation from varieties or inbred line which are the best performers in Cu accumulation. Therefore, for each of the analysed species, the two most promising genetic material for phytoremediation of slightly Cr and Cu polluted soils and for biomass production are the varieties Gommenzer 1 and SVP nr. 12 of *B. carinata*, Primus and Trowse of *B. juncea*, Buko and Cobra of *B. napus*, Nabo Amazela and Pegletta of *R. sativus*, the inbred lines 28 R MT and 3620 MT of *H. annuus*, and the cultivars G165 and G19 of *N. tabacum*.

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