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EVALUATION OF PHYTOREMEDIATION POTENTIAL AND PHISIOLOGICAL RESPONSE TO ACCUMULATION OF SEVERAL METALS AND METALLOIDS BY

Salix spp. , Brassica juncea, Phragmites australis **AND** *Populus trichocarpa*

Direttore della Scuola : Ch.mo Prof. MARIO ARISTIDE LENZI

Coordinatore d'indirizzo: Ch.mo Prof. TOMMASO ANFODILLO

Supervisore :Ch.mo Prof. MARIO MALAGOLI

Dottorando : MARCO PITTARELLO

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INTRODUCTION

Phytoremediation is defined as the use of green plants to remove pollutants from the environment or to render them harmless (Cunningham et al.,1993, Raskin et al.,1994).
The basic idea that plants can be used for environmental remediation is very old and cannot be traced to any particular source. However, a series of fascinating scientific discoveries combined with an interdisciplinary research approach have allowed the development of this idea into a promising, cost-effective, and environmentally friendly technology. Phytoremediation can be applied to both organic and inorganic pollutants, present in solid substrates (e.g. soil), liquid substrates (e.g.water), and the air. Phytoremediation is currently divided into the following areas:
phytoextraction: the use of pollutant-accumulating plants to remove metals or organics from soil by concentrating them in the harvestable parts;
phytodegradation: the use of plants and associated microorganisms to degrade organic pollutants;
rhizofiltration: the use of plant roots to absorb and adsorb pollutants, mainly metals, from water and aqueous waste streams;
phytostabilization: the use of plants to reduce the bioavailability of pollutants in the environment;
phytovolatilization: the use of plants to volatilize pollutants; and the use of plants to remove pollutants from air.
Phytoremediation of metals is being developed as a potential cost-effective remediation solution for thousands of contaminated sites all around the world. Its development is driven by the prohibitively high cost of the available soil remediation methods, which mainly involve soil removal and burial at a price of about $1 million per acre.

PHYTOREMEDIATION TRIALS ON POLLUTED SLUDGE DREDGED FROM MARGHERA HARBOR

INTRODUCTION

The effectiveness of phytoremediation has been demonstrated for many classes of pollutants, like oil hydrocarbons, polycyclic aromatic hydrocarbons (PAH), pesticides, chlorinated solvents, and heavy metals (Aprill and Sims 1990; Anderson et al. 1994; Newman et al. 1997; Salt et al. 1997; Chang and Corapcioglu 1998; EPA 2000; Nedunuri et al. 2000; Pivetz 2001; Schnoor 2002).

At low levels of soil pollution, phytoremediation can be the most cost-effective reclamation strategy (Jones 1991), particularly for long term treatments. Phytoextraction is one of the phytoremediation techniques that uses plants able to accumulate concentrations of metals from contaminated soil in their biomass, distinguishing between hyperaccumulating species (Kumar et al. 1995; Blaylock et al. 1997) and species with a high biomass production (Bregante et al. 2001).

Phytoextraction could also be an effective solution for the treatment of dredged sediments. The contamination of sediments from the dredging of rivers or coastal harbours represents a problem that has assumed a notable importance in terms of the number of cases and the quantitative and economic extent of the necessary activities of restoration (Mulligan et al. 2001; Lors et al. 2004; Meers et al. 2005). Due to the high quantities of sediments to be treated every year and the high costs of traditional reclamation techniques, the conventional treatments of the sediments are not in most cases economically sustainable (Meers et al. 2003) and phytoremediation techniques could play an important role in these situations. Nevertheless, sediments, and particularly marine sediments, have poor hydraulic and agronomic characteristics, so that mixing with selected materials is probably the only way to make phytoextraction an effective remediation technique.

In Marghera harbor, inside Venice lagoon there is one of most important Italian chemical pole. During last decades many kind of organic and non organic pollutants were dropped off onto lagoon heavily polluting sediments. These had to be dredged in a final amount of 8 milions of tons and classified as "C" kind that means they are unable to host crops or to be used in civil constructions. A possible solution for their remediation is phytoremediation.

In this work we were focused on seeking not only good heavy metals accumulators but also fast high biomass producer species. Recently, driving forces behind the development of phytoextraction have been giving emphasis on a link with short-rotation coppice (Mirck et al., 2005; Kuzovkina and Quigley, 2005; Pulford and Dickinson, 2005). For phytoextraction purposes, high-biomass plants with large metal concentration in shoots and high bioaccumulation factors are required (BF = ratio of metal concentration in shoots to metal concentrations in soils).

Salix matsudana, S. fragilis, S. babylonica, S. alba, Phragmites australis and Brassica juncea satisfy these characteristics and over that, they are tipical of Italian environment (or at last established in it since a long time), and easy to be recruited , propagated and harvested.

The *Salicaceae* family comprises a large number of woody, high-biomass species (Pulford and Dickinson, 2005). Some willow and poplar species have been proposed for use in remediation (Robinson et al., 2003) of contaminated land as they showed high metal tolerance and accumulation potential in screenings, field (Eltrop et al., 1991; Hammer et al., 2003; Klang-Westin and Eriksson, 2003; etc.) and pot experiments (Granel et al., 2002; Rosselli et al., 2003; Vyslouzˇilová et al., 2003). Advantages of trees include deep rooting and the establishment of continuous vegetation cover, resulting in site stabilization, reduced risk of erosion and leaching of contaminants, and metal extraction from deeper soil layers (Wenzel et al., 1999).

Phragmites australis is not hyperaccumulator; however, it is fast growing and high-biomass producer, has a deep root apparatus and can tolerate and/or accumulate a range of heavy metals in its aerial portion. Given this, it is often utilized to reduce the metal concentration of soils, sediments and waters, in both natural and constructed wetlands (Cooper et al., 1996). Several works report higher accumulation of heavy metals in the belowground parts of plants than in the aerial parts (Karpiscak et al., 2001;, Stoltz et al., 2002, Peverly et al.,1995,Mays et al., 2001). However, in the current study the content of Cr, Ni, Cu and Zn in rhizomes and stems of P. australis plants was almost similar. The relatively low accumulation of heavy metals in foliar tissues at most sampling times was probably due to the need of plants to prevent toxicity to the photosynthetic apparatus as suggested by other authors (Bragato et al., 2006, Stoltz et al., 2002., Landberg et al.,1996). However, heavy metal accumulation in leaves increased considerably during fall. It may be inferred that plants possess an efficient root-to-shoot translocation system, which is activated at the end of the growing season and allows the concentration of toxic elements in the senescent tissues. In this way, plants can eliminate heavy metals through phylloptosis (Bragato et al., 2009).

Brassica juncea (Indian mustard) has been widely used in phytoremediation. This species is able to take up and accumulate in its aboveground tissues appreciable quantities of heavy metals like cadmium, copper, nickel, zinc, lead, hexavalent chromium, and selenium (Dushenkov et al., 1995; Bañuelos et al., 2005; Le Duc and Terry, 2005).

In order to increase the efficiency of process two chelant agents were tested : EDTA coupled with *phragmites australis* and EDDS coupled with *S. babylonica.* In indoor trials, willows grew coupled with *paspalum vaginatum*, a northern American salt tolerant grass, already experimented by Masciandaro (2008) to evaluate its tolerance to EDTA. In our experiment its ability in Na accumulation and tolerance to EDDS were verified.

Ethylenediamine tetraacetate (EDTA) is probably the most efficient chelating agent in increasing the solubility of heavy metals in soil solution and thus in plant tissues (Blaylock et al., 1997; Huang et al., 1997; Ebbs et al., 1997; Wu et al., 1999). However, most syntethic chelators form chemically and microbiologically stable complexes with heavy metals that can contaminate

groundwater (Alder et al., 1990; Satroutdinov et al., 2000) . those complexes have high stability constants that are degraded slowly and, as demonstrated, they are relatively stable from a biological point of view, even under conditions favorable to biodegradation (Hong et al., 1999 ; Bucheli-Witschel et al., 2001). For these reasons, taking EDTA as a reference for its performances, we tested EDDS that is a structural isomer of EDTA and has two chiral carbon atoms and three stereoisomers (Vandevivere et al., 2001). Among them, only the (S,S) isomer is readily biodegradable. EDDS has the potential to be a substitute of EDTA in chelant-assisted phytoextraction as it is a strong chelators and, unlike EDTA, it is easily biodegradable. Indeed, mineralization of EDDS in sludge-amended soil was completed in 28 d with a calculated half-life of 2.5 d (Jaworska et al., 1999). In addition, several metal- EDDS complexes are readily biodegradable (Vandevivere et al., 2001).

MATERIALS AND METHODS

GREENHOUSE AND OUTDOOR TRIALS

Salix spp.

At the end of March 2007, branches of *S. matsudana, S. alba, S. fragilis and S. babylonica,* one or two years old were were chosen in order to obtain several cuttings 10 cm long, with a minimum of 3 buds and with an homogeneous diameter. Leaves were immediately cut off to limit water loss and branches were enveloped in aluminum foil and transported to lab into a plastic bag.

Only cuttings with diameter between 3.5 and 5 mm were selected. Each cutting was enveloped in a parafilm ring at the first third of own highness to avoid the submersion of 2 of 3 buds at least. Several 3 L plastic beckers were filled up with 2.5 L of tap water and enveloped with aluminum foil; on the top of each beacker a gummy circular module with open bottom was inserted in order to contain an aluminum little basin (tipically used for food preservation). Each basin was inserted and locked into the gummy module. With scissors 13 holes in the bottom of aluminum basin were done (four for each larger side, two for each short side and one in the center of basin to guarantee the right distance between cuttings). In each hole a cutting was placed and inserted at the depth allowed by parafilm ring. All the beakers with cuttings were left in the dark for a week to make easier root building up. Once root germination happened, tap water was replaced by modified Hoagland nutrient solution (deionized water plus KH_2PO_4 40µM, KNO_3 200 µM, $Ca(NO_3)_2*4H_2O$ 200 µM, KCl 200 µM, $CaCl_2*2H_2O$ 200 µM, $MgSO_4*7H_2O$ 200 µM, H_3BO_3 4.6 µM, $MnCl_2*4H_2O$ 0.9 µM, $CuCl_2*2H_2O$ 0.036 µM, $ZnCl_2$ 0.086 µM, $NaMoO_4*2H_2O$ 0.011) and the beackers with cuttings were placed for 10 days in growth chambers settled with this parameters : Light 16 h, T 25 °C, R.H. 60% ; Dark 8 h, T 18 °C, R.H. 80% ; light intensity : 280 µE/m^2/s . In this period Hoagland solution was replaced one time and water oxygenation was

guaranteed by bubbles of small air pumps. Then, 15 best rooted cuttings for each species were gently extracted from basin and placed in a 1 L plastic pot with a universal soil used for house gardening with this characteristics: pH 5.47, TKN 809 (mg N/Kg dw), NH_3 257.14 (mg NH_4^+/Kg dw), TOC 7.4 (% of dw) Cl^- 707.4 (mg/Kg dw), P tot 660.5 (mg/Kg dw). Plants were let grow for 2 more weeks.

Polluted substrate preparation : the experimental substrate was prepared by mixing sediments of Venice lagoon with river sand (1:1 volume). The sediments were digged out from different sites in the "oil channel", a large channel connecting open sea with the chemical industrial pole of Marghera Harbor, which is crossed daily by oil carrier ships. Mean characteristics of the sediment are : pH 8.47, TKN 687.8 mg N/ Kg dw, NH_4^+ 108.8 mg/Kg dw, TOC < 1 (% of dw), P tot 1670 (mg/Kg dw). The substrate was left to dry for a week on a plastic groundsheet on the floor of greenhouse. The substrate was then characterized: pH 8.16, TKN 34 mg/ Kg dw, NH_4^+ 29 mg/Kg dw, TOC 10.2 (% of dw), P tot 180 mg/Kg dw. After that, eleven 50 L plastic tanks were prepared as follows: a flexible plastic tube 50 cm long was connected to the bottom of the tank. The tube was placed to stay bent avoiding water loss between two water sampling or drainage . In the inner side of tube link a sackcloth filter was put to protect the tube from clogging. The tank bottom was covered until the tube hole with a 10 cm thick layer of expanded clay to ameliorate water drainage ; then tanks were filled up with 35 L of mixed substrate.

Willows planting : initially five individuals for each species were placed on tanks with naked roots. Although cuttings were very gently manipulated, tey quickly died, likely due to the direct contact with salt and pollutants plus the transplanting shock. A second series of plants were planted with soil bulk to avoid transplanting shock and allow an acclimation of root system. This trick permitted the surviving of *S. matsudana* only, and after a week, 9 more *S. matsudana* plants were placed in two tanks to have a total of 14, seven for each tank. In the same period 6 *S. matsudana* plants were transplanted in six 4 L pots with universal soil as controls. Indeed, the characteristics of the soil are different from the mixed substrate, but it was thought to have an idea of the normal development of a *S. matsudana* plants in comparison with stressfull growth conditions.

Willows growth : plants were left on tanks from the end of May, until the end of vegetative season (Mid-October). For the first 30 days tanks remained in a greenhouse, then, they were put outdoor under a gazebo, covered on the top with a transparent plastic large sheet to avoid an oversupply of rainy water. Modified Hoagland nutrient solution (0.5L) + tap water (0.5L) were added once a week for each tank . During summer regular weed killing action was done around tanks to limit danger of pest infestation and one time plants were treated successfully against young grasshoppers invasion.

Water sampling was done four times : the first one just before plants placement, then in July, August and September.

Willows harvesting : seven (of the starting 14) plants survived and at Mid-October were harvested with the six control plants. Root system was gently extracted and gently washed firstly twith tap water to remove the major part of substrate and then with deionized water for a fine

clean up. Roots and leaves were separated from stems and fresh weights were recorded for each plant. Then leaves, stems and roots were put in oven for 48 h at 105 °C and dry weights were recorded. All samples were ground with a pestle in liquid nitrogen. The samples were digested in nitric and chloridric acid on heating plate and. analyzed with ICP-AES method to detect concentration of Cd, Zn, Pb, Ni, Cu and Na. Analysis of each sample was repeated three times.

Phragmites australis

Thirty *P. australis* stems with rhizomes and thin roots were collected during March 2007 in the Ca' di Mezzo wetland close to the town of Codevigo, Padova, Italy. The rhizomes were collected with some muddy soil to maintain their moisture and put in a sealed plastic bag to transport them to the greenhouse. Once in greenhouse rhizomes were washed with tap water and planted into two tanks (ten for each tank) filled up with the mixed subtrate sand-sediment. Ten plants were planted in a third tank filled up with universal soil as control. *Phragmites* plants, differently from *Salix spp.* tolerated the direct contact with new substrate. After two months, the tanks were put under gazebo (as described above for willow plants).

Polluted substrate preparation : substrate and its preparation are the same described for willows.

Phragmites growth : during the experiment, once a week 0.5 L of modified Hoagland nutrient solution and twice a week 0.5 L of tap water were added to each tank. The control plants were supplied with the same volume of tap water only. The aim of this constant water supply was to reproduce as much as possible the conditions of soil saturation, typical of *Phragmites* environment. In July, once plants health conditions were considered enough stabile (increasing biomass production, absence of pests and chlorosis), in one tank 1 mmol/Kg dw of Ethilene Diammine Tetracetic Acid (EDTA) was added. EDTA was added in two times after a week without irrigation to favorite its absorption by rhizomes. During each addiction it was diluted in 100 ml of deionized water.

Water sampling were taken once before EDTA addiction, and in August, September October and November at the end of experiment. Only data of July, October and November were reported because no significant changes in metals concentrations happened between July and August. Every water sample was collected in a 50 ml falcon tube, then filtered and analyzed with ICP-AES method to detect concentration of Cd, Zn, Pb, Ni, Cu and Na.

Phragmites harvesting : two intermediate biomass samplings were done (1 in September and 1 in October) to detect variations in heavy metals and Na concentration in Phragmites stems (not for control), since Phragmites plants are known to transfer from roots to shoots potential toxic elements absorbed during the vegetative season . In November all stems and rhizomes were harvested and cleaned up two times with tap water to remove biggest pieces of substrate and then with deionized water for a fine clean up. Rhizomes and shoots were separated and fresh weights were recorded for each individual. Then, shoots and roots were left in oven for 48 h at 105 °C and dry weights were recorded. All samples were ground with a pestle in liquid nitrogen. About two grams of samples were digested in nitric and chloridric acid on heating plate and were

digested by Milestone Ethos 1600 microwave mineralizer. The samples were analyzed with ICP-AES method to detect concentration of Cd, Zn, Pb, Ni, Cu and Na. Analysis of each sample was repeated three times.

Brassica juncea

In April 2007 seeds of *Brassica juncea* cv.PI 426314 were planted in 0.1 L plastic pots filled up with universal soil (1 seed per pot) and left in greehouse. After germinations, seedlings 10 days old were transplanted in 0.5 L pots and after 2 weeks 30 plants (15 per tank) were transplanted with own bulk soil into the experimental substrate to minimize the double shock of transplanting and direct contact with the salty substrate. Remaining 10 plants were transplanted in 1 L pots (one per pot) with universal soil as control and supplied with tap water.

Polluted substrate preparation : substrate and its preparation are the same described above.

Brassica growth : plants were supplied with 0.25 L of modified Hoagland nutrient solution (as described above) two times a week in a range between 0.25 and 0.5 L for each tanks, depending on temperature variations.. The survival of plant wasn't homogeneous (11 in one tank and 4 in the second one). .

Water sampling and drainage : because the high water retaining of substrate, and probably a partial blockage of filter we were not able to make a regular and sufficient water sampling for ICP analysis.

Brassica harvesting : all Indian mustards survived were harvested the last week of July, after a life cycle of almost 3 months. Roots were washed two times with tap water and with deionized water then separated from shoots. Roots and shoots were put in oven for 48 h at 105 °C, and dry weights were recorded. All samples were ground with a pestle in liquid nitrogen. Two grams or more samples were digested in nitric and chloridic acid on heating plate, smaller than 2 grams samples like roots, were digested by Milestone Ethos 1600 microwave mineralizer. The samples were analyzed with ICP-AES method to detect concentration of Cd, Zn, Pb, Ni, Cu and Na. Analysis of each sample was repeated three times

GROWTH CHAMBER TRIALS

Willows hydroponic trial

Willows growth : Sampling and growing of *Salix matsudana, fragilis, alba* and *babylonica* were the same for greenhouse and growth chamber trials, until roots germination with tap water in dark room. For each species four beackers with 13 cuttings for each one were prepared. After root germination, beackers were placed in growth chamber settled with the following parameters : Light 16 h, T 25 °C, R.H. 60% ; Dark 8 h, T 18 °C, R.H. 80%. During the entire period of the

experiment in growth chamber, nutrients and oxygen supply were guaranteed respectively by modified Hoagland nutrient solution, weekly replaced, and small air pumps. At the beginning of the second week in growth chamber, experiment started.

Treatments : based on water sampling at the beginning of greenhouse trial, the concentration in hydroponic solution of five ions (Cd, Zn, Pb, Ni, Cu) four beackers (one for each species) was : Cd 0.1 µM as $CdCl_2$, Zn 0.5 µM as $ZnCl_2$, Cu 1 µM as $CuSO_4*5H_2O$, Pb 0.1 µM as $Pb(NO_3)_2$, Ni 0.1 µM as Cl_2Ni*6H_2O. Four more beackers were kept as control and, since the third week of experiment, other four beackers, previously supplied with metals cocktail, were added with NaCl 0.05 M. NaCl concentration was increased week by week from 0.05 to 0.1 to 0.2 M, in order to approach Na concentration detected in water collected from tanks avoiding at the same time, to use immediately a lethal saline concentration for plants. Increasing NaCl concentration brought plants to death 3 weeks before end of experiment.

Willows harvesting : after two months, (7 weeks for NaCl treated plants), willow plants were harvested. Fresh weight of leaves, stems and roots and maximum root length were recorded. All tissues were dried in oven for 48 h at 105 °C , then dry weights were recorded. The tissue accumulation of Cd, Zn, Pb, Ni, Cu and Na was analyzed as described above. Analysis for each sample was repeated three times.

Brassica juncea hydroponic trial

Brassica growth : Seeds (cv.PI 426314), collected from plants previously grown in greenhouse were put in a 15 ml tube to be sterilized by 1 minute in ethanol 70% and 30 minute in bleach 30%. After that, seeds were cleaned up from bleach by five 10 minutes long washes in sterile deionized water; during each wash tubes were put on the rocker to be gently shaked. Between each wash, water was replaced under sterile hood. Then sterilized seeds were put in big glass magenta boxes (50 -60 per box) under sterile hood using sterile forceps. The medium was agar added with MS salts and vitamins MS 5519. The medium was prepared as follows : (250 ml per box), 4.46 g/L of MS (M5519, Sigma)+ 30 g/L of sucrose + some distilled water. After all is dissolved, pH was brought to 5.8 with diluted KOH . Than solution was brought to final volume with distilled water. Agar gel 4 g/ L was added and the mixture put in glass boxes and then in autoclave for 21 minutes, then boxes were put under hood to cool down to ~ 55 °C . Boxes were put in growth chambers for 5 days; the cap was sealed with plastic foil and covered with aluminum foil. Seven 3 L plastic beckers were filled up with 2.5 L of deionized water plus modified Hoagland nutrient solution (as described above for willows) and a gummy circular module was put on the top of each becker; In each module a metallic grid (light diameter = 2 mm) was inserted. Brassica seedlings were gently extracted from agar gel and, after a quick root washing in deionized water, placed into each of seven grids with roots submerged and shoots over the grid surface. All beackers were put into grow chamber (settled as described for willows). Brassica seedlings were let to grow for a week.

Treatments : One beacker was filled up again with 2.5 L of deionized water plus modified Hoagland nutrient solution (as described above for willows) and kept as control, two beckers with Hoagland solution plus 1x metal cocktail (as described for willows), two beackers with Hoagland nutrient solution plus 1x metals cocktail and NaCl 0.05 M. Experiment was 45 days long. Nutrients and metals were replaced weekly and water was continuously oxygenated by air pumps. NaCl concentration in metals plus NaCl treated beckers was increased week by week from 0.05 to 0.1 to 0.2 M, in order to approach Na concentration detected in water collected from tanks avoiding at the same time, to use immediately a lethal saline concentration for plants. Increasing NaCl concentration brought plants to death 2 weeks before the end of experiment.

Brassicas harvesting : seedlings in control and metals added beackers were harvested after 45 days while only after 30 days the seedlings treated with metals and NaCl were harvested. Total fresh biomass of shoots and roots for each treatment was recorded. After 48 h at 105 °C , shoot and roots dry weights were recorded. Samples were digested by Milestone Ethos 1600 microwave mineralizer. The samples were analyzed with ICP-AES method to detect concentration of Cd, Zn, Pb, Ni, Cu and Na. Analysis of each sample was repeated three times.

Willows on sludge trial

Cuttings collection and growth : this steps were carried on exactly as described above for hydroponics and greenhouse trials. After hydroponic step (one week in the dark and ten days in growth chamber with modified Hoagland solution), 15 willows were transferred on polluted substrate.

Polluted substrate preparation : five 20 L plastic tanks were prepared removing the top with a knife and adding a sackcloth filter (to protect the tube from clogging) in the inner side of tap, placed in the bottom of one of shorter sides. From the bottom, tanks were filled up with a 7 cm thick layer of fine gravel to ameliorate water drainage, 20 cm of polluted sludge mixed with sand as described above and on surface, with a 6 cm thick layer of the same universal soil used to grow willows and place them on sludge for greenhouse trial.

Willows planting : In each tank three willow plants were planted. One species per tank (*S. matsudana, S. alba, S. fragilis and S. babylonica*). *Salix Babylonica* was planted in two tanks in order to evaluate its uptake performances also with help of Ethylenediamine disuccinic acid (EDDS). Willows were placed as a row in the middle of tank, surrounded by 8 plants of *Paspalum vaginatum* planted with their 12 cm^3 of universal soil. Before planting, fresh weight of each willow was recorded to compare it with final biomass increase at the end of experiment.

Willows growth : experiment was 50 days long. Once a week plants were supplied with 150 ml of modified Hoagland nutrient solution composed as described above. One week after planting, 3 mmol/Kg d.w. of EDDS (100ml with 30% of water) were addicted to fifth tank with second group of *Salix Babylonica*.

Water sampling : at the end of the second, fourth and sixth week, 50 ml of drained water were collected from each tank tap. Samples were filtered and analyzed with ICP-AES method to detect concentration of Cd, Zn, Pb, Ni, Cu and Na. Analysis of each sample was repeated three times.

Willows and *Paspalum* harvesting : After fifty days willows and *Paspalum* were harvested. Fresh weight of roots, leaves and stems was recorded. All paspalum biomass was divided in shoots and roots without distinctions between individual plants. Roots were washed two times with tap water and deionized water. All tissues were dried in oven for 48 h at 105 °C , then dry weights were recorded. All samples were ground with a pestle in liquid nitrogen. concentration of Cd, Zn, Pb, Ni, Cu and Na for willows and Na only for *Paspalum*. Analysis of each sample was repeated three times.

Willows root system deepening trial

Cuttings collection and growth : steps were carried on exactly as described above for hydroponics and greenhouse trials.

Polluted substrate preparation : twenty four 2 L plastic bottles were deprived of the top with a razor blade and a tap was added closest to bottom to drain exceeding water. Sixteen of this bottle were filled up, from the bottom with a 4 cm thick layer of fine gravel to allow water drainage, 20 cm of sludge mixed with sand and on the top, 4 cm of same universal soil used in previous experiments. Other eight bottles were filled up with fine gravel, 20 cm thick layer of soil from Experimental Farm and 4 cm of universal soil. These bottles were used as control. All bottles were enveloped with aluminum foil against light stress for external roots.

Willows planting : one willow was planted in each bottle. For each species three willows were planted on sludge and three on bottles kept as "control".

Willows growth : bottles were left in growth chamber (settled as described above) for 50 days. Once a week they were supplied with 50 ml of modified Hoagland nutrient solution. To avoid water accumulation on surface, every ten days a drainage of 20-30 ml was done.

Willows harvesting : after 50 days, willows stems were harvested, leaving root system in the bottle. After fresh weight recording, leaves and stems were put in oven at 105 °C for 48 h, then dry weights were recorded. Bottles with substrate and root systems were put in freezer at – 20 °C for 3 days. After freezing , bottles were sawed in four parts : the first corresponding to universal soil layer, the second one to the more narrow bottle section filled up with sludge (from 6 to 16 cm of depth), the third to the larger section of bottle filled up with sludge (from 16 to 26 cm of depth) and the fourth corresponding to gravel. The last section was always immediately discarded because never explored by roots; other parts of bottle were separated from external plastic layer, then roots, with water, were gently separated from substrate in the sink, being careful to maintain intact as more as possible the root system, using a basin to avoid polluted sludge dispersion. Once cleaned up, each part of root system, corresponding to a section of bottle was put on a transparent glass with a thin layer of water and scanned by Amersham image

scanner. Images captured were analyzed with Image capturer and analyzer KS 300 (ZEISS-Germany) to evaluate total root length of each layer and root diameter. After that, fresh weight and, after 48 h in oven at 105 °C, dry weight of roots were recorded.

Statistical analysis

For all experiments described, one –way ANOVA and T-TEST were performed by SPSS 17.0. Post hoc analysis were performed with SNK test $P > 0.05$.

RESULTS

Willows

Some authors, comparing hydroponic results with field/pot trial results underline the inconsistency of first one because performances in accumulations don't reflect real performances in soil and over that, performances change widely, depending on kind of soil (Dos Santos & Wenzel 2006). Starting from these considerations , we wanted in this work compare hydroponic and pot trials (indoor and outdoor) to evaluate real *Salix spp* ability in metal accumulation and surviving in soil characterized by high salinity. We focused on available ions contained in soil solution to evaluate the real effectiveness of phytoextraction in this specific conditions without paying attention to possible maximum remediation potential of species considered that are already reported in literature. Outdoor trial was carried on with *S. matsudana* only because of the quick death of other three species of willows.

Cadmium : (Fig 1,) hydroponic trial shows that in leaves, Cd-accumulation of *S. fragilis* is the most affected by Na accumulation, although concentration of this one in this tissue is the lowest of the group. Other three species shows similar Cd concentration in high salinity conditions, resulting less affected in Cd accumulation by Na. Trial in grow chamber in soil (Fig 4) shows data similar to Hoagland plus metal plus Na treatment in hydroponic, that means for Cd hydroponic has simulated pretty reliably conditions in soil solution. Cd concentrations in leaves are pretty low like in hydroponic. The unique significant difference is shown by S. fragilis that seems less affected by salinity in soil than in hydroponic.

In the stems of plants grown in hydroponic (Fig 2 PUO'), no significant differences were observed between presence and absence of Na for *S. fragilis* and *S. babylonica* , that are best performers in Cd accumulation, while *S. alba* accumulated in stem roughly 5 times more Na than in *S. matsudana, fragilis and babylonica*. Trial in soil (FIG 5) showed that there are no significant differences in accumulation between *S. alba, fragilis, babylonica* and *babylonica* EDDS- addicted. Only *S. matsudana* showed significantly lower level than the other ones. Hydroponic and soil trials shows similar Cd concentration values.

In roots of all species, grown in hydroponics (3) showed Cd uptake was heavily reduced by the presence of Na; also, every species showed similar concentrations of Cd when grown in presence of Na, except for *S. fragilis*. In soil trial, *S. matsudana* showed the lowest Cd concentration,

while the other three species showed similar values. In general Cd accumulation in root was similar in hydroponic and soil trial.

S. matsudana in outdoor trial (fig 7) shows very similar values in all tissues to *matsudana* grown in grow chamber.

Nickel : in hydroponic (Fig 1B) , Ni accumulation in leaves was strongly depleted by Na presence, except for *S. babylonica*. In soil trial (Picture 4 Graf B), concentrations are all closest to zero and even not EDDS could increase performance of *babylonica*. Two trials shows an easier Ni accumulation in leaves in favour of hydroponic.

For stems hydroponic (picture 2 B) shows no significant differences and very low concentrations. We just note only *S. babylonica* and alba depleted their accumulation in presence of Sodium. In soil trial too (Picture 5 Graf B) we found very low concentrations (< 0.5 mg/Kg D.W.) so we can say hydroponic gives back us a good picture of situation.

Roots of plant grown in hydroponic (Fig 3 B) roots do not accumulate, in presence of NaCl, more than 10 mg/Kg D.W. and values are all similar except for *S. fragilis* that has concentration closest to zero. In soil trial (Fig 6 B) concentrations are low and similar to hydroponic except for *S. alba* that accumulates almost 50 mg/Kg.

S. matsudana grown outdoor shows concentrations that are an average between hydroponic and indoor trial in soil. (picture 7)

Copper : hydroponic (Picture 1 D) shows leaf Cu contents, in plus Na treatment, similar for *S. matsudana, alba* and *babylonica* (the last two show Cu accumulation higher than control), while *fragilis* is again the more suffering Na interaction. In soil trials (Picture 4 C) leaf contents are similar for all species and comparable with hydroponic results. In this case EDDS is effective, enhancing accumulation five time more than not EDDS addicted *S. babylonica*.

Plants grown in hydroponic (Fig 2 D) show no significant differences in Cu accumulation between + Na and –Na treatments. *S. babylonica* shows higher concentration in +Na treatment (15.5 mg/Kg) although not significantly different from –Na treatment. Plants grown in soil (Picture 5 C) shows the same Cu accumulation in stems, with results very similar to hydroponic trial. EDDS enhanced significantly (two times) accumulation in *S. babylonica*, bringing up accumulation to a value comparable with +Na treatment in hydroponic.

In roots grown in hydroponic (Picture 3 Graf C) NaCl presence seems to improve significantly Cu uptake in *S. alba* (360 mg/Kg) and *S. matsudana*, while in *S. fragilis* uptake is depleted and in babylonica enhancement is not significant. Roots grown in soil (Picture 6 Graf C) shows values similar to hydroponic treatment without NaCl. EDDS doesn't improve accumulation.

In outdoor trial S. matsudana (Picture 7) uptake is two times higher than indoor trial in all tissues, while comparing it with hydroponic, roots uptake is less than 50%, while translocation to stems and leaves shows similar behavior.

Lead : plants in hydroponic (Fig 1 C) show leaf concentrations closest to zero in + Na treatments. In every cases Pb accumulation is very low and severely depleted by NaCl.

In plants grown in soil (Picture 4 Graf D), lead accumulation in leaves resulted completely absent except for EDDS treated *S. babylonica* that shows anyway very low concentration (2.5

mg/Kg). Considering very low values recorded for hydroponic we can consider them reliable in comparison with concentrations obtained in soil trial.

Plants in hydroponic show low Pb concentrations in stems (Picture 2 Graf D) with no differences between + Na and –Na except for *S. fragilis* in which salt enhanced two times accumulation (5.5 mg/Kg). Values are pretty similar for soil trials (Picture 5 Graf D) and EDDS didn't enhance babylonica accumulation.

In roots grown in hydroponic (Picture 3 Graf D) NaCl enhances strongly Pb uptake (190 mg/Kg in *S. alba*) except in *S. fragilis* in which Pb uptake is anyway low. Behaviour in soil (Picture 6 Graf D) was completely different with a maximum of 8 mg/kg and without effective action by EDDS.

Outdoor trial (Picture 7) shows a low translocation to stems and leaves and a higher uptake in roots in comparison with indoor trial while it is lower almost two times than concentration in hydroponic.

Zinc : in hydroponic (Picture 1 Graf E) leaves show a significantly depleted accumulation in a range between 20 (alba and babylonica) and 30% (matsudana) while in fragilis accumulation is severely affected by NaCl (10mg/Kg). In soil trial (Picture 4 Graf E) values are two times higher except for alba that shows concentration similar to hydroponic and EDDS treated *babylonica* increased accumulation 4 times compared with not EDDS addicted *babylonica* and 5 times compared with hydroponic that shows generally concentrations half than soil trial.

Plants grown in hydroponic show in stems (Picture 2 Graf E) a slightly depleted accumulation by Na in fragilis and alba, while in *babylonica* NaCl enhanced significantly Zn accumulation (130 vs 100 mg/Kg). Stems grown in soil (Picture 5 Graf E) show for *babylonica* and EDDS-addicted *babylonica* concentrations similar to hydroponic (140 mg/Kg) and other species too performed similar results comparing with hydroponic, but with values half time lower than *babylonica*.

About roots, in hydroponic (Picture 3 Graf E) only *S.fragilis* shows a severe depletion in Zn uptake by NaCl while other species accumulate between 100 and 200 mg/Kg. Similar ranges of accumulation are shown in roots grown in soil (Picture 6 Graf E). To be underlined is the difference between *matsudana* root system in water (100mg/Kg) and in soil (250 mg/Kg). EDDS doesn't act effectively on uptake.

Performances of *S. matsudana* in outdoor condition (Picture 7) are exactly the same of that ones in indoor condition and consequently not in according with hydroponic.

Sodium : in hydroponic (Picture 1,2,3, F) *S.alba* looks like as best Na accumulator and translocator from roots to leaves. All willows species are carachterized by a very high Na uptake (until 80000mg/Kg in *S. babylonica*) except *S. fragilis* that stay below 2500 mg/Kg. In soil (Picture 4,5,6) all species uptake and translocated many folds less Na than in hydroponic. While in hydroponic *S. alba* was best accumulator and translocator, in soil *S. matsudana* resulted to translocate Na to leaves more efficiently than other species.

Although leaf concentration in *S. matsudana* grown outdoor (Picture 7) are strongly higher (14000mg/Kg vs 3000 mg/Kg) than indoor ones, we can see the same behavior in translocating a great amount of Na to leaves.

Biometric parameters and accumulation indexes : all willows species tested in hydroponics suffered a great depletion in fresh biomass (about 60% except for *S. fragilis* that shows a depletion of 80% compared with control) caused by excess of NaCl (Picture 8). Root lengths of *S. fragilis* and S.*babylonica* seem lightly suffering Na interaction while S.*alba* and S.*matsudana* show a depletion in root length of 60%.(Fig. 9)

In indoor trials carried on in soil, *S. alba* shows the greatest dry biomass production and *S. babylonica* shows to be negatively affected by EDDS addiction. BF(Bioaccumulation Factor) and TF (Translocation Factor) suggests that EDDS in *S. babylonica* is effective regarding bioaccumulation of Zn and Cu only but it increases translocation to canopy of all elements in all *Salix spp* those grown indoor show good BF for Na and Cd while Cu, Ni, Pb and Zn remain below 1. TF was high (~ 3) only for Na. Dry biomass production is similar to indoor trial.(data not shown).

Root deepening trial : *Salix alba* and *S. babylonica* are less suffering because, as found by Vamerali et al. 2008 (data not published), root thickness increases with deepening into sludge layers. Root length shows an opposite behavior so root biomass is the summary of these two opposite forces. *S. matsudana* and *s. fragilis* developed root system mainly in first layer filled up with house gardening soil while roots was strongly shorter than control in deeper layers of bottle. At the opposite, *S. alba* and *S. babylonica* show a behaviour similar to the control plants. (data not shown).

 S. matsudana is the most suffering because the highest root biomass in first layer and biggest diameter in third layer coupled with a low biomass production (Fig 12). S. *alba* seems again the best performer : its root system was mainly developed in third layer both for length and biomass.

Effects of EDDS on soil solution : chelant agent affects positively metal availability in soil solution except for Cd and Zn. Na availability seems decreased by EDDS (10000mg/Kg vs 6000 mg/Kg of EDDS treated soil)

Phragmites australis

Phragmites was grown only outdoor . As demonstrated by Bragato et al (2006) *Phragmites* at the end of grow season excludes from rhizomes toxic elements translocating them to shoots. In this experiment this behavior is not so clear, probably because of the low uptake of heavy metals. EDTA affects negatively and significantly only Na accumulation in stems, cutting it off for a 50% since October (Fig 13). In rhizomes (Fig 14) the chelant agent was effective only on accumulation of Pb and Cu while it reduced Na level considerably (500 mg/Kg vs 3500 mg/Kg). No differences in fresh and dry biomass production between EDTA and non EDTA addicted *Phragmites* plants. No differences are noted in the water leached from different tanks containing the four willows species. Looking to bioaccumulation factor (Picture 15) it's clear how EDTA causes a severe depletion of Na uptake that in turn allows a better bioaccumulations of heavy metals although BF never pass the value 1. EDTA enhance translocation factor of Na more than two times but the result is fake because the lower Na accumulation in rhizomes by EDTA-treated *Phragmites*. Behaviour of Zn, Na and Ni in absence of EDTA and that one of Zn, Na and Cd in

presence of EDTA are that expected because they shows a regular increase in translocation to shoots across months.

In soil solution EDTA increase availability only of Zn, although its concentration remains low (4 ppm vs 0.5 ppm) while Na availability was depleted more than two times (10000 mg/L vs 22000mg/L).

Brassica juncea

Hydroponic trial carried on for a maximum of 45 days (30 for NaCl treatments) shows no significant differences in biomass between control, metal added solution and metal + NaCl solution. In roots Ni and Cd levels were low, both in presence and in absence of Na (<100mg/Kg) while Pb, Zn and Cu, in presence on Na, suffered a depletion of more than 50% (Cu ~200mg/Kg ; Zn ~ 125 mg/Kg ; Pb ~400mg/Kg). In stems metals concentration is depleted of a 50% by presence of Na in nutrient solution, too. Translocation to shoots both in absence and in presence of Na does never pass ratio 1 and for Cu and Pb is lower than 0.5. Na uptake is around 35000 mg/Kg in roots and 25000 in shoots.

In soil trial carried on outdoor, uptake of all metals is in average ten times lower than in hydroponic : less than 20 mg/Kg for all metals considered in roots and less than 6 mg/Kg in stems. BF in soil is impressive for Na (~25) and good for Cd (>1) while it is less than 1 for all other metals. TF is higher than 1 only for Na (2.4) and Zn (1.2). (Data not shown)

DISCUSSION

About the general plant capacity of metals accumulation and translocation in high salinity conditions, the hydroponic experiments give us back a reliable picture of pure phytoextraction potential in this specific situation. Infact, although Na accumulation is usually ten times higher in hydroponics than in soil, competition between Na and metals brings to results considered very similar to that obtained in soil, in the majority of cases. These data allow us to make reliable considerations on performances of the tested willows species and particularly to see the differences between ideal remediation potential with the metal concentrations in soil solution and real effectiveness considering the salinity action.

Considering heavy metals uptake in conditions of low salinity and metals availability reported in literature(Pulford and Dickinson 2005 ; Dos Santos and Wenzel 2006) we can see toxic concentrations in roots and aboveground tissues are almost never touched both in hydroponic plus salt and in soil; the concentrations in aboveground tissues are particularly low, while in roots, only Cu and Zn for all species and Ni for *S. alba* reached concentrations that could damage plant metabolism. Certainly, further investigations can be useful to determine more accurately the metals interactions, but, in this case, it is pretty clear the main action of sodium in depleting accumulation performances indirectly by pH increase (pH ~ 8.5) and directly by reducing biomass production and by competition with metals. Competition acts also in metal translocation

and it is clearly detected after EDDS addiction. In *S.babylonica,* it enhances metal translocation to stems and mostly to leaves, although does not increase fitoextraction.

Nevertheless, having taken care all details of transplantation of willows in 50 L tanks with their bulk of house gardening soil, only *S. matsudana* survived until the end of trial while S.*fragilis*, *S.babylonica* and *S.alba* died quickly. It should suggest a leading line to select a phytoremediation plant *in situ* for these polluted sludge : the main differences between outdoor trials and indoor trials are the variability of T, humidity, evaporation and hours of light in outdoor conditions. It is well known that in a soil characterized by high salinity root system enhances Na concentration locally by both Na ions extrusion and water uptake (Van Genuchten 1987). This phenomenon could become stronger when in a situation of water deficiency, roots explore only certain points lowering water potential together with Na concentration increase (Amato 1991). Considering that soil is already heterogeneous in Na concentration, *S. babylonica* seems unable to survive in salty soils (Yangh et al. 2004 ; Kennedy et al., 2003), *S. fragilis* needswell drained and aerated soils, and *S. alba* seems tolerate salinity in field conditions only when hybridized and *S. matsudana*, as reported by Zhang 1998 shows high salt tolerance, we can explane high difficult to keep alive willows in not controlled conditions in which there is possibility of unexpected water deficiency because quick air and T variation.

Paspalum vaginatum plants showed high tolerance and accumulation of Na together with high translocation factor. Therefore, it could, become one of possible best choice for Na extraction, also thanks to the easy harvesting of the aerial part. because its high tolerance to cut and easy harvesting. Over that, we found it tolerates EDDS addiction like EDTA as proven by Masciandaro et al., (2008)

Phragmites australis plants seem to tolerate pretty well salinity and its yield and metal accumulation performaces are not so far from data recorded in not salty wetlands (Bonanno 2009). EDTA addiction does not modify significantly metals uptake and translocation to shoots but depletes both Na availability and uptake in favour of metal accumulation in roots. Probably salt tolerance derived not only from the capacity of this species but also from a dilution of soil solution given by water supply to keep wet soil in order to simulate as much as possible the original environment of the plant species.

Brassica juncea plants show a completely different behavior between hydroponic and soil trial. Performances in soil do not leave space for a possible use of Indian mustard on this sludge both because low biomass production and low metals uptake.

CONCLUSIONS

Looking at the selection of a remediation plant, we are forced to decide if either start immediately with a slow phytoremediation process because high Na concentration, or focus first on Na extraction. The second choice appears the most proper, using *Paspalum vaginatum* as best Na extractor and *S. matsudana,* that shows the better ability in Na translocation to shoots

and leaves and a good tolerance to drought stress, coupled with a good biomass production. Since these two species occupy different ecological niches they can be used together. Once Na concentration has become enough low to allow an effective phytoextraction, *Salix babylonica* and S.*fragilis,* that showed good rates of metal accumulation, and *S. alba* that showed the best biomass production and the most tolerant root system in well watered conditions, could be utilized.

Phragmites australis should be a good solution considering its biomass production and salt tolerance. Its ability to survive in a wide range of environmental conditions coupled with good performances in pollutants uptake become it a good candidate in sludge remediation.

FIGURES

Figure 1 : ions content in leaves of willows in hydroponic trial. X axis : metal. Y axis : ion concentration measured in mg on Kg of dry biomass. M = *Salix matsudana* ; F = *Salix fragilis* ; A = *Salix alba* ; B = *Salix babylonica*. C= control ; m = Hoagland nutrient solution + metals ; Na = Hoagland nutrient solution + metals + Na (0.05 M then 0.1 then 0.2 M). Different letters mean significant differences.
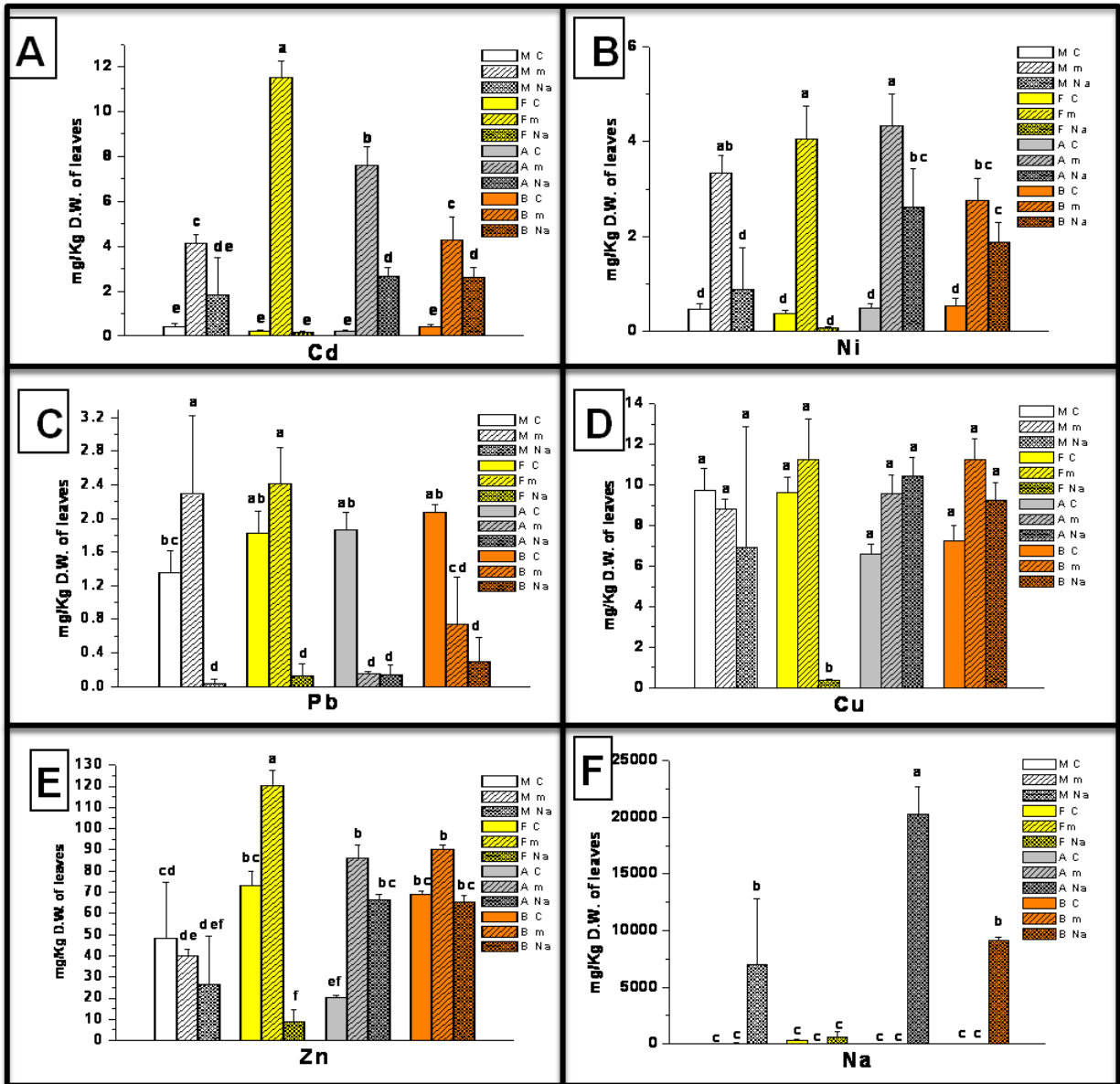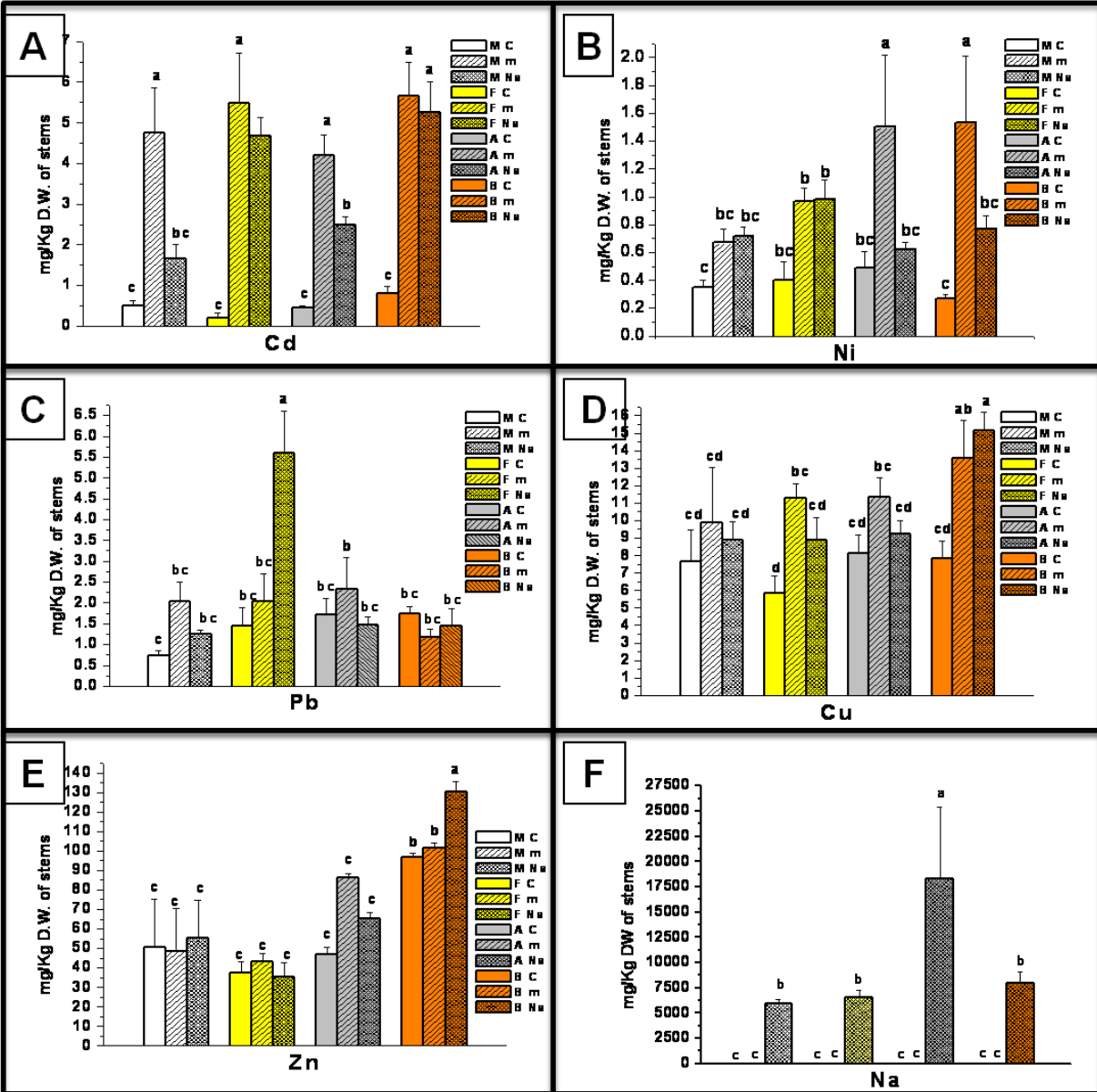
Figure 2 : ions content in stems of willows in hydroponic trial. X axis : metal. Y axis : ion concentration measured in mg on Kg of dry biomass. M = *Salix matsudana* ; F = *Salix fragilis* ; A = *Salix alba* ; B = *Salix babylonica*. C= control ; m = Hoagland nutrient solution + metals ; Na = Hoagland nutrient solution + metals + Na (0.05 M then 0.1 then 0.2 M). Different letters mean significant differences.

Figure 3 : ions content in roots of willows in hydroponic trial. X axis : metal. Y axis : ion concentration measured in mg on Kg of dry biomass. M = *Salix matsudana* ; F = *Salix fragilis* ; A = *Salix alba* ; B = *Salix babylonica*. C= control ; m = Hoagland nutrient solution + metals ; Na = Hoagland nutrient solution + metals + Na (0.05 M then 0.1 then 0.2 M). Different letters mean significant differences.
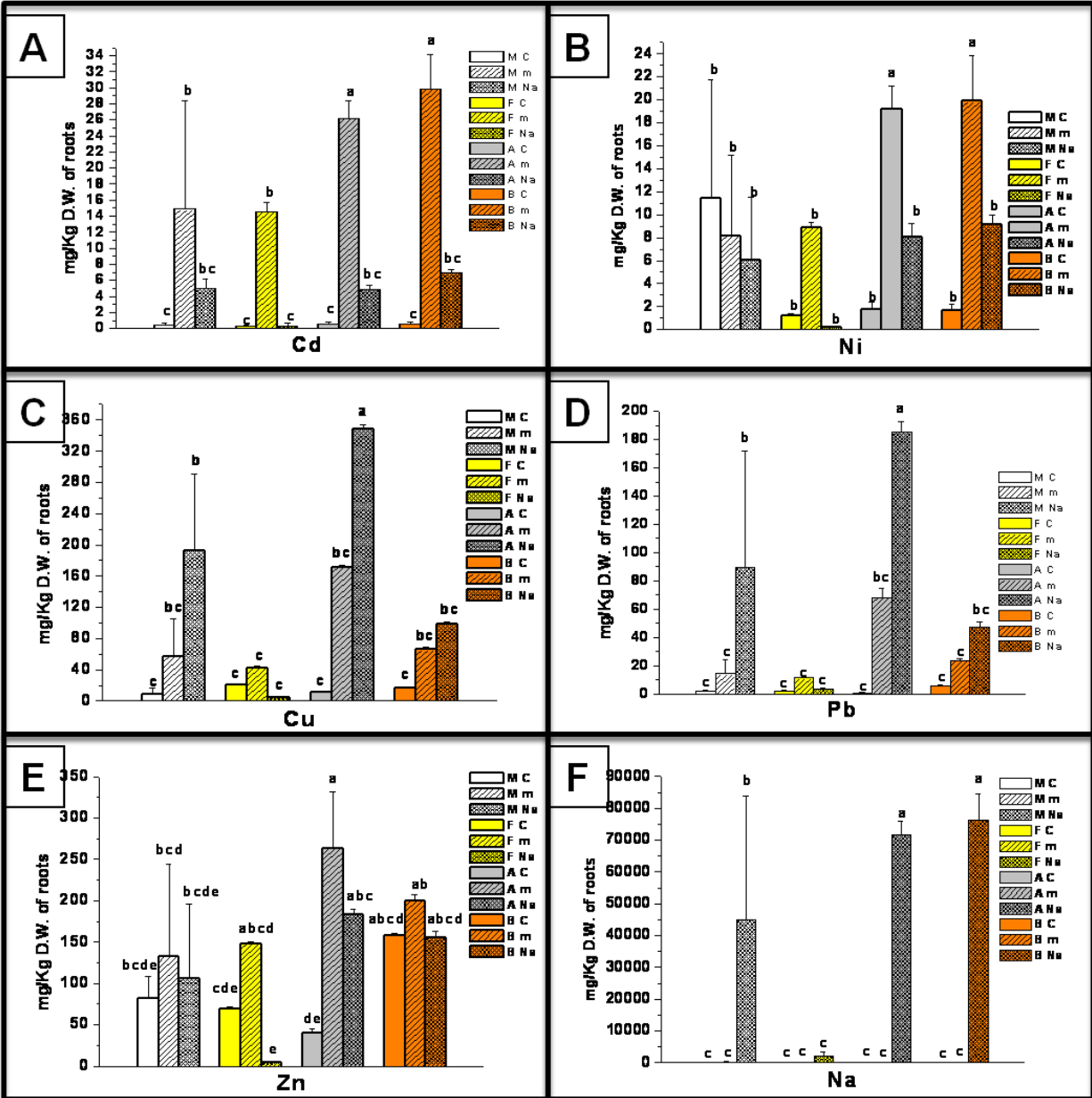
Figure 4 : ions content in willows leaves in soil trial carried on in grow chamber. X axis : metal ; Y axis : mg on Kg of dry biomass. M = *S. matsudana* ; A = *S. alba* ; F = *Salix fragilis* ; B = *Salix babylonica* ; EDDS = ethylen diamin disuccinic acid addicted to soil in a concentration equal to 3 mmol/Kg of soil. L = leaves.). Different letters mean significant differences.
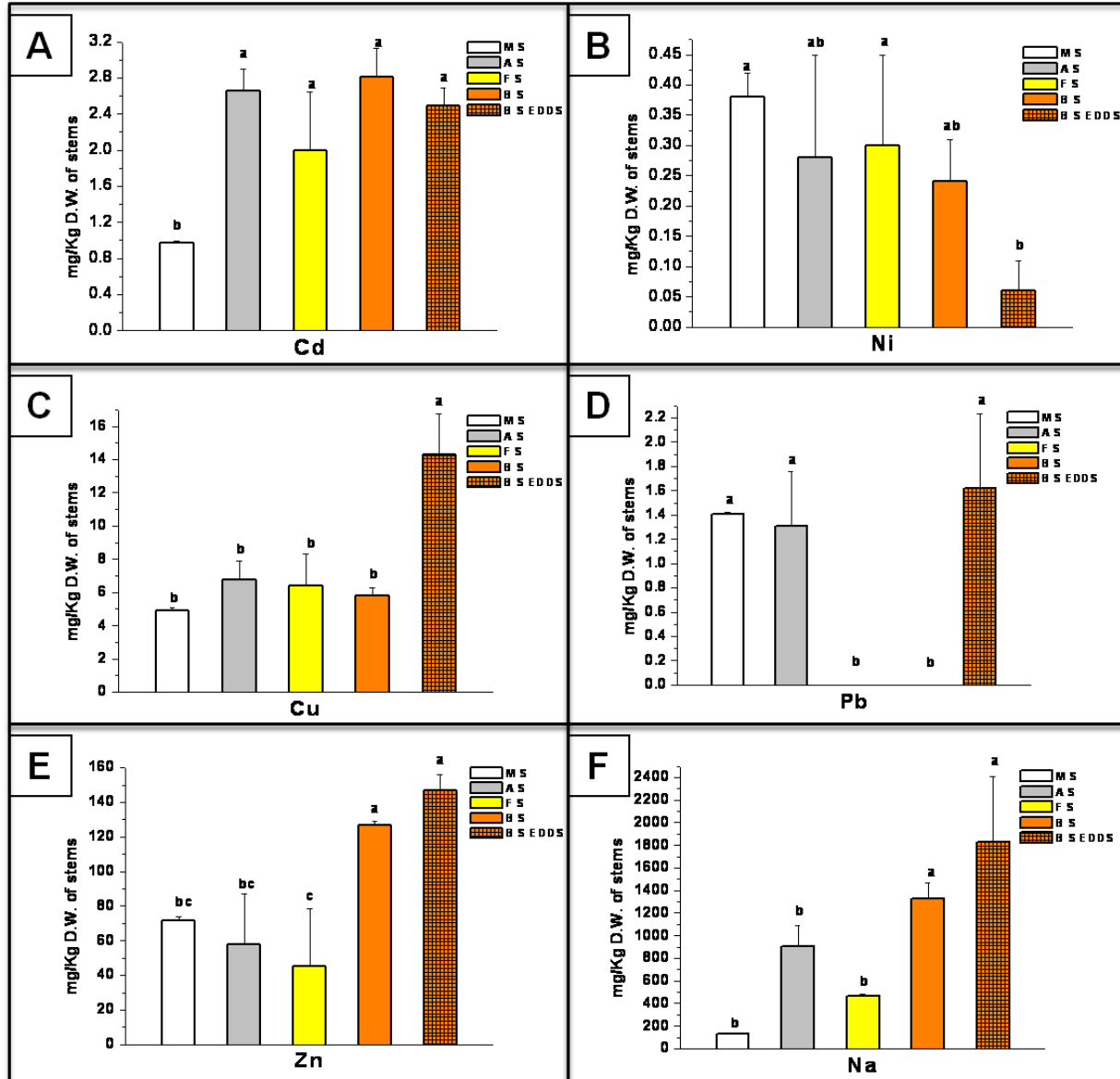
Figure 5 : ions content in willows stems in soil trial carried on in grow chamber. X axis : metal ; Y axis : mg on Kg of dry biomass. M = *S. matsudana* ; A = *S. alba* ; F = *Salix fragilis* ; B = *Salix babylonica* ; EDDS = ethylen diamin disuccinic acid addicted to soil in a concentration equal to 3 mmol/Kg of soil. L = leaves.). Different letters mean significant differences.
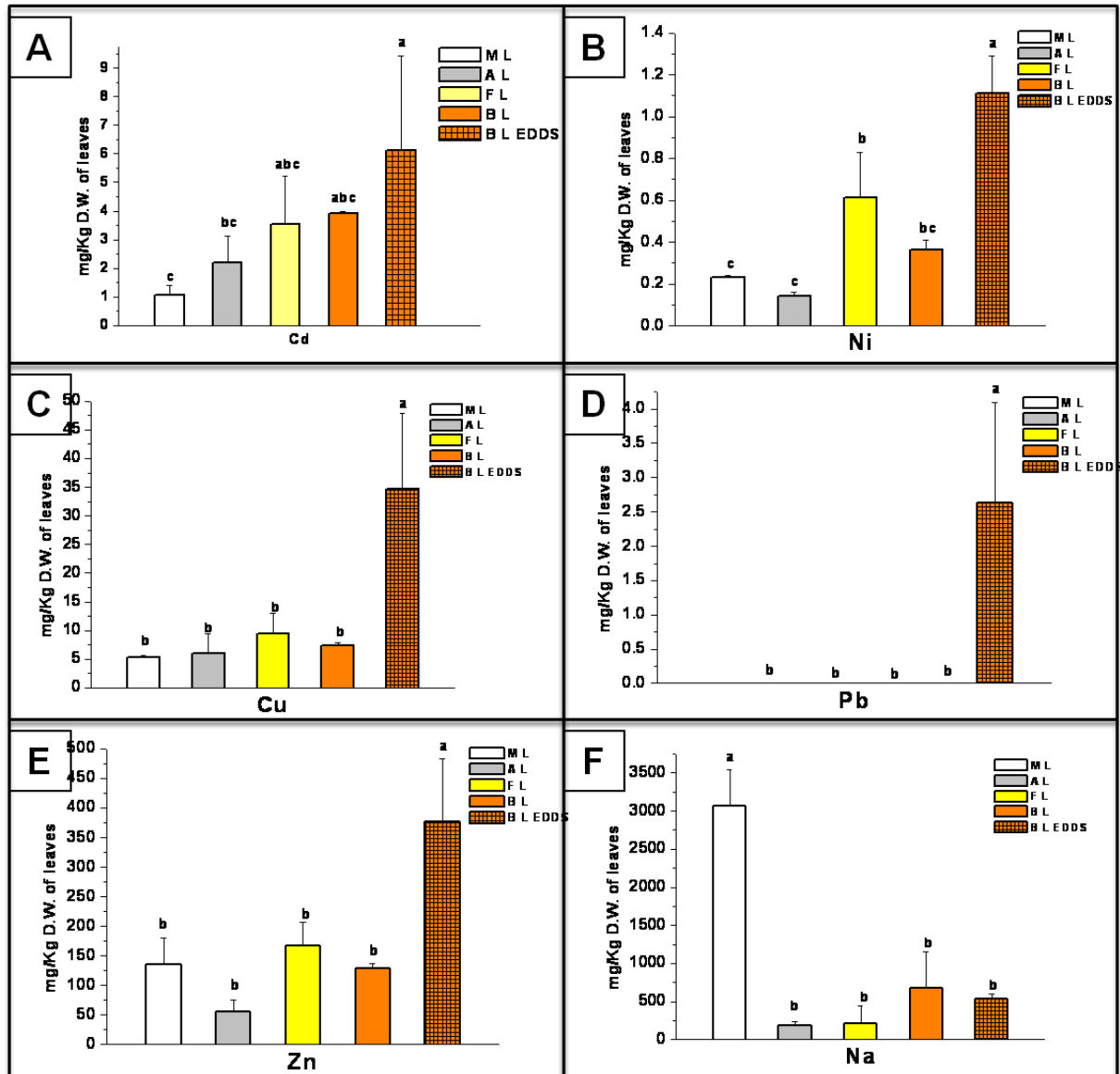
Figure 6 : ions content in willows roots in soil trial carried on in grow chamber. X axis : metal ; Y axis : mg on Kg of dry biomass. M = *S. matsudana* ; A = *S. alba* ; F = *Salix fragilis* ; B = *Salix babylonica* ; EDDS = ethylen diamin disuccinic acid addicted to soil in a concentration equal to 3 mmol/Kg of soil. L = leaves.). Different letters mean significant differences.
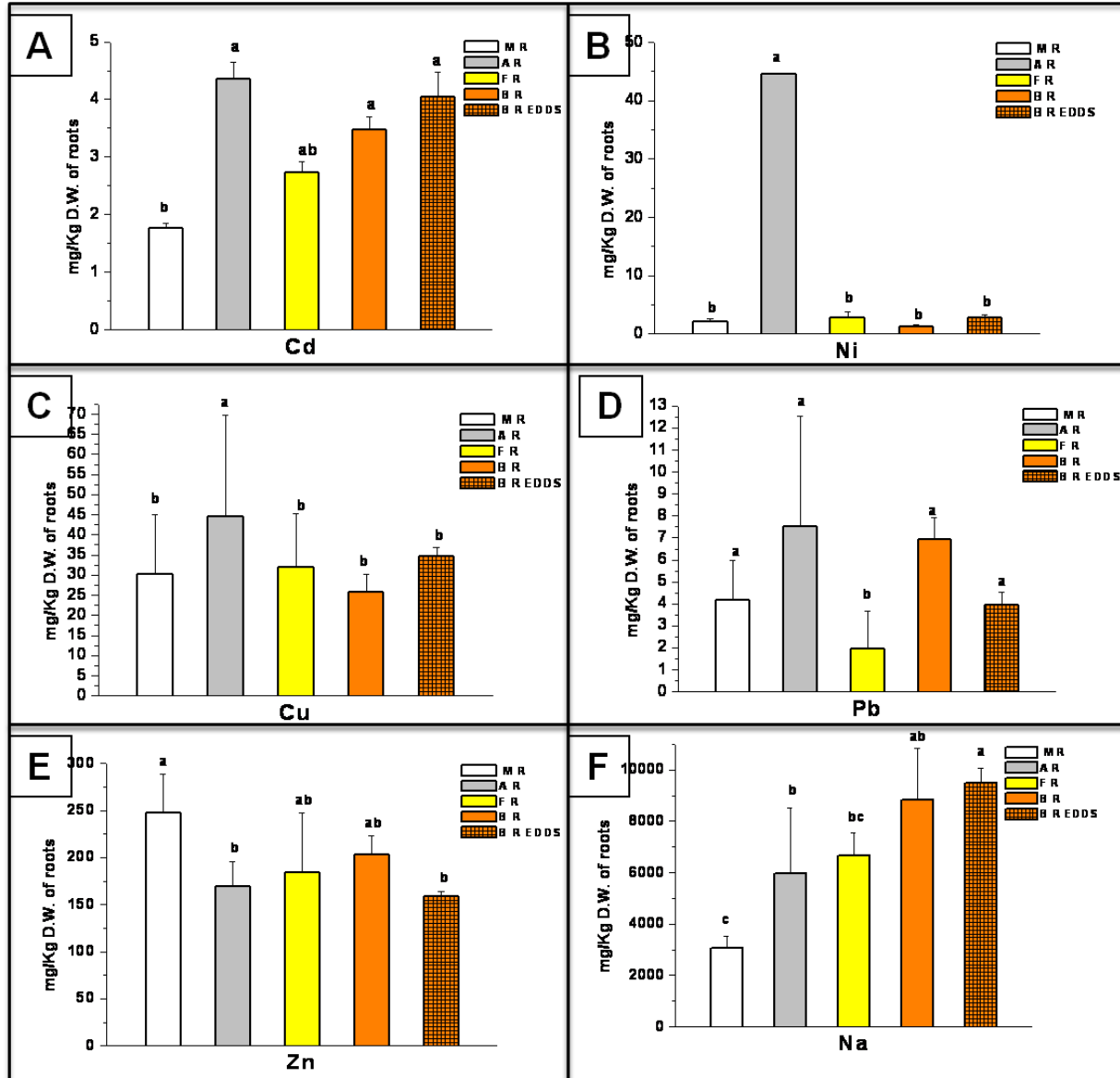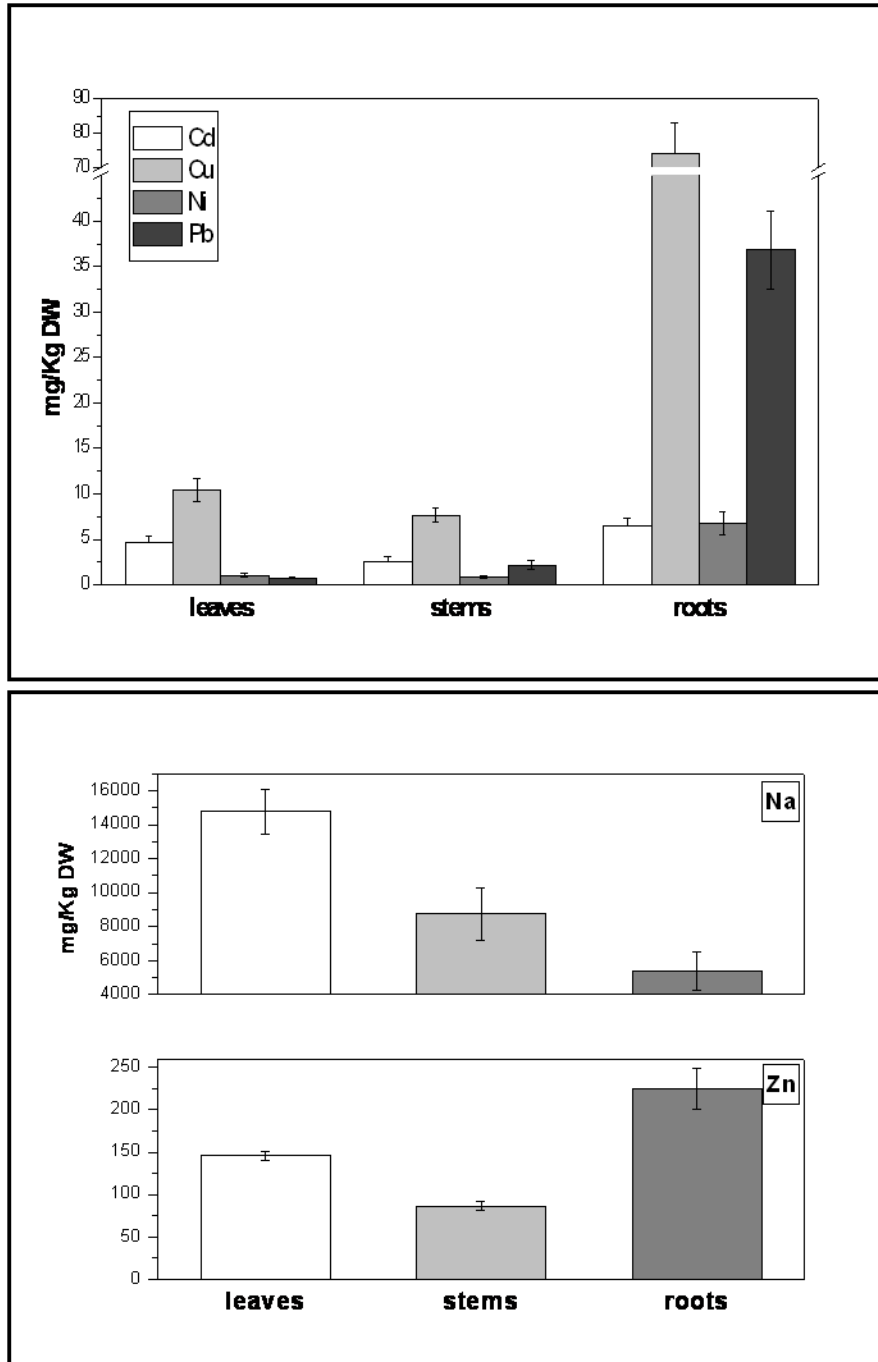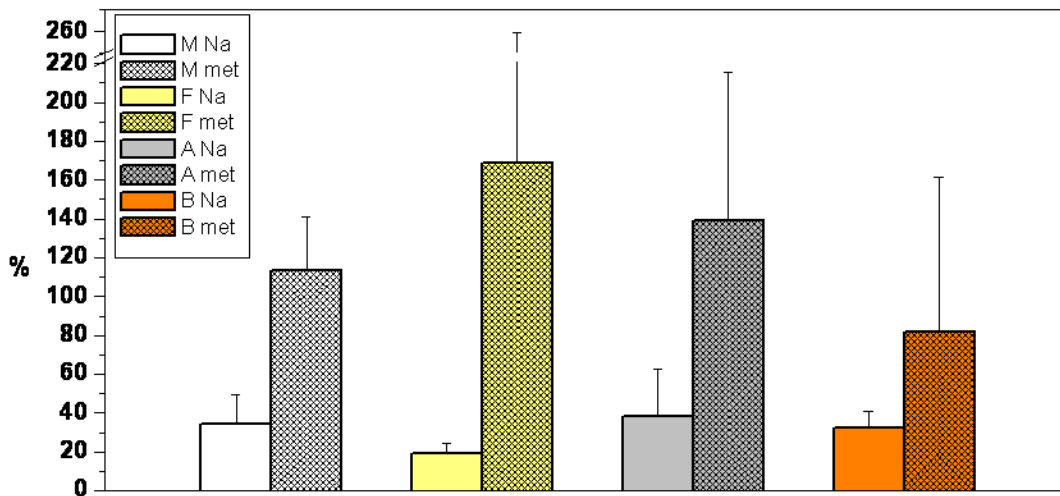
Picture 7 : metals and sodium accumulation in *S. matsudana* (in soil outdoor trial). X axis : plat tissues (leaves, stems and roots) Y axis : mg on Kg of dry biomass. Error bars = Standard deviation.

Figure 8 : Fresh biomass depletion (%) of plants treated with metals and plants grown in hydroponic + metals + Na (0.05 M then 0.1 then 0.2 M). Error bars indicate Standard deviation. X axis : willows fresh biomass in treatment with metal (darker bars) and with metal + sodium (lighter bars). M = *S. matsudana* ; F = *S. fragilis* ; A = *Salix alba* ; B = *Salix Babylonica* ; met = treatment with Ni, Pb, Cu, Cd, Zn. Na = treatment plus Ni, Pb, Cd, Zn plus Na. Y axis : % of depletion in comparison with control.
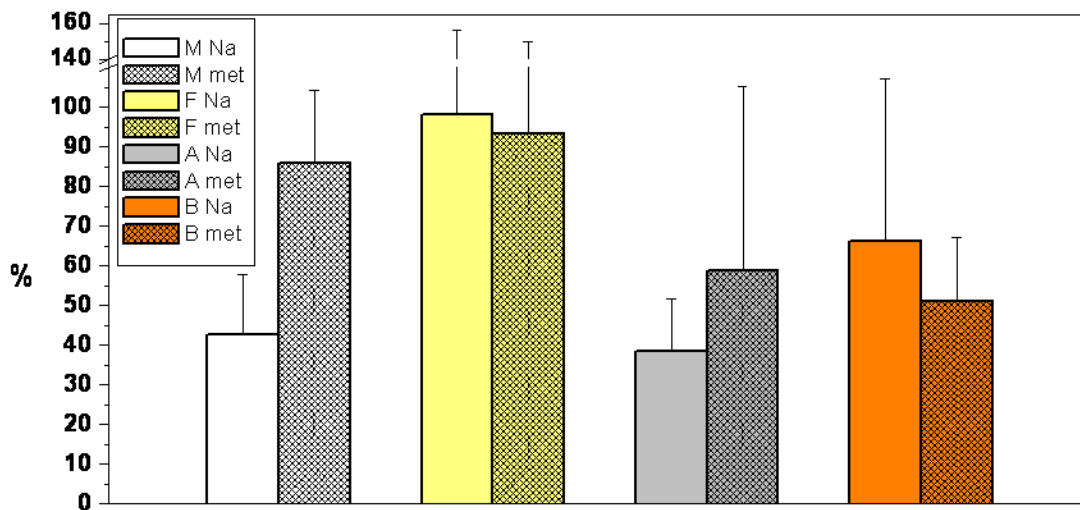
Figure 9 : root length depletion (%) of plants grown in hydroponic + metals and plants grown in hydroponic + metals + Na (0.05 M then 0.1 then 0.2 M). Error bars indicate Standard deviation. X axis : willows fresh biomass in treatment with metal (darker bars) and with metal + sodium (lighter bars). M = *S. matsudana* ; F = *S. fragilis* ; A = *Salix alba* ; B = *Salix Babylonica* ; met = treatment with Ni, Pb, Cu, Cd, Zn. Na = treatment plus Ni, Pb, Cd, Zn plus Na. Y axis : % of depletion in comparison with control.
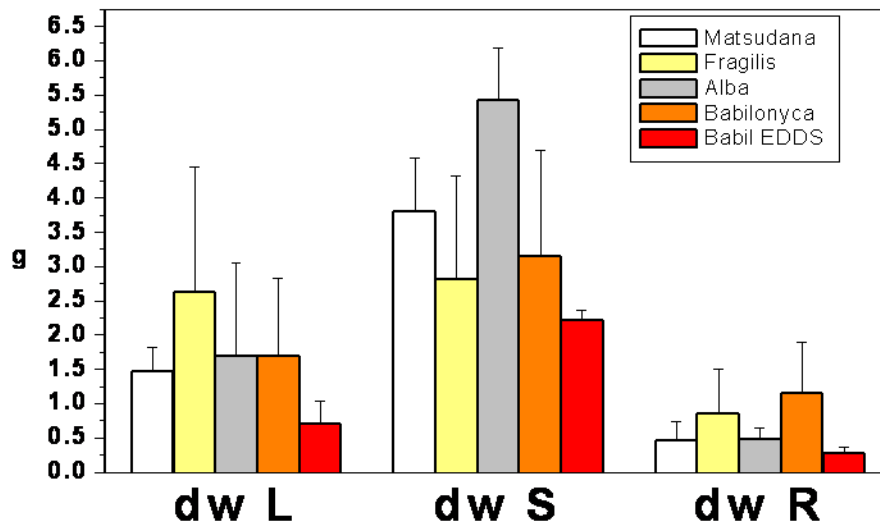
Figure 10 : dry biomass of willows grown in grow chamber on soil. X axis : dry biomass. Y axis : g of dry biomass. dw L = leaf dry biomass. Dw S = stem dry biomass. Dw R = root dry biomass. Error bars = Standard deviation.
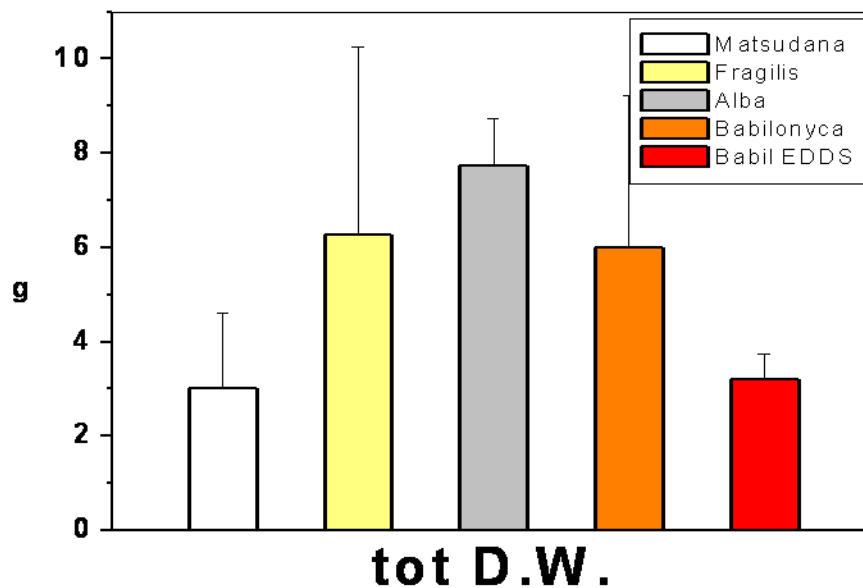
Figure 11 : total dry biomass of willows grown in grow chamber on soil. X axis : dry biomass. Y axis : g of dry biomass. Error bars = Standard deviation.
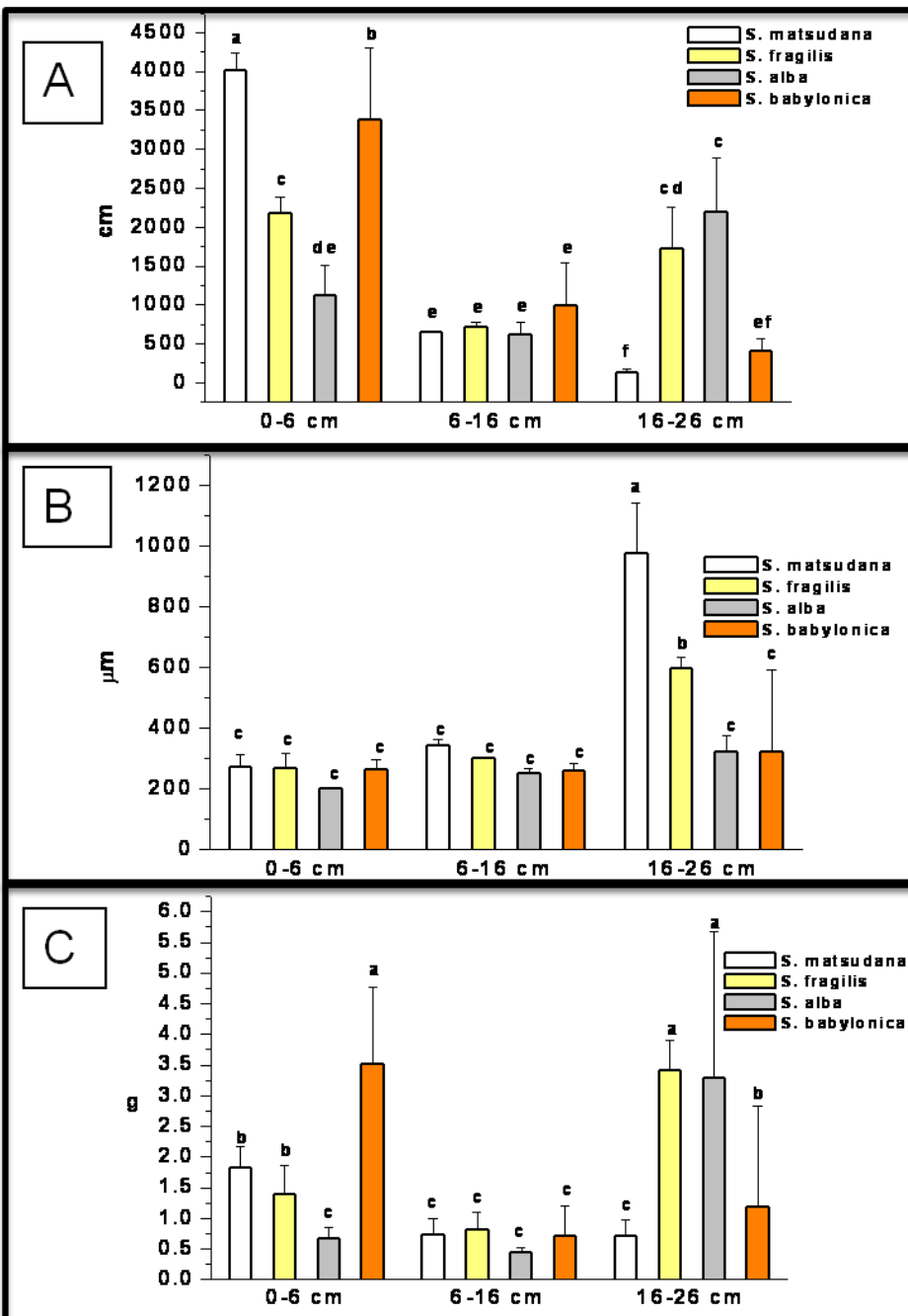
Figure 12 : Graph A : X axis : root lenghts grouped depending on layer (0-6 cm = soil for house gardening ; 6-16 = sludge ; 16-26 = sludge. Y axis : length in cm. Different letters mean significant differences between bars. Error bars = Std

Graph B : X axis : root diameters grouped depending on layer (0-6 cm = soil for house gardening ; 6-16 = sludge ; 16-26 = sludge. Y axis : diameter in μm. Different letters mean significant differences between bars. Error bars = Std

Graph C : X axis : root biomass grouped depending on layer (0-6 cm = soil for house gardening ; 6-16 = sludge ; 16-26 = sludge. Y axis : biomass in grams. Different letters mean significant differences between bars. Error bars = Std
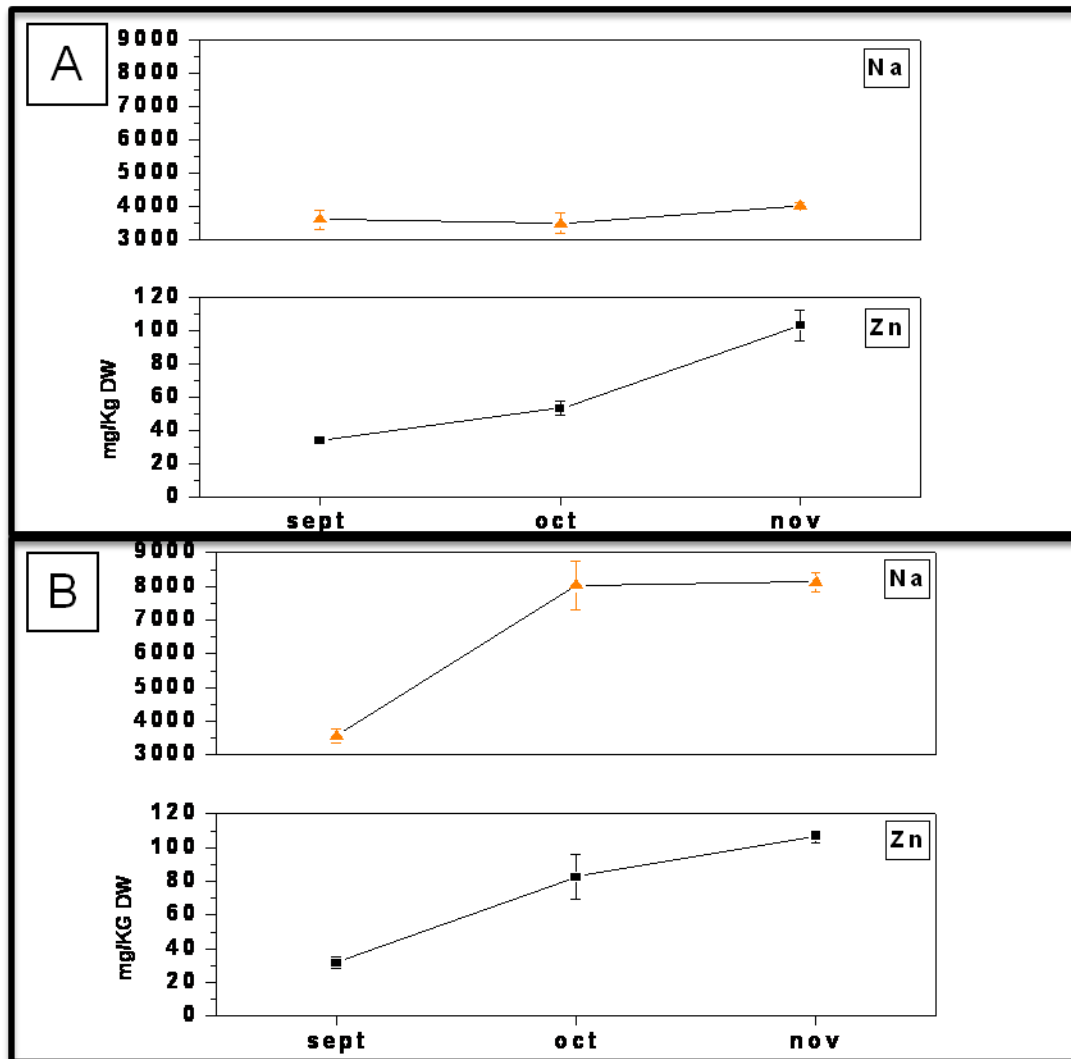
Figure 13 : Zn and Na accumulation in Phragmites stems .
Graph A : Na and Zn accumulation in presence of EDTA. X axis : time of sampling. Y axis : mg of Zn or Na on Kg of dry stem biomass. Error bars = Std
Graph B : : Na and Zn accumulation in absence of EDTA. X axis : time of sampling. Y axis : mg of Zn or Na on Kg of dry stem biomass. Error bars = Std
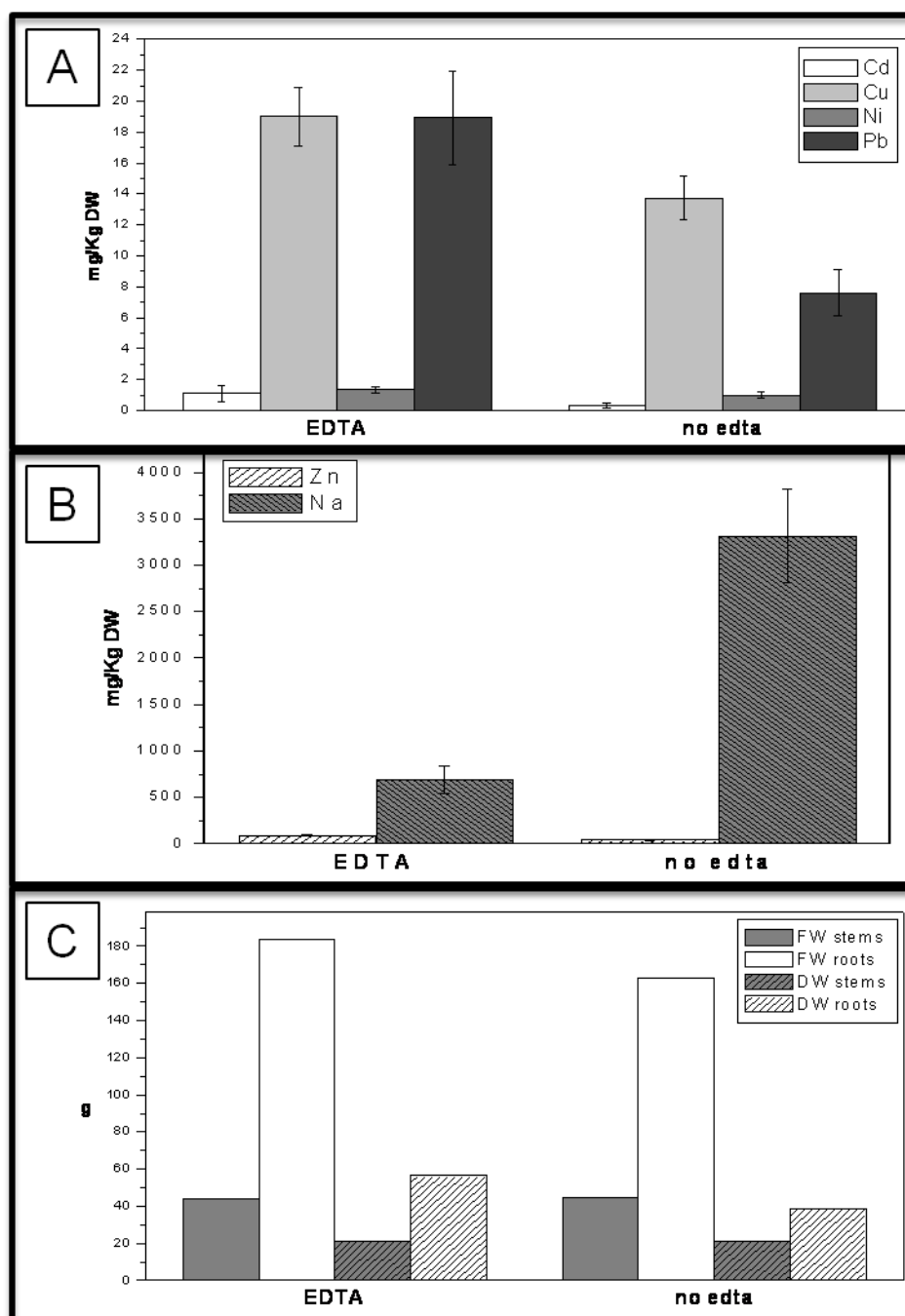
Figure 14 Metal accumulation in rhizomes and biomass production.
Graph A : Cd, Cu, Ni and Pb accumulation. X axis : metals in presence and absence of EDTA. Y axis : mg/ kg of dry biomass. Error bars = Standard deviation.
Graph B : Zn and Na accumulation. X axis : Zn and Na in presence and absence of EDTA. Y axis : mg of Zn or Na /kg of dry biomass. Error bars = Standard deviation.
Graph C : biomass production. X axis : dry and fresh biomass of shoots and rhizomes in presence and absence of EDTA. Y axis : grams of fresh and dry biomass.
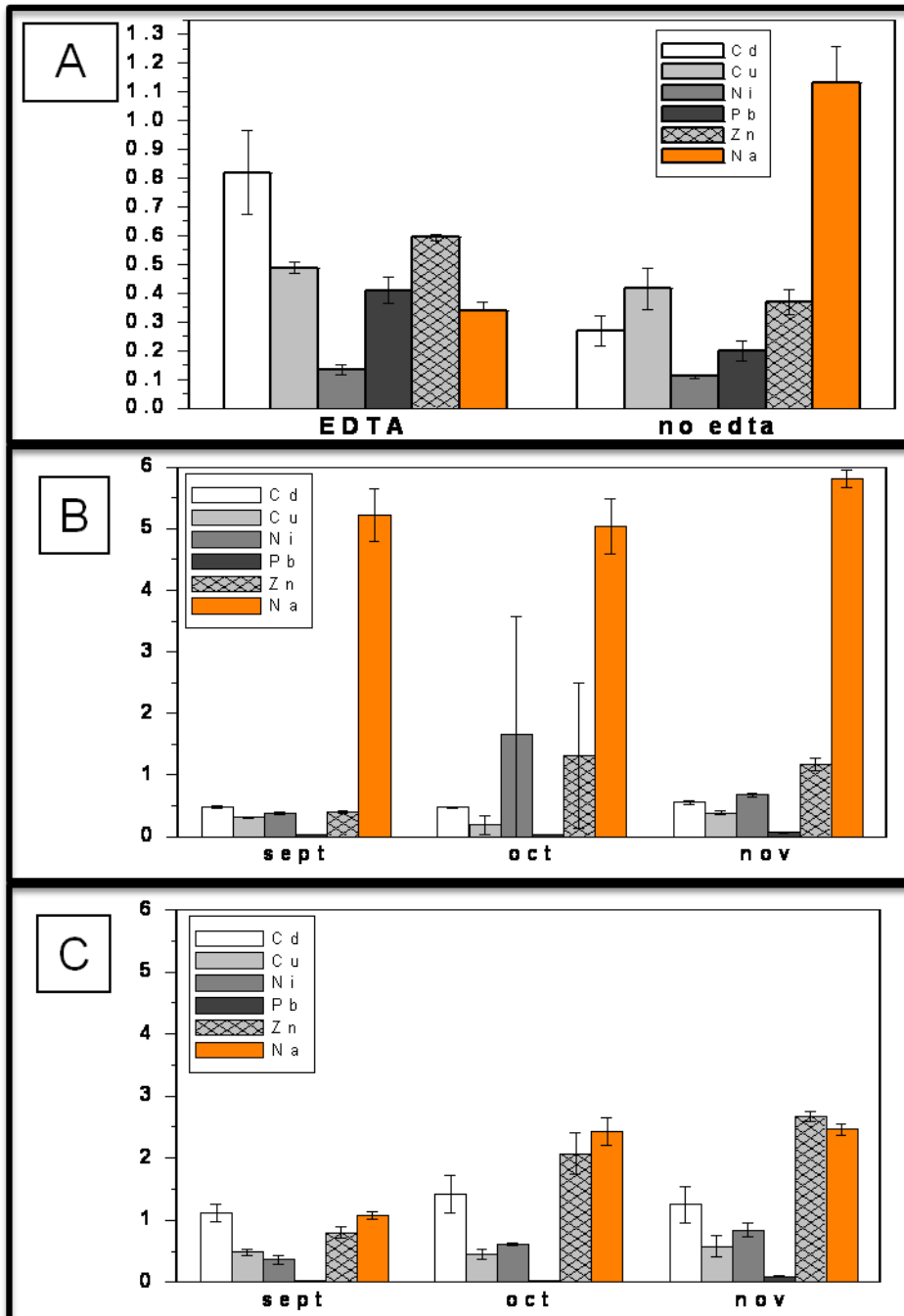
Figure 15 Graph A : bioaccumulation factor(concetration in plant/ concentration in soil). X axis : metals and sodium in presence and absence of EDTA. Y axis : BF ratio. Error bars = Standard deviation.
Graph B : Translocation Factor (concentration in shoots/ concentration in rhizomes) in presence of EDTA X axis : TF for each metal and Na across the final part of growing season.
Graph C : Translocation Factor (concentration in shoots/ concentration in rhizomes) in absence of EDTA X axis : TF for each metal and Na across the final part of growing season.

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Comparison of the ability of two species of *Astragalus*, Se hyperaccumulator *A. racemosus* and non-accumulator *A. convallarius* to accumulating several others metals and metalloids

INTRODUCTION

Hyperaccumulators are plants with ability to accumulate extraordinarily high concentrations of metals and metalloids, even from low external concentrations (Baker and Brooks, 1989). Usually these plants accumulate these elements 100 or 1000 folds more than those found in other species from the same site. Elements that can be hyperaccumulated include arsenic (As), cobalt (Co) cadmium (Cd), copper (Cu), manganese (Mn), Nickel (Ni), Selenium (Se) and zinc (Zn); some species are able to hyperaccumulate more than one of these elements (Reeves and Baker, 2000 ; Guerinot and Salt 2001). At last 400 plant species in 45 plant families are hyperaccumulators and this have been found in many different geographic locations (Reeves and Baker, 2000). In particular Se hyperaccumulators contains greater than 1000 mg Se Kg^{-1} DW and tipically occur on seleniferous soils, such as those found in the western USA (Beath et al 1939 ; Feist and Parker 2001). Some species of Astragalus and Stanleya both native to the western USA accumulate upwards of 1000 mg Se Kg^{-1} DW from soil containing 4-10 mg Se Kg^{-1} DW whereas in nonaccumulators Se concentrations are usually <20 mg Kg^{-1} DW (Shrift 1969 ; Feist and Parker 2001; Pickering et al. 2003).Se hyperaccumulators are characterized by a high leaf Se concentration, a higher Se : S ratio and a higher shoot/root Se concentration (Galeas et al. 2007 ; White et al. 2007) indicating altered regulation of sulfate transporters and presence of (more) specific selenate transporters. Moreover Se-hyperaccumulators show a pronounced and S-indipendent seasonal variation in Se concentration in different plant organs suggestive of Se flow from roots to young leaves in early spring, from older to younger leaves and reproductive tissues in summer and from shoot to roots in the fall (Galeas et al. 2007). Thus, whole plant level Se fluxes seem to be specialized in hyperaccumulators and separate from S movement. Further studies revealed that both hyperaccumulators and non accumulators seem to access the same labile pools of Se in soil ; therefore, Se-hyperaccumulators might be no better at accumulating Se from a relatively low-available-Se soil than are non accumulators and root proliferation in Se-enriched soil (positive chemotaxis) might contribute to Se hyperaccumulation (Goodson et al, 2003).

<u>Astragalus spp</u> : The genus *Astragalus* (Leguminosae) contains app. 375 species in North America, 180 of which are located in intermountain region of western US, and approximately 1600 worldwide (Barneby 1989). Variable pod characteristics in intermountain species include size, outline, thickness of pericarp , texture, colour, number of locus and composition. The genus

is also chemically diverse with some species highly toxic because of selenium accumulation (Rosemfeld and Beath 1964). *Astragalus racemosus*, that's the Se-accumulator species investigated, extents its habitat from North to South of USA, from North Dakota to Northern Texas across Wyoming, Colorado, Nebraska, Kansas, Oklaoma ,Utah and New Mexico. It is included in the Bisulcati group : although it frequently occurs in the same area with other species, in this group were not documented at the same locale and this taxon does not appear to hybridize in the wild (Heidel 2003).

Selenium (Se) :Selenate is the predominant form of Se in alkaline and well-oxidized soils with pH from acidic to neutral. Predominantely Se exists as selenite (Elrashidi et al. 1987) Se is an essential element for mammals (Stadman 1990) and Se- hyperaccumulating plants may be a useful source of dietary Se (Ellis and Salt 2003; Freeman et al 2006). However, Se is toxic at higher levels, and ingestion of hyperaccumulators is responsible for a loss of livestock valued at $ 330 milion annually in the USA (Rosenfeld and Beath 1964 ; Wilber 1980). Selenium toxicity is thought to result from its chemical similarity to sulfur, leading to inadvertment uptake and replacement of S by Se in proteins and other redox-important S compounds (Stadtman 1990). It is unlikely that Se serves an essential role in higher plants, but elevated levels of Se are toxic to plants (Anderson 1993). As a result of its similarity to sulfate, selenate is incorporated into selenocysteine (SeCys) via the sulfate assimilation pathway. SeCys replaces cysteine in proteins, resulting in a loss of protein function ; this process is in part responsible of selenium toxicity (Stadtman 1990; Anderson 1993). Se hyperaccumulators have evolved a tolerance to Se methylating SeCys into methylselenocysteine (MeSeCys) which is not easily incorporated into proteins (Brown and Shrift 1981).

Chromium (Cr) : Chromium is found in all phases of the environment, including air, water and soil. Naturally occurring in soil, Cr ranges from 10 to 50 mg kg^{-1}depending on the parental material. In ultramafic soils (serpentine), it can reach up to 125 g kg^{-1} (Adriano, 1986). In fresh water, Cr concentrations generally range from 0.1 to 117 µg L^{-1}, whereas values for seawater range from 0.2 to 50 µg L L^{-1}. Cr concentration varies widely in the atmosphere, from background concentrations of 5.0^{10-6}–1.2^{10-3} µg m^{-3} in air samples from remote areas such as Antarctica and Greenland to 0.015–0.03 µg m^{-3} in air samples collected over urban areas (Nriagu, 1988). Cr(VI) is a strong oxidant with a high redox potential in the range of 1.33–1.38 eV accounting for a rapid and high generation of ROS and its resultant toxicity (Shanker et al., 2004a,b).

The stable forms of Cr are the trivalent Cr(III) and the exavalent Cr(VI) species, although there are various other valence states which are unstable and shortlived in biological systems. Cr(VI) is considered the most toxic form of Cr, which usually occurs associated with oxygen as chromate (CrO$_4$) or dichromate (Cr$_2$O$_7$) oxianions. Cr(III) is less mobile, less toxic and is mainly found bound to organic matter in soil and aquatic environments (Becquer et al., 2003). The impact of Cr contamination in the physiology of plants depends on the metal speciation, which is responsible for its mobilization, subsequent uptake and resultant toxicity in the plant system. Cr toxicity in plant is observed at multiples levels, from reduced yield, through effects on leaf and

root growth, to inhibition on enzymatic activities and mutagenesis. Although some crops are not affected by low Cr concentration (3.8 10^{-4}μM) (Huffman and Allaway, 1973a,b), Cr is toxic to most higher plants at 100 μM kg^{-1} DW (Davies et al. 2002). The first interaction Cr has with a plant is during its uptake process. Cr is a toxic, nonessential element to plants; hence, they do not possess specific mechanisms for its uptake. Therefore, the uptake of this heavy metal is trough carriers used for the uptake of essential metals for plant metabolism. The toxic effects of Cr are primarily dependent on the metal speciation, which determines its uptake, translocation and accumulation. The pathway of Cr(VI) transport is an active mechanism involving carriers of essential anions such as sulfate (Cervantes et al, 2001). Fe, S and P are known also to compete with Cr for carrier binding (Wallace et al., 1976). About Cr in crops, Skeffington et al., (1976) from radioactive tracer studies using ^{51}Cr reported that Cr mainly moved in the plants xylem. Golovatvj et al. (1999) have shown that Cr distribution in crops had a stable character which did not depend on soil properties and concentration of this element ; the maximum quantity of element contaminant was always contained in roots and a minimum in the vegetative and reproductive organs. In bean, only 0.1% of the Cr accumulated was found in the seeds as against 98% in the roots (Huffman and Allaway, 1973a). The reason of the high accumulation in roots of the plants could be because Cr is immobilized in the vacuoles of the root cells, thus rendering it less toxic, which may be a natural toxicity response of the plant (Shanker et al., 2004a). Since both Cr(VI) and Cr(III) must cross the endodermis via symplast, the Cr(VI) in cells is probably readily reduced to Cr(III) which is retained in the root cortex cells under low concentration of Cr(VI) which in part explains the lower toxicity of Cr(III). Although higher vascular plants do not contain Cr(VI)-reducing enzymes, they have been widely reported in bacteria and fungi (Cervantes et al., 2001).

Arsenic (As) : with an average concentration of 5-6 ppm in the continental crust is relatively scarce ; however, individual areas show particularly high concentrations. Anthropogenic inputs such as smelters, mining processes, fuel utilization and pesticides are generally responsible for the high arsenic concentrations found in those areas. (Heikens et al. 2007). In south east of Asia, for instance, millions of people have been exposed to high levels of arsenic in ground water because the contamination of drilled wells (Christen 2001). Studies on arsenate (the dominant form of arsenic phytoavailable in aerobic soils) toxicity have shown that plant species not resistant to arsenic are highly stressed by exposition to arsenate with symptoms ranging from inhibition of root growth through to death (Macnair & Cumber 1987 ; Meharg & Macnair 1991 ; Paliouris & Hutchinson 1991; Barrachina et al 1995). The exposure to inorganic arsenic species results in the generation of reactive oxygen species (ROS) (Hartley-Whitaker et al, 2001a). This probably occurs through the conversion of arsenate to arsenite, a process which readily occurs in plants and leads to the synthesis of enzymatic antioxidants like superoxide dismutase (SOD) and catalase and non enzymatic antioxidants, for example gluthathione and ascorbate (Alscher, 1989; Mylona et al., 1998 ; Dat et al., 2000; Hartley-Whitaker et al., 2001a). Arsenate is toxic also because acts as a phosphate analogue and is transported across the plasma membrane via phosphate cotransport systems (Ullrich-Eberius et al., 1989). Once inside the cytoplasm it

competes with phosphate, for example replacing phosphate in ATP to form unstable ATP-As and leads to the disruption of energy flows in cells (Meharg 1994). However, Bertolero et al. (1987) point out that because Arsenate is rapidly reduced to arsenite in plant tissue, arsenate will not normally so much concentrated to exert toxicity. Because Arsenate is linked with phosphate nutrition, an increased phosphate status leads to reduced arsenate uptake, through suppression of the high- affinity phosphate/arsenate uptake system (Meharg & Macnair 1991 1992). For the same reason non resistant plants can become more resistant to arsenate by rising their phosphorus status (Meharg et al. 1994b) infact the phosphate/arsenate trasporter has a higher affinity for phosphate and if external phosphate status is high phosphate will be taken up more efficiently than arsenate (Meharg & Macnair 1994).

Nickel (Ni) : it is the twenty second most abundant element in the earth's crust (Nielson 1987 ; Sunderman et al., 1991). Generally naturally occurring concentrations of Ni in soil and surface waters are lower than 100 and 0.005 ppm respectively (McIlveen et al. 1994 ; McGrath et al. 1995). Ni is also released into the environment by anthropogenic activities such as metal mining, smelting, fossil fuel burning, vehicles emissions, disposals of household, municipal and industrial wastes, fertilizer application and organic manures (Alloway 1995 ; Salt et al., 2000). Ni^{2+} concentrations may reach 26000 ppm in polluted soils (Alloway 1995 ; McGrath 1995) and 0.2 mg/L in polluted surface waters (Amstrom et al.,1996 ; Zwolsman et al., 2007) 20 to 30 times higher than found in unpolluted areas.

Ni is essential for plants (Eskew et al., 1983 ; Ragsdale 1998) but generally the concentration in plants is very low (0.05 to 10 mg/kg DW) (Nieminem et al.,2007). With increasing Ni pollution toxic effects in plants for excess are more frequently reported than others linked to deficiency (Alloway 1995 ; Salt et al., 2000) for example reduction in plant growth (Molas 2002) and adverse effects on fruit yeld and quality (Gajewska 2006) ; Extremely high soil Ni concentrations can forbid completely crops fruits and vegetables growth (Duarte et al., 2006). The Ni uptake in plants is carried out mainly by root systems via passive diffusion and active transport (Seregin et al., 2006). The ratio of uptake between active and passive transport varies with species, Ni form and concentration in the soil or nutrient solution (Dan et al., 2002 ; Vogel-Mikus et al., 2005). For example, soluble Ni compounds can be absorbed via the cation transport system. Since Cu^{2+} and Zn^{2+} inhibit Ni^{2+} uptake competitively, these three soluble metal ions seem to be absorbed by the same transport system (Cataldo et al., 1978 ; Kochian 1991). Over 50% of the Ni absorbed by plants is retained in the roots (Cataldo et al., 1978). This may be due to sequestration in the cation exchange sites of the walls of xylem parenchyma cells and immobilization in the vacuoles of roots (Seregin et al., 2006). Furthermore over the 80% of Ni in the roots is present in the vascular cylinder, while less than 20% is present in the cortex , maybe due to an high Ni mobility trough the vessels (Marschner 1995 ; Page et al., 2005 ; Riesen et al., 2005). Responses to toxicity differ according to plant species, growth stage, cultivation conditions, Ni concentration and exposure time (Krupa et al., 1996 ; Marschner 1995; Sheoran et al., 1990 ; Xylander et al., 1994; Kabata-pendias 2001; Assuncao et al., 2003). In general critical toxicity levels are > 10 mg/kg DW in sensitive species (Kozlow 2005), > 50 mg/kg DW in

moderately tolerant species (Bollard 1983; Ascher 1991) and > 1000 mg/kg DW in Ni hyperaccumulator plants like *Alyssum* and *Thlaspi* spp. (Kupper et al., 2001 ; Pollard et al., 2002) .In sensitive species (for example, barley, water spinach and wheat), chlorosis and necrosis of leaves can appear after plants are treated with Ni at very low concentrations (≤ 0.2 mM or 11.74 ppm) for less than a week (Gajewska et al., 2006, Sun et al., 1998,Rahman et al., 2005). Plants grown in Ni contaminated soil and media show various responses and toxicity symptoms including retardation of germination (Nedhi et al., 1990), inhibition of growth (Madhava et al., 2000 ; Gajewska et al., 2006; Boominathan et al., 2002), reduction of yield (Madhava et al., 2000 ; Molas 1997; Balaguer et al., 1998 ;Pandey et al., 2002), induction of leaf chlorosis and wilting (Gajewska et al., 2006; Pandey et al., 2002), disruption of photosynthesis (Molas 2002; Gajewska et al., 2006; Molas 1997;Szalontai et al., 1999 ; Gajewska et al., 2007) inhibition of CO_2 assimilation (Kozlow 2005; Molas 1997), as well as reductions in stomatal conductance (Sheoran et al., 1990 ; Bethkey et al., 1992).

As written above Ni hyperaccumulator plants can sequester high levels of Ni in tissues (from several thousands of mg/kg up to 5% of dry biomass) without exhibiting phytotoxicity (Baker et al., 2000; Prasad 2005 ; Milner et al. 2008). The family with the most such species is the Brassicaceae, with more than 80 species which are capable of accumulating Ni to concentrations as high as 3% of shoot dry biomass (Reeves et al., 2000; Brooks et al., 1979). These species have higher requirements for Ni (e. g., up to 500 mg Ni/kg) than normal plants (Kupper et al., 2001)Stackhousia tryonii Bailey (Stackhousiaceae), an herbaceous species from Australia, has been shown to accumulate Ni in dry leaves at concentrations exceeding 4% (Batianoff et al., 2002; Bathia 2003).

Vanadium (V) : V has a mean crustal abundance of 150 mg/kg, that places it as the fourteenth most abundant element in the earth crust (Hansen 1983). V is more widely dispersed than other elements such as Co, Zn, Cu and Mo, so it is more readily accessible by plants than those elements which tend to be concentrated in discrete bodies. Several authors have presented a compilations of plant tissue vanadium levels (Bengtson and Tyler 1976; Bertrand 1950; Hopkins et al., 1977). Generally from unpolluted sources, plants accumulate V in a range between 0.5 and 2.0 μg /g DW. Elevated V concentrations has been reported from plants growing in high mineralized soils of the Colorado plateau (USA) with plants of the genus *Astragalus* containing up to 144 μg/g DW. Even some non accumulator plants were found to contain high V levels when cultivated closest to highly mineralized soils. Brenchley (1932) in experiments on phosphate fertilization found that fine grinding of the fertilizer tended to reduce yield because the presence of V. Later work indicated that levels of 1-2 μg/g of V in the nutrient solution could produce reductions in shoot biomass. Warington (1951, 1954) demonstrated that V could relieve symptoms of excess of Mn in soybean, but causing at the same time symptoms of V toxicity such as apical chlorosis and abnormal root growth. A supply of 10^{-4} M of vanadate to plants caused a reduction in dry matter production (Wallace et al., 1977) ; in these experiments V tissue level increased according to the rate of supply ; the major part of this was stocked in root system. Also

Hara et al. (1976) found retention of V in cabbage roots up to 2500 µg/g DW that was the 95-98% of the total V absorbed.

Tellurium (Te) : With an abundance in the Earth's crust comparable to that of platinum, tellurium is one of the rarest stable solid elements in the Earth's crust but is widely distributed. Its abundance is about 1 µg/kg ; nevertheless in Ely mining district (Nevada) were found concentrations up to 100 mg/kg of rocks (Gott and McCarthy 1966). Te is known to be chemically similar to Se but also the behavior of this two elements once inside organisms is similar : they tend to be methylated and / or form Se /Te amminoacids both in animals and microorganisms (Terry et al., 2000 ; Chasteen et al., 1990). About Te in plants little is known. Beath et al. (1935) found in *Atriplex nuttallii* and in *Opuntia polyacanta* Te concentrations from 2 to 25 mg/kg DW; in addition in greenhouse experiments he found in young wheat plants, in soil amended with 10 mg/kg of Te as Te dioxide, 11 mg/kg DW; when the same amount of Te was added as K salt, he found up to 507 mg/kg DW. In twigs of *pinus flexilus* Hubert (1971) found 0.05 mg/kg of Te and in wood 0.005 mg/kg. Duke (1970) analyzing vegetables, grains, tuber crops and fruit eaten by panama and Colombia indigenous groups, found Te contents ranged from 0.9 mg/kg in *Phaseolus vulgaris* to 0.001 mg/kg in *Musa sapientium* , the banana. Based on DW, generally vegetables and grains contains more Te than crops and fruits. Cowgill (1988) found in different species of the genus *Astragalus* from different locations of Nevada, Te concentrations from 1.2 µg/kg of *A. asclepiadoides* to 62.1 µg/kg of *A. bisulcatus*. Interestingly the higher values corresponded to species that are Se hyperaccumulators, to underline the similarity of this two elements although the Se concentrations in hyperaccumulators was many folds higher than Te concentrations. He found also that in those investigated Nevada locations, vegetation fails to grow when Te soil concentration is higher than 10 mg/kg.

Molybdenum (Mo) : Molybdenum is an essential micronutrient for plants, bacteria, and animals. Mo-deficient plants exhibit poor growth and low chlorophyll and ascorbic acid content (Marschner, 1995). Mo is also a component of some bacterial nitrogenases, and therefore is especially important for plants that live in symbiosis with nitrogen-fixing bacteria. The major metabolic function for Mo in eukaryotic organisms is as an essential component of the mononuclear Mo enzymes (Hille, 1996), which play roles in many key metabolic processes such as sulfur detoxification, purine catabolism, nitrate assimilation, and phytohormone synthesis in plants (Stallmeyer et al., 1999). These enzymes usually (but not always) catalyze two-electron redox reactions that are coupled to the transfer of an oxygen atom to or from substrate and the metal, during which the Mo cycles between the Mo(VI) and Mo(IV) oxidation states. In these systems, Mo is bound to the dithiolene of a novel pyranopterin cofactor that is known as molybdopterin (Hille, 1996). Although a low-*M*r species containing both Mo and molybdopterin has never been properly characterized, this complex is known as the Mo cofactor (Moco). These enzymes are unrelated to the Mo-containing nitrogenase of nitrogen-fixing bacteria that contain a Mo-iron-sulfur cluster.

In plants, Mo is readily mobile in xylem and phloem for long-distance transport (Kannan and Ramani,1978), though little is known of the mechanisms involved in Mo homeostasis.

Molybdate competes with sulfate for uptake at the root surface, suggesting a common uptake mechanism (Stout et al., 1951). However, it has also been suggested that molybdate is taken up by a phosphate transporter (Heuwinkel et al., 1992).

The Mo content of plants is a direct reflection of the bioavailability of Mo in the soil. The relation of Mo influx versus concentration is approximately linear in soil systems (Barber, 1995). Bioavailability of Mo is positively correlated with soil pH (Karmian and Cox, 1978). In acidic soils, Mo deficiency in plants is common.

On the other hand, in some soils of high pH, plants may accumulate enough Mo for ruminants feeding on the plants to develop molybdenosis. Molybdenosis is caused by an imbalance of Mo and copper in the ruminant diet, which induces a copper deficiency (Stark and Redente, 1990). Plants supplied with adequate Mo usually contain 1 to 2 mg /kg Mo; plant material containing an excess of 5 mg/kg Mo is sufficient to cause molybdenosis in ruminant animals (Barber, 1995). Molybdenosis in livestock occurs in the western U.S., often in soils with poor drainage and high organic matter (Gupta and Lipsett, 1981).

About Mo toxicity in plants, they appear quite tolerant of high soil molybdenum concentrations. There is no record of molybdenum toxicity under field conditions. In greenhouse studies, tomato leaves turned golden-yellow and cauliflower seedlings turned purple. Animals fed foliage high in molybdenum may need supplemental copper to counteract the molybdenum (Vitosh et al., 1994).

Mo pollution due to mining and stainless steel industry poses a serious environmental problem at several locations in the U.S., including several Superfund sites (polluted sites in the U.S. designated by the U.S. Environmental Protection Agency for high priority remediation). Plant samples from a mining site near Empire, CO were found to contain up to 400 mg kg^{-1} Mo (Trlica and Brown, 2000). Phytoremediation, the use of plants to remediate environmental pollution, may prove to be a viable strategy for remediating Mo in these areas, either via phytoextraction (accumulation in harvestable plant parts) or phytostabilization (in situ immobilization).

Tungsten (W) : The earth's crust, by far the most significant source of tungsten in the ecosphere, is estimated to contain 0.00013% W on a mass basis(Smith, 1994), equivalent to an average (background) W concentration of 1.3 mg kg^{-1}. Based on this figure, W occupies the 57th/18th positions in the overall element/metal abundance lists. Surface rocks reportedly contain W at levels in the range of 1.0–1.3 mg kg^{-1}(Penrice 1997, Krauskopf 1972). In terrestrial systems, W exists almost exclusively in the form of oxo-rich tungstate minerals either as *scheelite* ($CaWO_4$) or *wolframite* ($[Fe/Mn]WO_4$). Literature assessing background W concentration levels for soil systems globally is not available and only a limited number of studies exist on a local scale. Studies in the European Union have reported W concentrations in soils (0.5–83 mg kg^{-1} dry mass) and surface soils (0.68–2.7 mg kg^{-1} dry mass) (Senesi et al., 1988,Senesi et al., 1999). The same studies have reported W content levels (in mg kg^{-1}) in the lithosphere (0.1–2.4), phosphate and phosphorite rocks (30–270), limestones and carbonate rocks (0.2–0.8), sewage sludge (1–100), manure (8–2800) and a variety of fertilizers (ND-100). Elevated values in agricultural soils from New Zealand (1.9–21.4 mg kg^{-1}) may be attributed to agrochemical practices (Quin 1972).

Conversely, values in the range of 0–2 mg kg^{-1} reported for agricultural soils in Iowa are in close agreement with the background concentration of 1.3 mg kg^{-1} (Fu et al.,1988). W can be transported to biotic systems from terrestrial, atmospheric or aquatic systems via several mechanisms including uptake by plants, trees and vegetative matter, bioaccumulation in microbial and animal organisms, sorption/desorption, formation of organotungsten compounds and subsequent partitioning in organic matter, and excess metal sequestration.

Plants are known to take up (possibly in anionic form,WO_4^{2-} (Wilson et al., 1966]) and accumulate tungsten in substantial amounts. The extent of accumulation appears to be directly related to the W content of the soil irrespective of the source nature (natural or anthropogenic) and varies widely depending on the plant genotype. Certain plants (e.g. tomatoes) act as excluders of W, particularly in the presence of Mo (Kishida et al., 2004). Concentrations of 5–100 mg kg^{-1} were found in trees and shrubs in the Rocky Mountains (Shacklette et al., 1978), whereas values below 1 mg kg^{-1} appear to be most common in other plant types (Kabata Pendias et al., 1992). Elevated values of W have been reported in the leaves (13.6 mg kg^{-1}), young stems (2.9 mg kg^{-1}) and old stems (4.3 mg kg^{-1}) of eucalyptus trees (*Eucalyptus melanophloia*) in the vicinity of an inactive wolframite mine in North Queensland, Australia compared to control values of 0.1, 0.0 and 0.1 mg kg^{-1}, respectively (Pyatt et al., 2004).W detected in the leaves of European beech trees (*Fagus sylvatica*) varied in the range of 7–50 mg kg^{-1} and was attributed primarily to atmospheric deposition (Tyler 2007) A Czech study reported a mean W concentration of 0.775 mg kg^{-1} in oak tree bark (range 0.129–4.79 mg kg^{-1}) (Bohm et al., 1998) Information has been presented earlier on elevated values of W in tree ring samples taken from Fallon, Nevada and other all struck communities (Sheppard et al., 2003) Limited literature exists on food crops. W was detected in onions harvested in various sites (Denmark) at a mean level of 16.7_g kg^{-1} fresh mass (*n* = 64; range, 6.3–39_g kg^{-1}) (Bibak et al., 1998), in two wild berry species in Sweden at levels in the range of 0.22–7.2 mg kg^{-1} (Roduskin et al. 1999) and in cabbage (0.2–1.7 mg kg^{-1}) grown in fly ash-amended soils (Furr et al., 1976).

Although certain plants are capable of acting as W accumulators or excluders, plant toxicity has also been reported in the literature. Accumulation and toxicity of W on rye grass was reported recently (Stingul et al., 2005). The accumulation increased as the W soil concentration increased from 0.1 to 10,000 mg kg^{-1}. At the highest soil content level, toxicity became apparent throughout the life of the plant and resulted in death, approximately 4 weeks after germination. Accumulation of W, investigated using three species of *Brassica* (fast plants, red cabbage and Indian mustard), was attributed to anthocyanins (Kerry et al., 2002). The same study concluded that replacement of Mo in nitrate reductase by W is a likely toxicity mechanism and that sequestration of excess metals in the peripheral cell layers appears to be a common mechanism of plant metal accumulation. Sequestration of metals in tissues where these substances are less harmful to the plant, or plant defense against pathogens or herbivores are two plausible explanations.

MATHERIAL AND METHODS

Plant growth : seeds of *Astragalus racemosus* and *A. convallarius* were obtained from Western Native Seeds Co., Colorado (USA). To improve germination, seeds of *A. racemosus* and A. *convallarius* were scarified by scratching the coat with sandpaper. After the seeds were scarified under a microscope with a scalpel, removing piece of the seed coat to facilitate water entrance, the seeds were put in a 15 ml tube to be sterilized by 1 minute in ethanol 70% and then 30 minute in bleach 30%. After that, seeds were rinsed five times 10 minutes in sterile deionized water; during each wash tubes were put on the rocker to be gently shakedn. Between each wash, water was replaced under a sterile hood. The sterilized seeds were put in magenta boxes (3 or 4 per box) under a sterile hood using sterile forceps. The medium was prepared as follows : (50 ml per magenta box), 4.46 g/L of MS (M5519, Sigma)+ 30 g/L of sucrose + some distilled water. After all is dissolved, pH was brought to 5.8 with diluted KOH . Than solution was brought to final volume with distilled water. We added agar gel 4 g/ L and put the mixture in autoclave for 21 minutes, then flask was put under hood to cool down to ~ 55 °C . Before the medium was solidified heavy metal solution were added as follows: Na_2SeO_4 (16 ppm), K_2TeO_4 (8 ppm), $KCrO_4$ (4 ppm), Na_2HAsO_4 (16 ppm), Na_2WO_4 (32 ppm), $NiSO_4·6H_2O$ (8 ppm), $(NH_4)6Mo_7O_{24}$ (32 ppm) and $NaVO_3$ (16 ppm).We prepared 3 boxes for each treatment, so , at least 10 seeds per species per treatment. Once medium was solidified, we put on agar surface with sterile forceps 10 seeds per species per treatment, so roughly 3 seeds per box. All magenta boxes were put for 3 months in grow chamber settled as follow : 150 µmol m^{-2} s^{-1} PAR, 70% of humidity, 16 h of day light, 24 °C. After three months, plants were kept out from agar, extracting gently roots with forceps and/or gloves. Roots were washed for some seconds with distilled water in order to remove pieces of agar gel and metal ions adsorbed on root surface. Then plants were placed on a black plastic surface with a thin layer of water (avoiding roots could become dry and sticky) to measure roots and stems length with a ruler. Finally with scissors stems and leaves were separated from roots. For each species and treatment, the total shoot fresh weight and root fresh weight were separately recorded. For each species and treatment some leaves of the same age were put immediately in -80 °C freezer After that, stems of each treatment and species were grouped in a small plastic tray and put in the oven at 55 °C for 48 h. Then the total dry weight was recorded. We have crumbled dry stems and roots to homogenize the sampling and weighted between 30 and 60 mg of that - depending on the amount available of plant tissues- preparing three replicats per treatment (for each species as usual). Following Zarcinas method,(Zarcinas et al. 1987) the crumbled tissues, with 1 ml of 95% nitric acid were put into a 30 cm long glass tubes that were inserted in a digestor under hood. The digestion program was : 2 hours at 60 °C and 8 hours at 120 °C. The mineralized samples were brought to 10 ml with deionized water, than they were analyzed by ICP –AES (Inductively coupled plasma atomic emission spectroscopy) machine to determine heavy metals concentration.   *XANES(X ray Absorption Near Edge Structure):* to understand if there are favorite sites of accumulation for Cr, Se, W, V and As in Astragalus spp leaves , we have processed leaves of *A. racemosus* and *convallarius*

previously kept frozen in -80 freezer. The analysis was conducted by beamline 10.3.2 at the Advanced Light Source of the Lawrence Berkeley Lab in Berkeley, California. The XANES method consists in scanning leaf samples kept frozen at -30 °C with X-ray settled in a range between 2.5 keV and 17 keV .

RESULTS AND DISCUSSION

Selenium (Se)

Looking at Tolerance Index (Figure1) and biomass production (Figure 3) *A. racemosus*, as expected, shows higher Se accumulation and tolerance than *A. convallarius*. The latter shows a root length inhibition close to 90% in comparison with the control treatment, while inhibition was around 60% in *A.racemosus*. *A. racemosus* shows a translocation factor (TF) (figure 4) of 3.12; such a high value is typical for a hyperaccumulator. Se shoot concentration (231 mg/Kg d.w.) is in the range of good Se accumulator, although not of a best one (figure 2). Biomass production indicates *A.convallarius* is heavily affected by Se particularly at the root level; roots were often almost absent and the shape was shorter and thicker, all symptoms of stress. The surprisingly high Se concentration detected in roots and shoots of *A. convallarius* may be explained in part by the very low amount of dry biomass used for ICP analysis because of the low dry biomass available. At very low sample weights the concentrations tend to be overestimated due to the large conversion factor. This insight is confirmed by XAS analysis : Se concentration in *A. racemosus* seems clearly higher than in *A. convallarius* (Picture 1) ; Se in *A. racemosus* seems also widely and homogeneously distributed in leaf tissue and in particular in leaf tip and borders. This distribution is not only different from non accumulator *A. convallarius* but also from that of hyperaccumulator *A. bisulcatus* which stores Se mostly in trichomes (Freeman et al., 2006, Plant Physiology).

Chromium (Cr)

Tolerance index (Figure1) shows a severe inhibition of root growth in *A. convallarius* (almost 80% lower than control) while *A. racemosus* shows a reduction of about 45% in comparison with its control. *A. racemosus* seems to have accumulated in roots 750 mg/kg d.w. but it did not translocate to shoot in which we find only 25 mg/kg d.w. *A. convallarius* shows an opposite behavior with high translocation factor (53.9) and almost 350 mg/Kg d.w. in shoots vs roughly 10 mg/kg in roots. *A. racemosus* seems suffer less than *A. convallarius* in term of root biomass depletion while *convallarius* stem biomass seems increased by Cr. Because CrVI, supplied as $KCrO_4$, is more mobile than CrIII both in soil and inside plant, we should hypothesise that in *A. convallarius* CrVI is efficiently reduced to Cr III not in roots but in stems, given that we don't see any particular damages in shoots . Cr hyperaccumulators like *Salsola kali* and *Leersia exandra* accumulate Cr VI mostly in leaves respectively up to 2900 mg/Kg (Gardea et al., 2005)

and 2978 mg/kg (Zhang 2009). Because of, as reported in literature, a real Cr-hyperaccumulator stocks Cr as Cr VI mostly in leaves we should assume that *A. convallarius* shows the same behavior of an hyperaccumulator. On the other side, the severe depletion of root growth leads to concern about a passive uptake of CrVI by *A. convallarius* stems due to a fade filtering and reducing action of root system.

Finally *A. racemosus* can be considered a secondary Cr-accumulator because its good tolerance and Cr uptake in roots, while *A. convallarius* shows unclear responses. In any case, it is intriguing to try to understand why Cr increases shoot biomass production and decreases root biomass at the same time.

Tellurium (Te)

Several Se accumulator species of genus *Astragalus* are also Te accumulators and shows same performances in accumulating Se and Te; the same is for non Se accumulator (Cowgill 1988). *A. racemosus* and *A. convallarius* show no significant differences in tolerance index (Figure1) , accumulation and translocation factor ; biomass production only could indicate A. convallarius is more suffering than A. racemosus due to a − 65% in root biomass versus − 50% of A. racemosus and a -85% in stem biomass compared with − 55% of A. racemosus.

Te accumulators studied in Ely district (Nevada) show ranges of accumulation between 100 and 10,000 mg/Kg d.w. (Cowgill 1988) Both *A. racemosus* and *A. convallarius* stay in this range. They can be considered good Te accumulators, although , differently from Se accumulators, they store Te mostly in the root system, showing very low translocation factors.

Arsenic (As)

Both species considered shows a biomass depletion closest to 75%. This could mean they suffer competition between As and P in using P transporters and in forming ATP molecules. Although both of them accumulate impressive amount of As, only A. racemosus that accumulate in stems 7500mg/Kg d.w. with a translocation factor major than 5 could be considered a real hyperaccumulator (best As accumulator known is *pteris vittata* that accumulates up to 5131 mg/Kg in two weeks in media added with 50 ppm of arsenate). We need further investigation to dissolve question if A. racemosus can only survive or is really an hyperaccumulator.

Nickel (Ni)

Real Ni hyperaccumulator plants, can accumulate from several thousands of mg/kg up to 5% of dry biomass without exhibiting phytotoxicity (Baker et al.,2000; Prasad et al.,2005; Milner et al.,2008)A. racemosus and A. convallarius don't accumulate more than 10 mg/kg in roots and than 23 mg/Kg in stems. Sensitive species shows symptoms of Ni toxicity with 5-15 ppm in medium.(111, 112, 118). Because Ni concentration used in this trial is 8 ppm, a depletion of -

60% (racemosus) and -65% (convallarius) of root length and -75% (racemosus) and -60% (convallarius) in root and stem biomass leads us to consider these two species as sensitive species.

Vanadium (V)

Severe root length (-70% for convallarius and -85% for racemosus) (Figure1)   and biomass depletion (around -80% for both species) (Figure 3), and a translocation factor widely lower than one, suggests us *A. racemosus* and *A. convallarius* are not V accumulators, infact high V concentration found in root system could be the result of passive uptake and/or competition with Mo, that could explane biomass and root system reduction. According to ICP results that show a V concentration in convallarius shoots two times higher than in A. racemosus shoots, XAS shows V mostly accumulated in borders of convallarius leaf while racemosus leaf does not show any presence of this metal(Picture 3,4). Accumulation in leaf borders or thricomes seems to be a common behavior of metals and metalloids accumulators as seen for Se in racemosus and in A. bisulcatus. For all that reasons further investigations have to be done on interaction between *Astragalus spp* and V.

Tungsten (W)

Tolerance index (Figure1)   shows a less severe W-related reduction in root length for *A. convallarius* (-55%) than for *A. racemosus*(-77%). *A. convallarius* shows no depletion in biomass production (Figure3), while *A. racemosus* shows a decrease in biomass production close to 50%. Shoot W concentration was around 20,000mg/kg DW (Figure2)   in roots for both of species: this value is two times higher than the maximum level recorded in literature (Kerry et al., 2002). To this surprising concentration in roots does not correspond ah high Translocation factor although 299 mg/Kg and 151 mg/Kg respectively for *A. convallarius* and *A. racemosus* seems in average with some of highest concentration recorded in literature in aerial parts of several species (Piyatt et al., 2004 Shacklette et al., 1978 ,Tyler et al.,2005). XAS analysis shows a more marked presence of W in convallarius leaf (confirming ICP data) (Picture 5,6) and in particular it confirm again, for *A. convallarius*, the supposed tipical behavior of accumulators, that is metals and metalloids accumulation in leaf borders and or tips. Based on these data we should classify *A. convallarius* as a W hyperaccumulator.

Molybdenum (Mo)

Tolerance index (Figure1)   shows -20% in root length depletion for *A. racemosus* and -40% for *A. convallarius*. Mo concentration in roots of both species passed widely concentrations recorded for plant samples coming from mining districts in Colorado : 400 mg/Kg (Trlica and Brown, 2000) while in shoots the average is between 150 mg/Kg (*convallarius*) (Figure2)   and 350 mg/Kg (*racemosus*). Biomass depletion is around 50% for *A. racemosus* and less than 30% for

A. *convallarius*. Finally, both species should be considered Mo accumulators but considering that Mo is essential for plants and deficiency symptoms are more clear than toxicity symptoms, more investigations are probably necessary to better classify the accumulation potential of these two species.

FIGURES
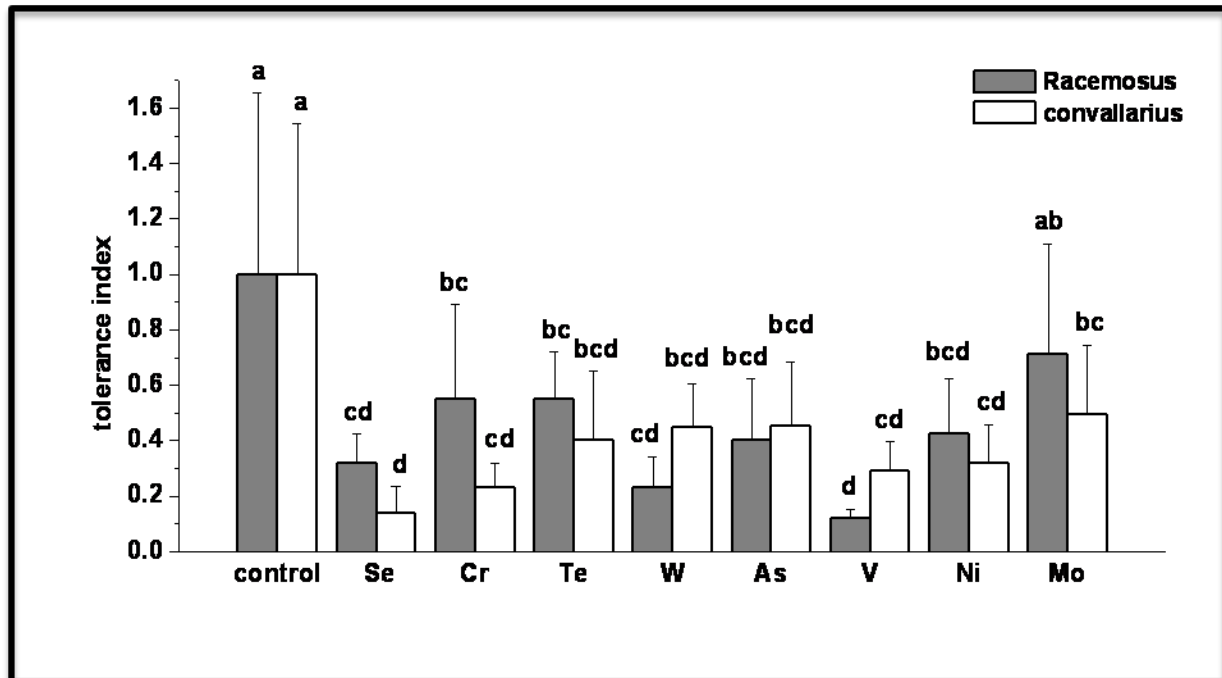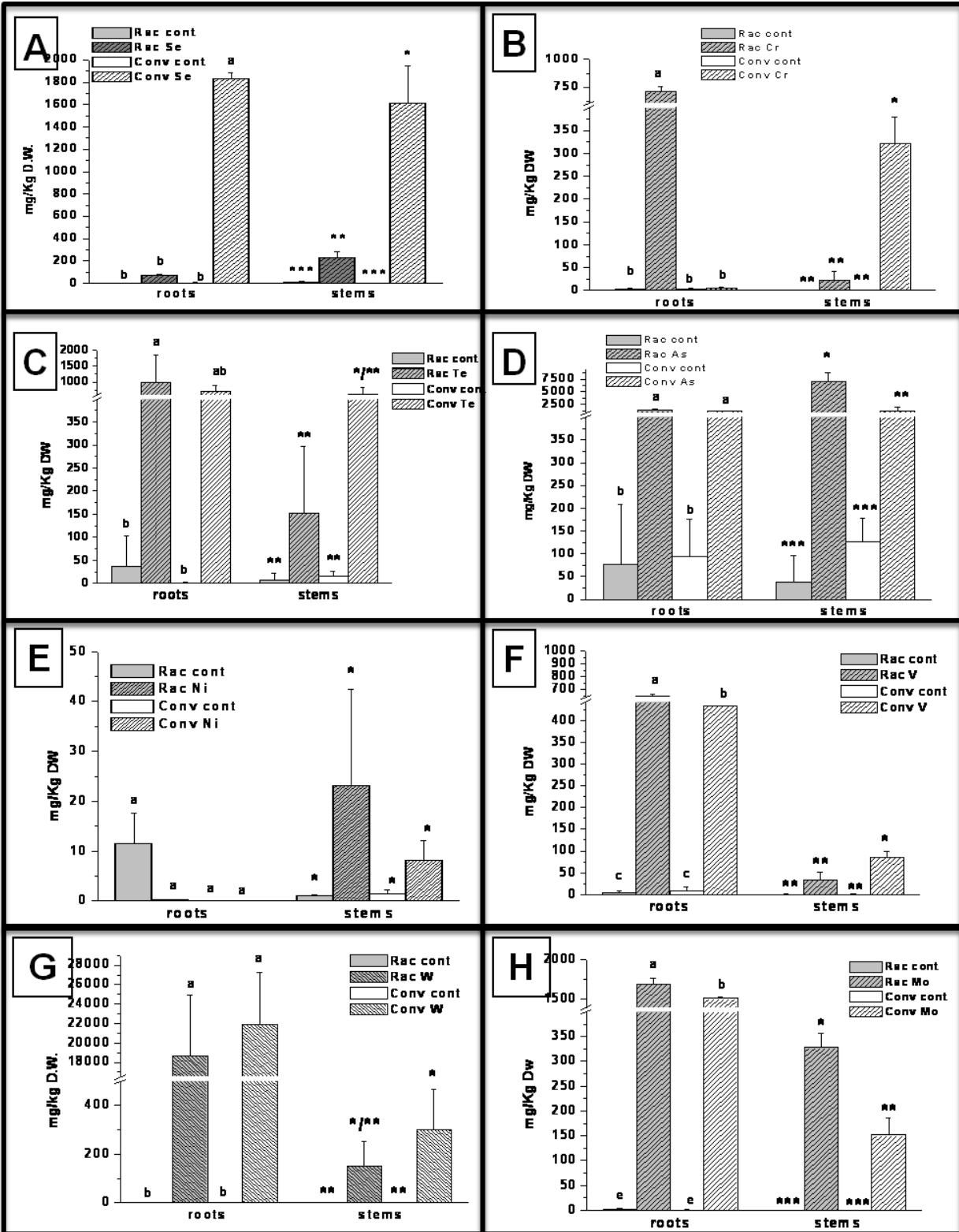

Figure 1 Tolerance index (average root length of treatment / average root length of control) of *Astragalus racemosus and A. convallarius* . Error bars : Std deviation. Different letters mean significant differences between bars.

Figure 2 metals and metalloids accumulation in roots and shoots of A. racemosus and A. convallarius grown in agargel amended time by time with Na_2SeO_4 (16 ppm), K_2TeO_4 (8 ppm), $KCrO_4$ (4 ppm), Na_2HAsO_4 (16 ppm), Na_2WO_4 (32 ppm), $NiSO_4 \cdot 6H_2O$ (8 ppm), $(NH_4)6Mo_7O_{24}$ (32 ppm) and $NaVO_3$ (16 ppm). Error bars : Std. Different letters and stars mean significant differences between bars.
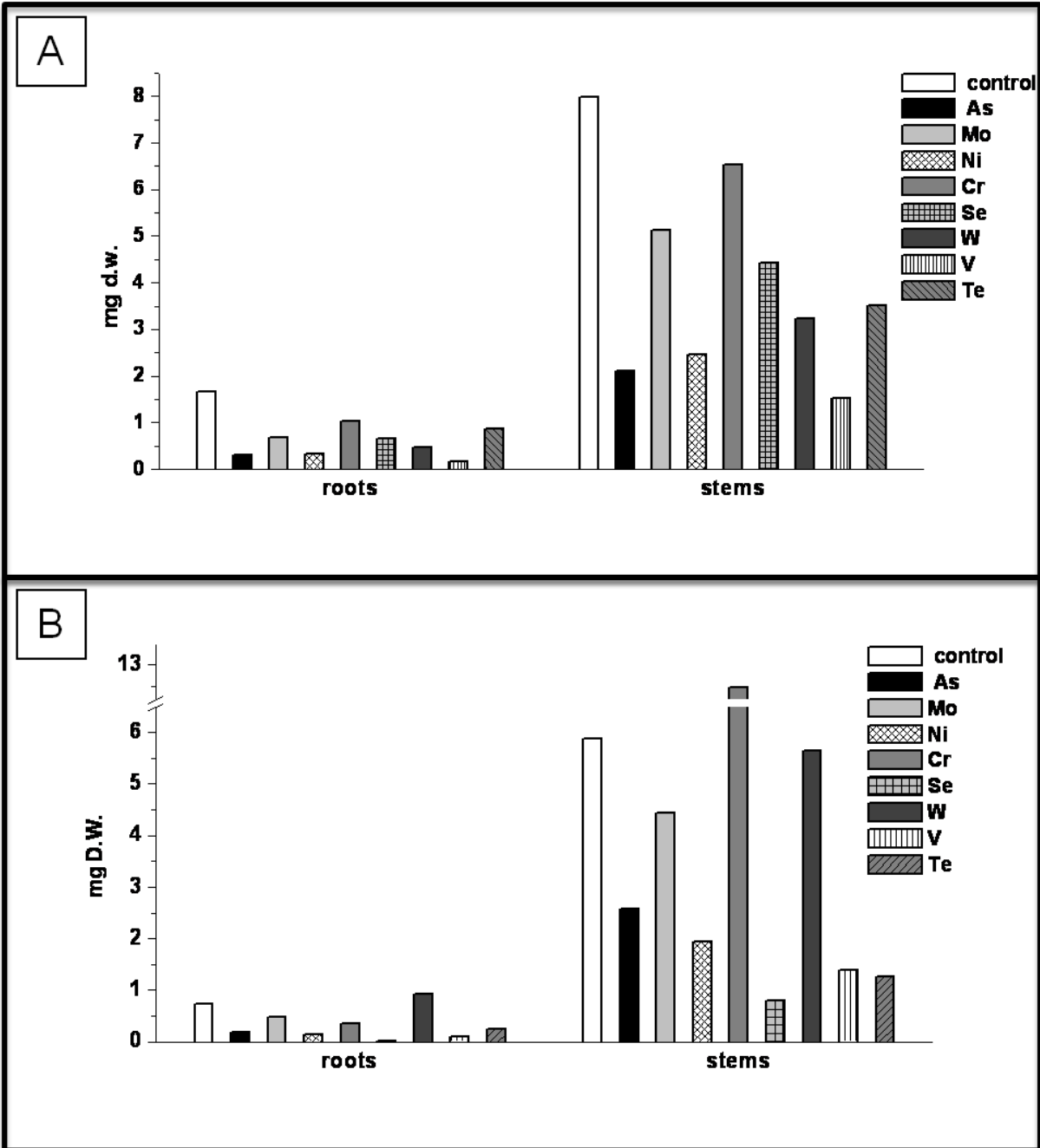
Figure 3 average biomass of roots and shoots of *A. racemosus* (A) and *A. convallarius* (B)
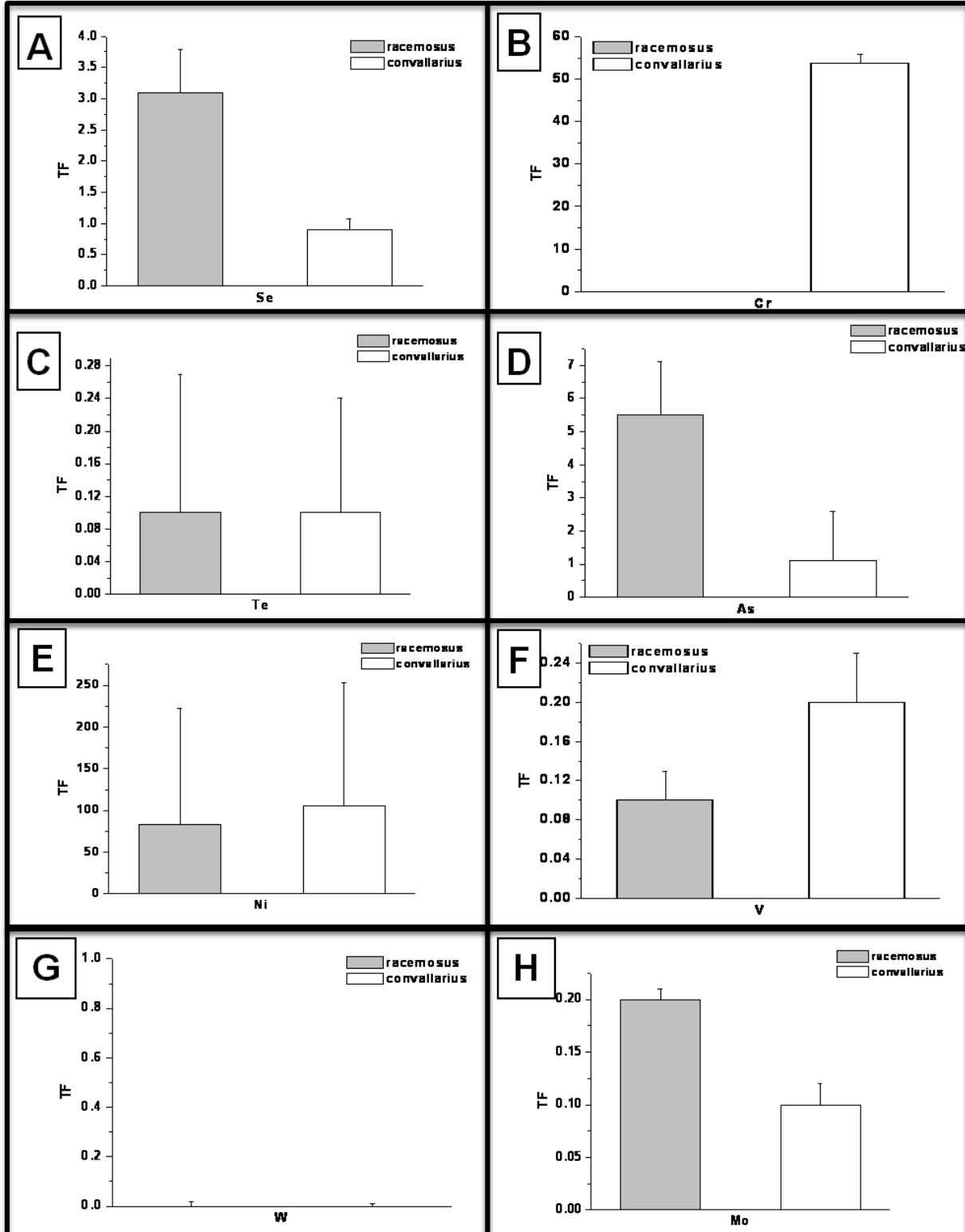
Figure 4 : Translocation factor calculated for each element investigated. Error bars : Std
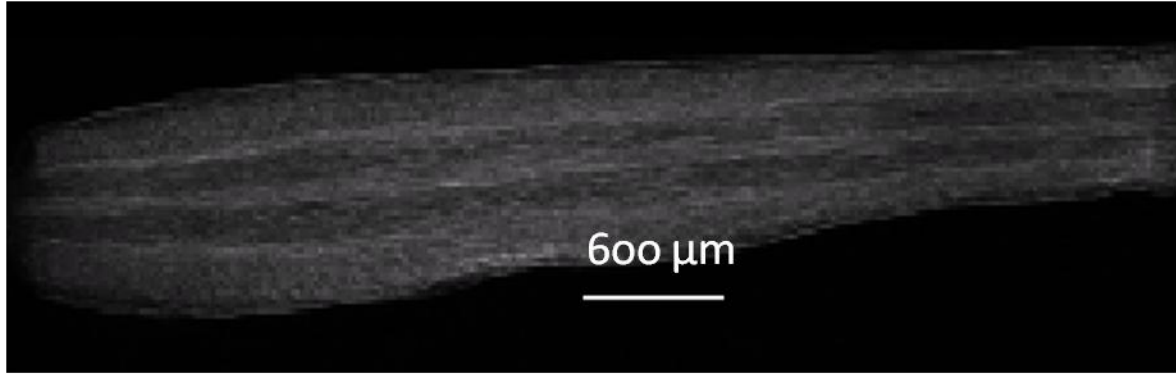
Picture 1 : Se detection in leaf of *A. convallarius* by XAS
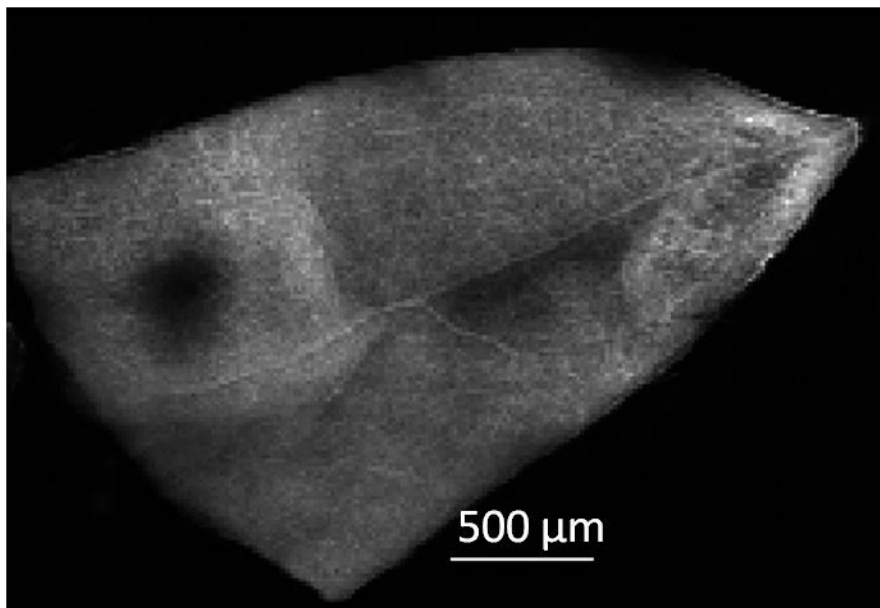
Picture 2 Se detection in leaf of *A. racemosus* by XAS
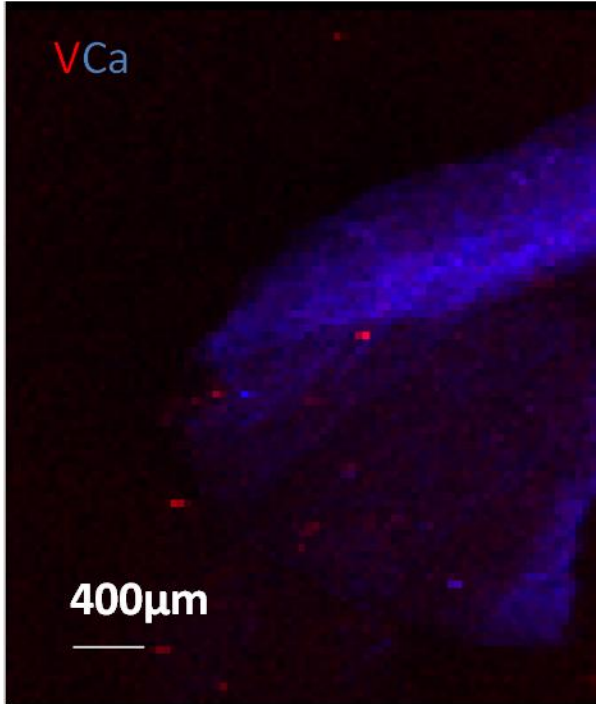
Picture 3 V detection (pink) in leaf of A. racemosus by XAS
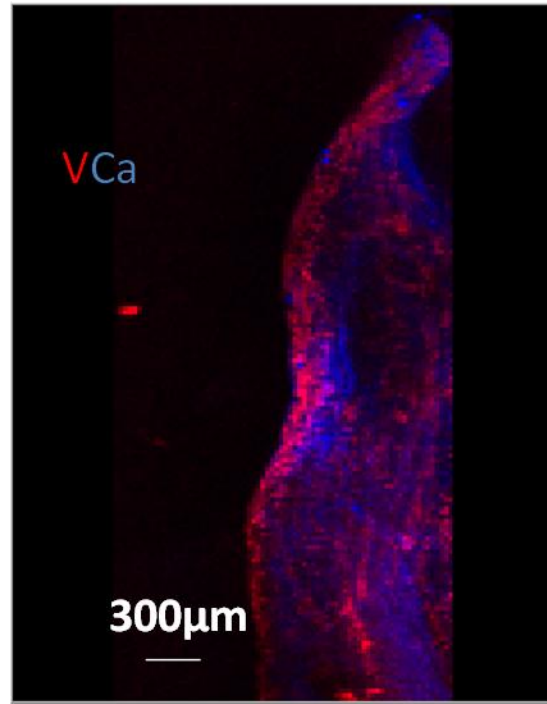
Picture 4 V detection (pink) in leaf of A. convallarius by XAS
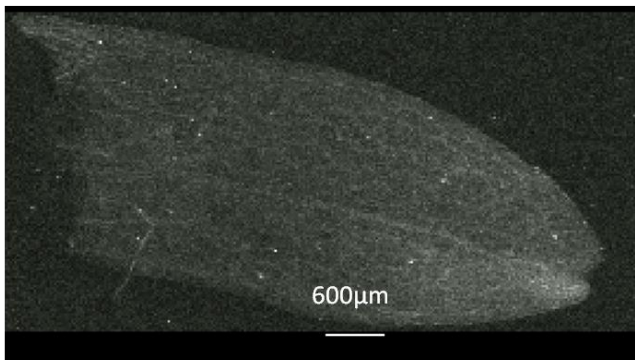
Picture 5 : W detection in leaf of *A. convallarius* by XAS
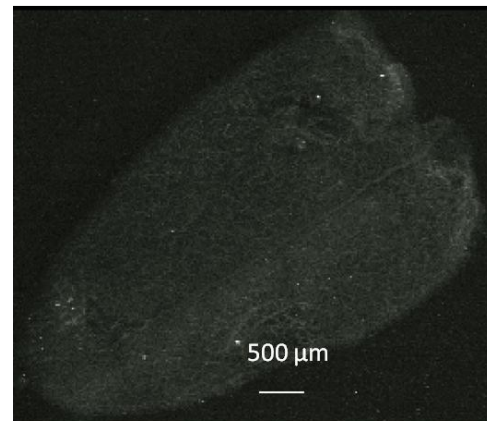
Picture 6 W detection in leaf of *A. racemosus* by XAS

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A LIKELY ROLE OF SELENIUM ON DROUGHT STRESS TOLERANCE IN

BRASSICA JUNCEA AND *STANLEYA PINNATA*

INTRODUCTION

Several hypotheses have been put forward to explain the functional significance of elemental hyperaccumulation in plants (Boyd et al., 1992; Mc Nair 2003). These include allelopathy, drought resistance, and defense against herbivory or pathogens. Most evidence to date supports the third, elemental defense hypothesis. Hyperaccumulated Zn was shown to protect plants from invertebrate herbivory (Pollard et al., 1997, Jhee et al.,1999), and Ni hyperaccumulation can protect plants from caterpillar herbivory as well as fungal and bacterial infection (Boyd et al., 1994 ; Boyd et al. 1999, Boyd et al. 2002 ; Martens et al., 2002; Gadherian et al., 2002). Selenium also protects plants from herbivory, both by vertebrates and invertebrates (Franke et al., 1936; Freeman et al., 2006) and, for some authors it seems to have a positive role in drought stress (Kuznetsov et al. 2003; Djanaguiraman et al. 2005). However, to our knowledge, there has been limited effort to understand the role of Se in plants under drought condition.

In order to make clear physiological consequences of adaptation to Se accumulation and because drought stress is considered to be one of the most important agricultural problems and is likely increasing in the future (Chaves et al. 2002), we have further investigated a possible role of Se in increasing drought stress tolerance in *Stanleya pinnata* and *Brassica juncea*.

Stanleya pinnata is a perennial species of *Brassicaceae* family and it is considered a Se hyperaccumulator. In Colorado *S. pinnata* populations grow on seleniferous soil in a semiarid environment. *S. pinnata* can accumulate in leaves up to 3750 mg/Kg d.w. as reported by Galeas et al. (2007).

Brassica juncea is a secondary Se accumulator (probably because it is an avid S accumulator and in a condition of S deficiency Se that has similar steric characteristics to that ones of S, can be uptake by S-transporters) of family of *Brassicaceae*. In this class of accumulator Scientists have placed all species that can accumulate in average up to 1000 mg/Kg of Se (Frankenberger and Benson, 1994).

Hyperaccumulators may tolerate 1000 fold more selenium without exhibiting toxic effects than non-accumulators. The mode for tolerance in hyperaccumulators may be by one of several mechanisms. The first involves compartmentalization of selenate or seleno-amino acids in vacuoles (Terry et al., 2000). : this strategy could change Ψw and Ψs inside the vacuole allowing plant to limit water loss in a drought stress condition. Dimonstration of this hypothesis is the target of drought stress simulation in hydroponic.

Bathia et al. (2005) demonstrated that in *Stackhousia tryonii* Bailey, a Nickel hyperaccumulator, the removed green canopy is replaced with a new one characterized by Nickel concentration directly correlated with the severity of applied drought stress. Because Stanleya pinnata is a Se hyperaccumulator, our focus is reveal a similar behavior in Se management, removing canopy

and treating with three levels of drought stress (25% field capacity = no stress ; 15% f.c. ; 7% f.c.) two groups of Stanleya grown one supplied for 15 months with selenate and a second one grown without selenate.

To investigate accurately the likely role of Se in drought stress tolerance, a second group of Stanleya were treated with same tre level of drought stress, leaving the original canopy and measuring during 30 days water and osmotic potential and chlorophyl fluorescence.

MATERIALS AND METHODS

Drought stress simulation in hydroponics

Brassica juncea seeds from Coloradoan populations were put in a 15 ml tube to be sterilized by 1 minute in ethanol 70% and 30 minute in bleach 30%. After that, seeds were cleaned up from bleach by five 10 minutes long washes in sterile deionized water; during each wash tubes were put on the rocker to be gently shacked. Between each wash, water was replaced under sterile hood. Then, sterilized seeds were put in magenta boxes (30 per box) under sterile hood using sterile forceps. The medium was agar added with MS salts and vitamins MS 5519. The medium was prepared as follows : (50 ml per magenta box), 4.46 g/L of MS (M5519, Sigma)+ 30 g/L of sucrose + some distilled water.   After all is dissolved, pH was brought to 5.8 with diluted KOH . Than solution was brought to final volume with distilled water. We added agar gel 4 g/ L and put the mixture in autoclave for 21 minutes, then flask was put under hood to cool down to ~ 55 °C . Sterilized seeds were left to germinate and grow for a week in growth chamber settled as follows: 150 µmol m^{-2} s^{-1} PAR, 70% of humidity, 16 h of day light, 24 °C. After that, plants were removed from agar, gently extracting the roots with forceps. Roots were washed for some seconds with distilled water to remove pieces of agargel then seedlings were gently put into a holed table placed on a 6 L plastic tray (one seedling per hole), filled up with 1/5 strenght Hoagland nutrient solution. Oxygen exchange was guaranteed by air pumps. Seedlings were placed to have roots submerged and shoot over the table surface.

Plants were grown at room temperature under artificial light (150 µmol m^{-2} s^{-1}). Seventeen seedlings were grown in nutrient solution only for first and second week and in Hoagland plus 20 µM of Na_2SeO_4, in the third and fourth week. Other seventeen ones were grown for a month with Hoagland nutrient solution only. This preliminary step allowed + Se plants to accumulate Se before drought stress trial was started.

At the beginning of fifth week, in both +Se and –Se tray polyetilenglycol (m.w. 10000- by SIGMA-Aldrich) was added to nutrient solution in order to bring down water potential to – 0.6 MPa (considering 0.0125 g/ml to have -0.1 MPa of decreasing). Week by week water potential was brought down until -1.3 MPa, value at which all plants wilted. At the end of each step, water and osmotic potential (with psycrometer HR 33T by Vescor ,USA) and chlorofyll

fluorescence (to determine PSII efficiency at 45 µE and 450 µE with Hansatech Fluorometer FMS2 (Hansatech Instrument,Norfolk, UK) were measured. Dried leaves (in oven at 55 °C for 48 h) were crumbled to homogenize the samples and weighted between 30 and 60 mg of that - depending on the amount available of plant tissues- preparing three replicats per treatment. Following Zarcinas method (Zarcinas et al. 1987), the crumbled tissues, with 1 ml of 95% nitric acid were put into a 30 cm long glass tubes that were inserted in a digestor under hood. The digestion program was: 2 hours at 60 °C and 8 hours at 120 °C. The mineralized samples were brought to 10 ml with deionized water, than they were analyzed by ICP –AES (Inductively coupled plasma atomic emission spectroscopy) machine to determine Se concentration.

The second drought stress simulation was carried on in the same way except for tray size and drought stress increase. 6 seedlings were placed in six holes of a plastic cap on a 0.6 L plastic tray; one tray for +Se plants and one for –Se plants. Drought stress was brought directly from 0 to -1.3, to avoid possible physiological adaptations happening at intermediate stress conditions. Then, week by week drought stress was increased to -1.6 MPa until final wilting. All measurements were done as described for first experiment.

Drought stress simulation in agargel

Brassica juncea seeds coming from 2 populations, one grown in a Se addicted soil and one in a not Se addicted soil, were surface-sterilized as described for previous experiments. Six magenta boxes were filled up with agargel plus MS salts. Four of them were added with two different amount of PEG 10000 to obtain two drought stress levels: -0.6 MPa and -1.2 MPa. Each box hosted 36 seeds. Boxes were placed in growth chamber (150 µmol m^{-2} s^{-1} PAR, 70% of humidity, 16 h of day light, 24 °C) for five days. After that, root length, stem length and total fresh biomass were recorded for each treatment. Previously, some seeds coming from same two populations were ICP-AES analyzed to measure Se content.

Experiment with Stanleya pinnata in green house

Stanleya pinnata 15 months old plants, grown in greenhouse in gravel were recruited for this experiment. Thirty plants were grown in Scott Peters nutrient solution supplemented with 20 µmol Se (as Na_2SeO_4) and thirty plants were grown without Se. Each plant grew in a 0.4 L plastic pot. Fifteen +Se and fifteen –Se plants were deprived of green biomass, then each pot weight plus gravel and plus stem, was recorded. +Se plants and –Se plants were divided in 3 groups corresponding to three levels of drought stress : 5 +Se plants were supplied weekly with 100 ml of nutrient solution, 5 with 60 ml and 5 with 30 ml. Second group of thirty plants were left unclipped and divided in same groups described above with same levels of drought stress. Experiment was eight weeks long for clipped plants (July 1st – August 29) and five weeks long for unclipped plants (July 7-August 16). Each week, chlorophyll fluorescence (to determine PSII efficiency at 45 µE and 450 µE with Hansatech Fluorometer FMS2 -Hansatech

Instrument,Norfolk, UK) and water and osmotic potential of whole plants were measured. At the end of trial, clipped plant pots were weighed; new canopy was harvested and fresh and dry weight were recorded; after that Se content of old and young leaves was detected by ICP-AES machine. Unclipped plant pots were weighed. Canopy of each plant was harvested and fresh and dry weight were measured. Se content was detected by ICP –AES method.

Stanleya pinnata detached leaves

three leaves of a 15 months old Stanleya pinnata grown in gravel and supplied with 20 µmol Se (as Na_2SeO_4) addicted Scott Peters nutrient solution and three leaves of a Stanleya pinnata not supplied with Na_2SeO_4 were detached from plants, weighed and left in the dark at room temperature (26 °C, 50% R.H.) for four hours. Weight was recorded after 20 min. , 40 min., 1 h, 2 h and 4 h.

RESULTS

Drought stress simulation in hydroponic

In the first experiment of drought stress simulation by polyethilenglicol   no significant differences between + Se and − Se plants about water and osmotic potential were observed (Figure 1 A,B). The greatest gap between +Se and −Se plants is detectable at the end of first week of treatment at -0.6 MPa of simulated drought stress. Increasing hydroponic water potential up to -1.3 MPa, eliminated differences completely. Se content in Selenate-added *Brassica* plants increased up to 1200 mg/Kg at the end of second week of drought stress, then it decreased to level before start of experiment (600mg/Kg) (Figure 1 graph C). Chlorophyll fluorescence didn't show significant differences. Fv'/Fm' (the efficiency of the open reaction centers in the light) seemed lightly affected only close to final wilting, until the day before water potential increased up to -1.3 MPa (Figure 2 A). NPQ (non photochemical quenching) is significantly higher than in − Se plants in first steps of experiment, then differences became less evident (Figure 2 B)

In the second experiment Se content decreased from about 2200 mg/Kg to 1000 mg/Kg showing the same behavior of first experiment probably due too quick leaf turn over accelerated by drought stress (Figure 3 C). To avoid physiological adaptation that could hiding a role of Selenium, plants were brought up immediately to a stress level equal to -1.25 MPa, so only a 0.05 MPa less the limit recorded in the previous trial. Both + Se and −Se holded the stress and survived some hours up to -1.6 MPa, but + Se plants wilted quicker than − Se ones, differently from that happened in first trial in which + Se group showed at -1.3 MPa a general better condition from a visual point of view. Both + Se and −Se shows very similar water and osmotic potential across entire experiment, while Se concentration in + Se, despite this time concentration was almost two time higher than that one of first trial (probably due too the week

of grow in Hoagland + Selenate addicted), shows the same behavior of first trial with an initial increase and a following decrease of Se concentration. Like in previous experiment Fv'/Fm' (Figure 4 A) doesn't show any differences both between + Se and – Se and inside groups during the entire experiment, NPQ (Figure 4 B) shows same behavior of previous trial with an initial higher ratio in favour of + Se group and a following arrangement to higher and similar values.

Drought stress simulation in agargel

Brassica seeds containing Se and other without Se, germinated in PEG added agar gel, shows to suffer equally the drought stress in term of root and stem length, while in term of biomass production +Se seedlings shows a fresh biomass 30% greater than –Se in absence of drought and at -0.6 MPa. No differences at -1.2 MPa. (Figure 8 A,B)

Drought stress applied to *Stanleya pinnata* in green house

Stanleya pinnata clipped plants in +Se did not show an increase of Se concentration in new canopy, related with severity of stress (Figure 7 B). + Se plants produced in well watered condition 22.3% of fresh green biomass more than –Se group, while at 15% and 7% of field capacity no differences were noted (Figure 7 graph A).

Whole plants (not clipped) did not show significant differences in water and osmotic potential between +Se and –Se (comparison done by T test) (Figure 5 A,B,C,D,E,F). Analysis of chlorophyll fluorescence, about Fv'/Fm' did not produce significant differences between +Se and –Se neither between start and end of trial (Figure 6 graphs A,C,E). Reguarding NPQ we can note values in +Se plants in average higher than in –Se group in well watered and medium stress condition, while at 7% f.c. + Se and –Se are similar (Figure 6 graphs B,D,F).

Stanleya pinnata detached leaves

From the same populations of previous experiment three leaves from +Se group and three from –Se plants were detached and left to dry. After 4 hours –Se leaves have lost roughly 5% of their fresh weight while +Se leaves have lost 20% (Figure 9).

DISCUSSION

Summarizing the results of all experiments we can conclude that Se accumulation has a positive role on plant growh in well watered or light stress conditions, as reported for wheat seedlings by Xiaoquin et al (2009). Clipped *Stanleya pinnata* plants grown in gravel with Se and +Se *Brassica juncea* seedlings showed a fresh biomass production respectively 22.3 and 30% greater than the same plants and seedlings grown without Se. Not clipped *Stanleya pinnata* plants in green house and *brassica juncea* in hydroponic, seems confirm a light positive effect of

Selenium in mild stress conditions. We can note it in the first hydroponic experiment at -0.6 MPa and -0.8 MPa, while in the second experiment, probably because of the severe drought stress (-1.25 MPa), +Se and –Se plants don't show any significant differences. Chlorophyll fluorescence analysis don't shows particular trends and differences about Fv'/Fm' ratio (the efficiency of the open reaction centers in the light) while NPQ (non photochemical quenching) could be avoided in case of marked differences in Fv'/Fm' ratio between +Se and –Se groups, but our Fv'/Fm' values needs a further investigation to understand if and how much drought stress has damaged photosynthesis. It's important to underline that an increase in NPQ, that is a measure of heat dissipation, report a result either of processes that protect the leaf from light-induced damage or the damage itself. In first hydroponic trial, although not always supported by statistic significancy, we can see a lower vaule in fv/fm ratio in +Se plants, corresponding to a higher NPQ value in comparison with –Se group, that could be evaluated as heavier damage to photosystem by drought stress ; over that it's interesting to note how this phenomenon is more marked before drought stress in all chlorophyll fluorescence measures (for *Brassica* in hydroponic and *Stanleya* on soil), suggesting a possible negative effect of Se on photosynthesis. A quantification of some carotenoids like zeaxanthyn, involved in efficiency of heat dissipation, should be done in order to confirm or discard the hypothesis of a negative effect of Se on photosynthesis or a positive effect of that one on heat dissipation process.

Measure of detached leaves fresh biomass, should confirm, like water and osmotic potential data, that Se has not a positive role in cutting off water loss.

Finally we can say Se seems to be not effective in drought tolerance but seems to enhance plant health in well watered conditions and tolerate light drought stress conditions.
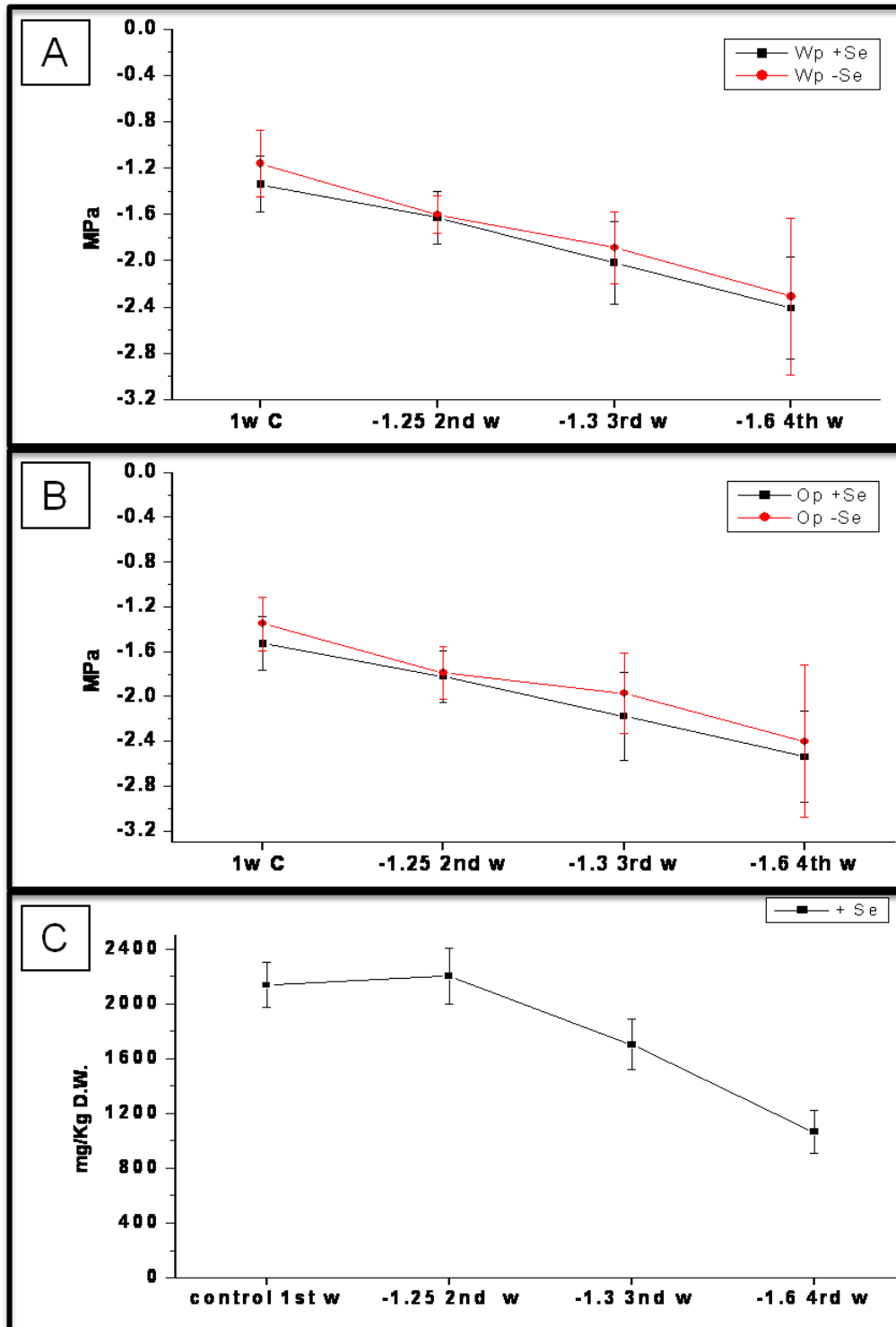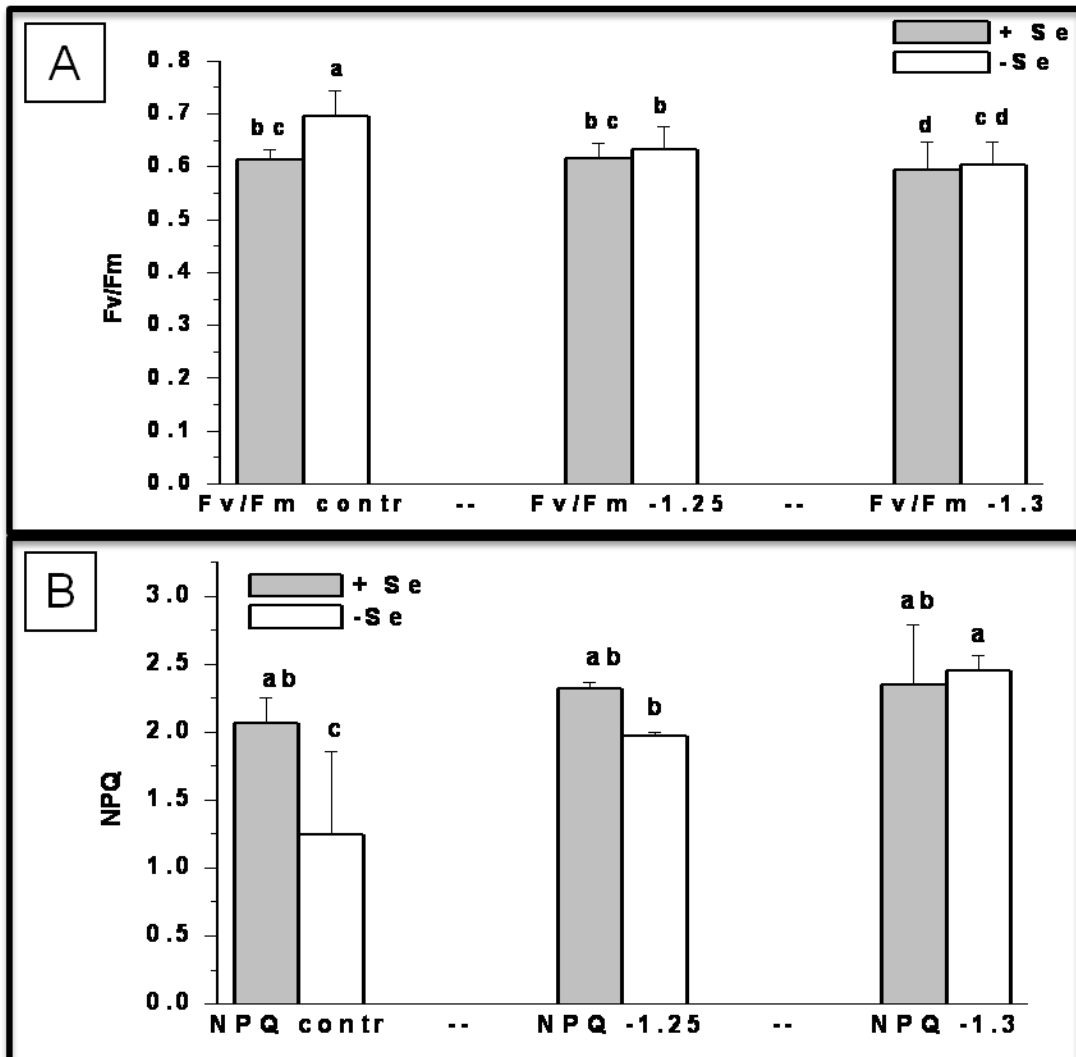
FIGURES
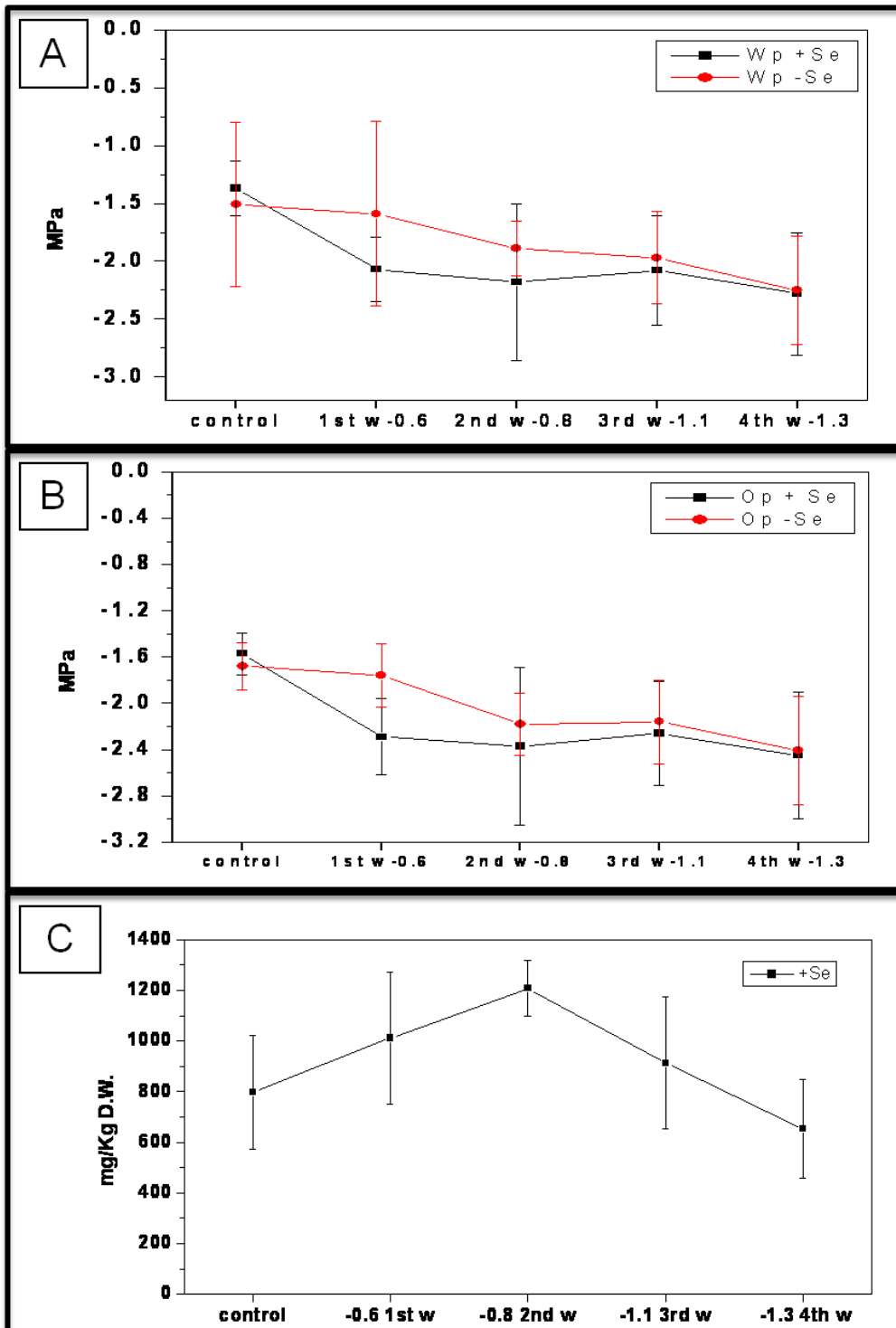
Figure 1 Drought stress in hydroponic by addiction of Poly ethilen glycol 10000. Graph A : X axis : weeks of treatment (control = week zero ; 1st w = first week ; 2nd w = second week ;3rd w = third week ; 4th w= fourth week. -0.6 = Water potential in hydroponic nutrient solution by poly ethilen glicol 10000. Y axis : Water potential in leaf cells. Error bars = Std .Graph B : X axis : weeks of treatment (control = week zero ; 1st w = first week ; 2nd w = second week ;3rd w = third week ; 4th w= fourth week. -0.6 = Water potential in hydroponic nutrient solution by poly ethilen glicol 10000. Y axis : Osmotic potential in leaf cells. Error bars = Std .Graph C : Se concentration in Brassica leaves across weeks. : X axis : weeks of treatment and level of drought stress .Y axis : mg/kg d.w. of Se in Brassica leaves.
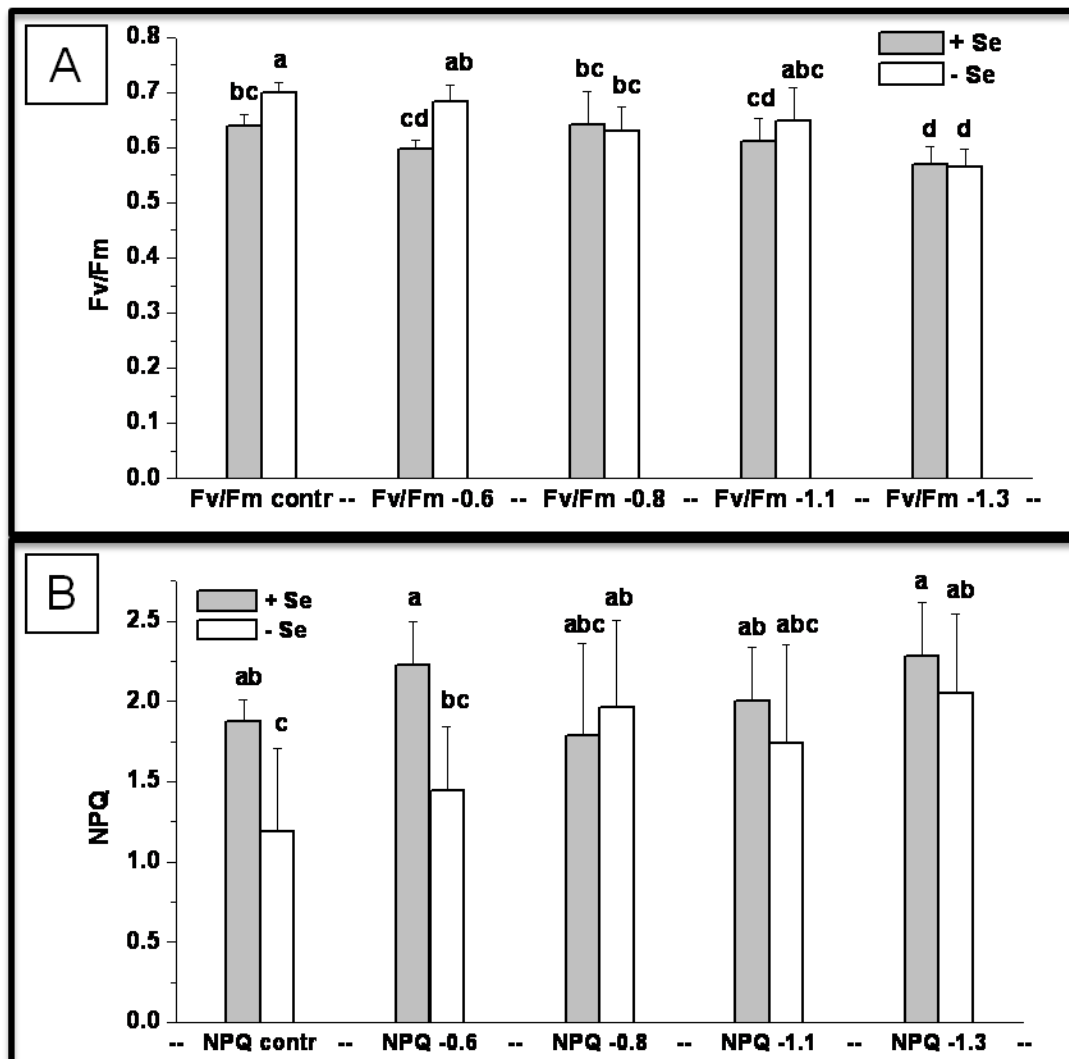
Figure 2. Analysis of fluorescence in first drought simulation with PEG 10000. X axis : weeks of treatment at different simulated drought stress. Y axis : Fv/Fm (Graph A) and NPQ (Graph B). Error bars = Std. Different letters mean significant differences between values.

Figure 3 second drought stress simulation in hydroponic with **PEG 10000.** X axis : weeks at different hydroponic water potential given by **PEG. W = week. (Graph A, B, C).** Y axis : Graph A : water potential measured in leaf cells for Se supplied brassicas (Black) and no selenium amended Brassicas (red). Graph B : : water potential measured in leaf cells for Se supplied brassicas (Black) and no selenium amended Brassicas (red). Graph C : Se concentration in $20\mu M$ Selenate treated Brassicas leaf , measured across the weeks of experiment.

Figure 4 Analysis of fluorescence in second drought simulation with PEG 10000. X axis : weeks of treatment at different simulated drought stress. Y axis : Fv/Fm (A) and NPQ (B). Error bars = Std. Different letters mean significant differences between values.

Figure 5 A, C, E : *Stanleya* leaf water potential measured at 25%, 15% and 7% of field capacity, across the month of experiment. X axis : times of measures. Y axis : leaf water potential in Mpa. Black = Se amended *Stanleya pinnata*. Red = not Se amended *Stanleya pinnata*. Error bars = Std. B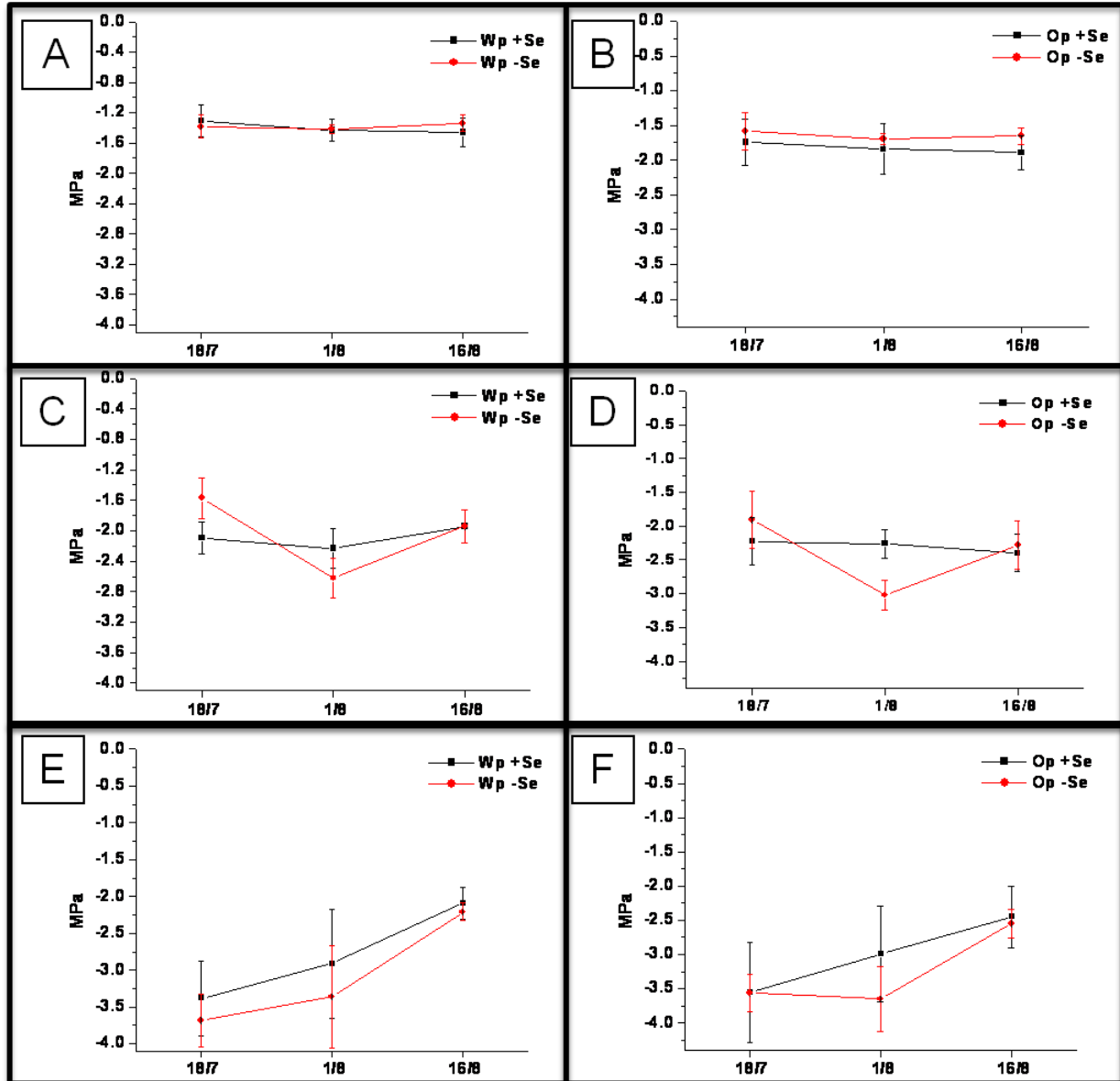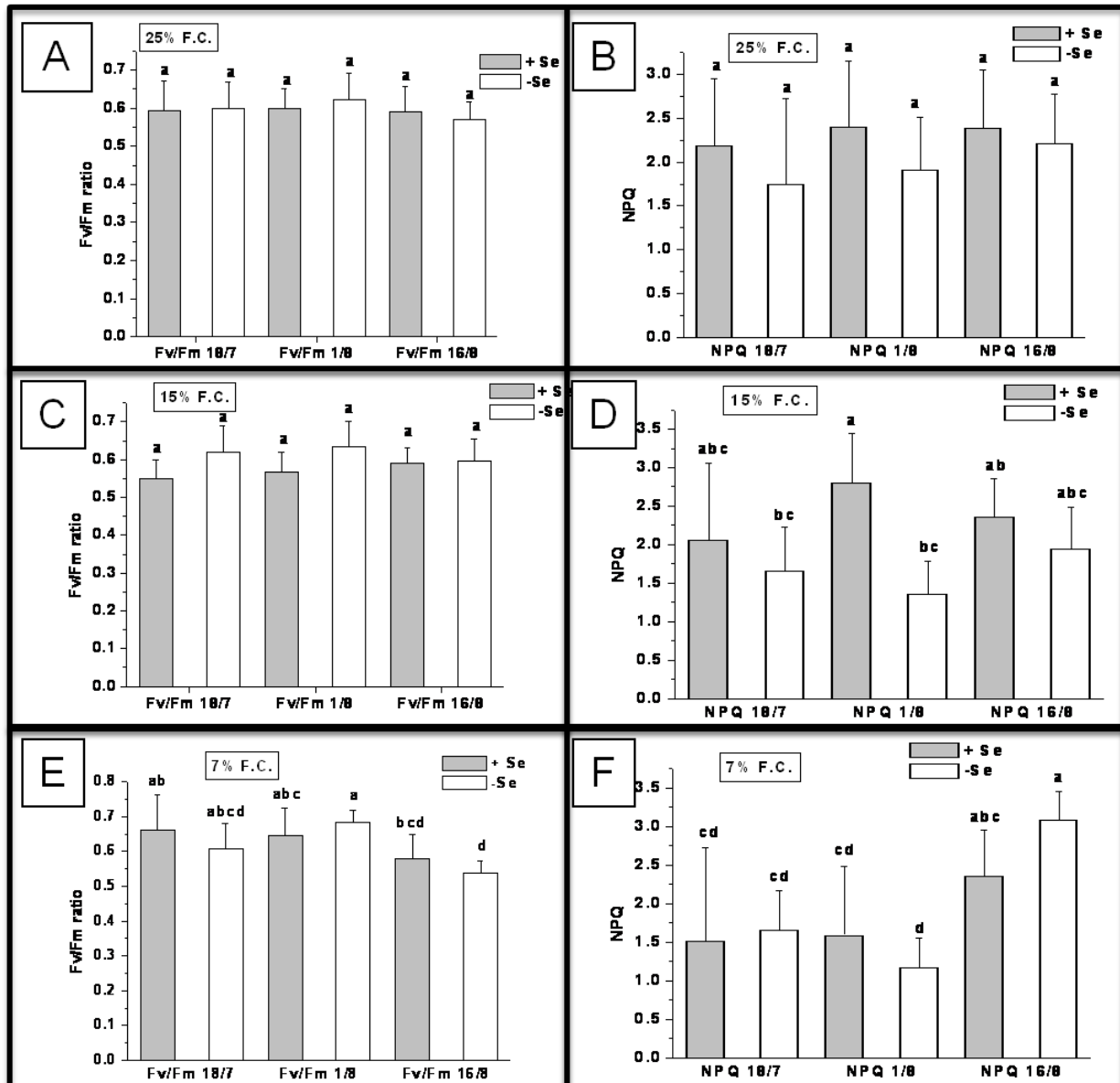,D, F = *Stanleya* leaf osmotic potential measured at 25%, 15% and 7% of field capacity, across the month of experiment. X axis : times of measures. Y axis : leaf water potential in Mpa. Black = Se amended *Stanleya pinnata*. Red = not Se amended *Stanleya pinnata*. Error bars = Std.

Figure 6 Graph A,C,E : analysis of fluorescence. Fv/Fm ratio measured at 25%, 15% and 7% of field capacity, in Se amended Stanleya pinnata leaf (grey bars) and not amended (white bars). Error bars = Std. Different letters mean significant differences between values. X axis = time of measures. Y axis : Fv/Fm values.

Graph B,D, F : analysis of fluorescence. NPQ measured at 25%, 15% and 7% of field capacity, in Se amended Stanleya pinnata leaf (grey bars) and not amended (white bars). Error bars = Std. Different letters mean significant differences between values. X axis = time of measures. Y axis : NPQ values.
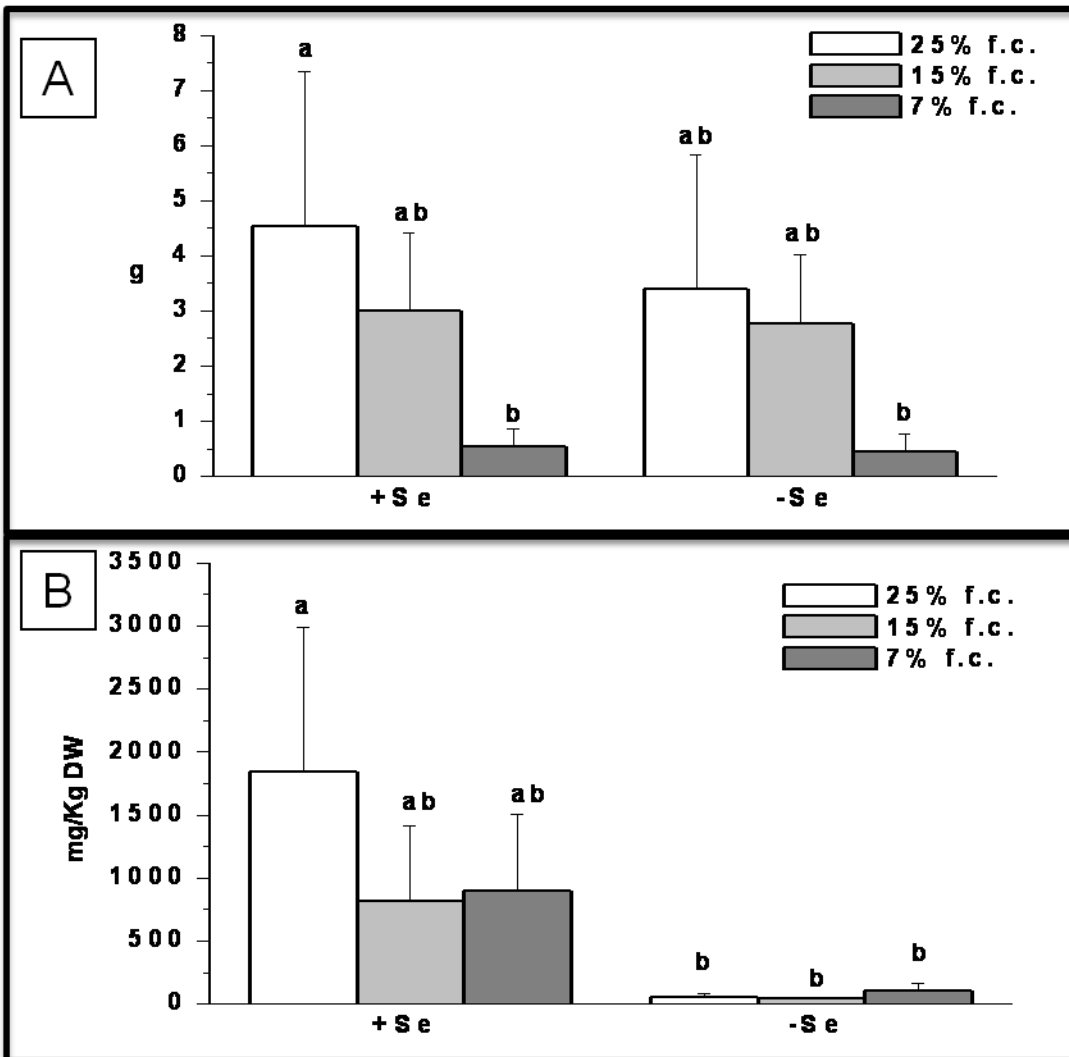
Figure 7 Drought stress applied to Stanleya pinnata in two different levels. 25% of field capacity is considered control condition. X axis : two groups of plants (Se amended and not amended) subjected to 3 different levels of water supply. Y axis : Se concentration in leaves. Error bars = Std. Different letters mean significant differences between bars
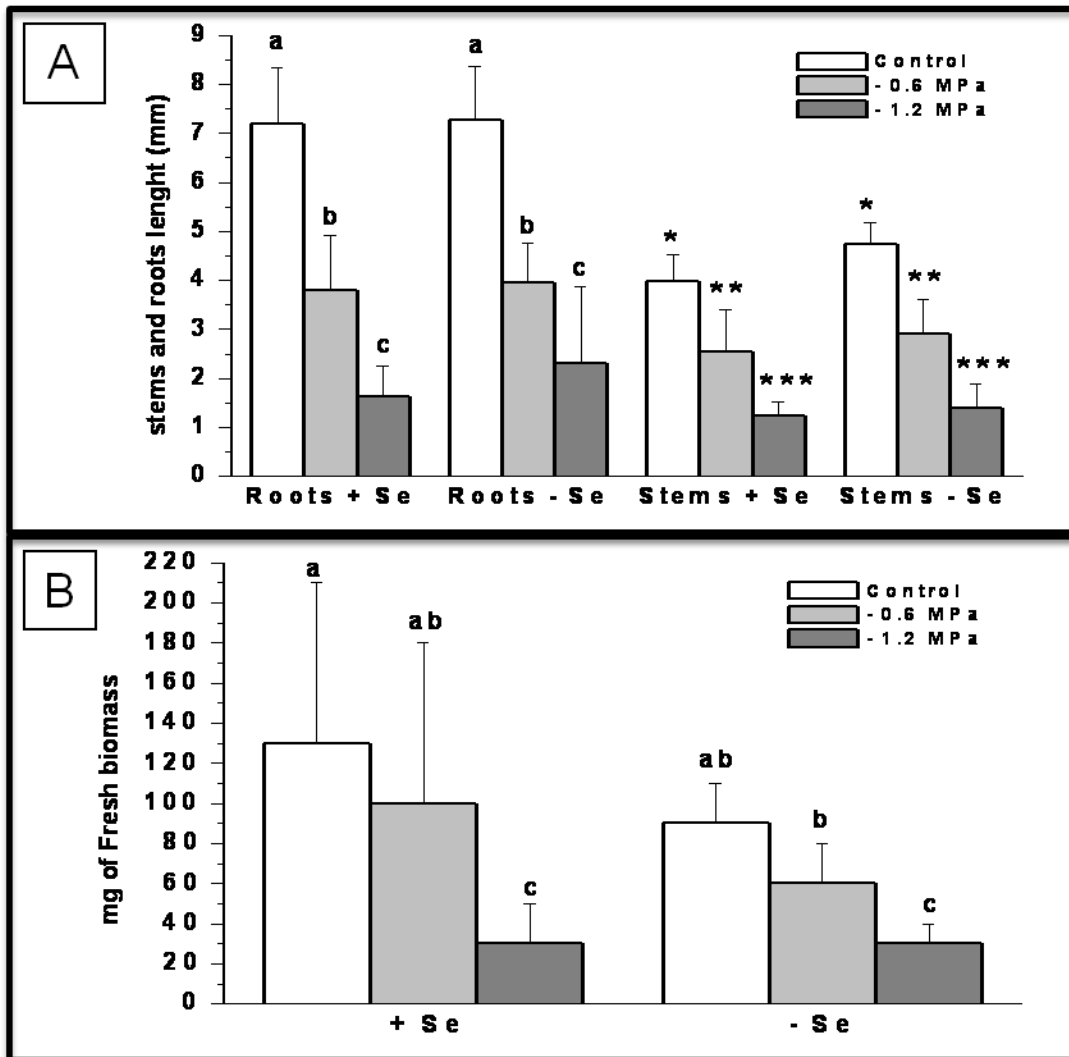
Figure 8 Drought stress simulated in agar gel by PEG 10000. Graph A : X axis : Brassicas seedlings tissues. + Se = seeds containing Se. −Se = seeds not containing selenium. Y axis : root length and stem length in mm. Control = agar without PEG. -0.6 = agar mixed with PEG to simulate a water potential = -0.6 Mpa ; -1.2 = agar mixed with PEG to simulate a water potential = -1.2 Mpa. Error bars = Std. Different letters and numer of stars = significant differences between values. Graph B X axis : + Se = + Se = seedlings containing Se. −Se = seedlings not containing selenium. Y axis : fresh biomass in mg. Error bars = Std. Different letters = significant differences between values.
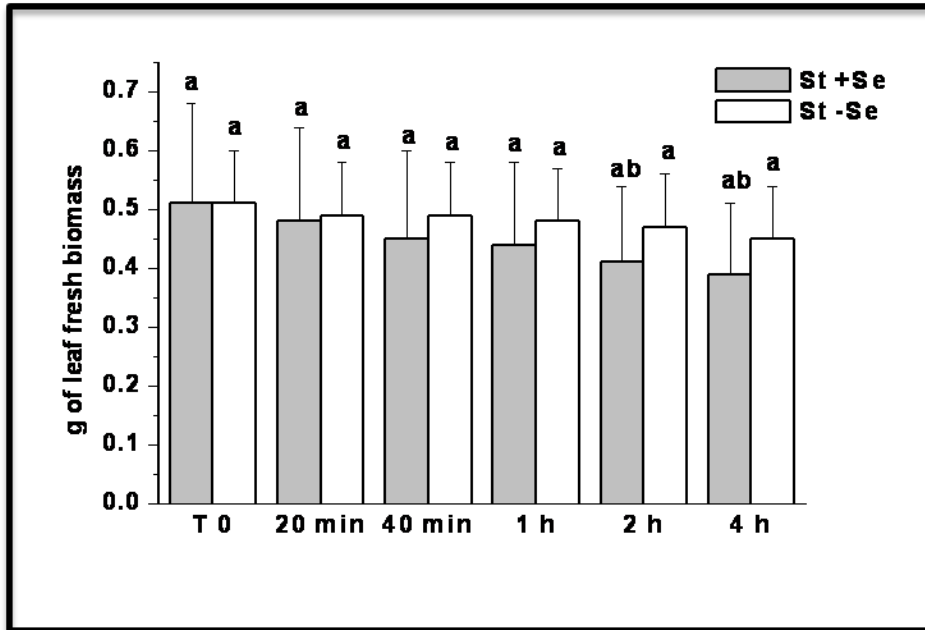
Figure 9 Water loss of Stanleya pinnata leaves measured across 4 h. X axis : time of wheight Y axis : fresh weight of Se amended leaves (grey bars) and not Se amended leaves (white bars). Error bars =Std. Different letters mean significant differences between values.

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Interaction of selenate and molybdate with the transport and assimilation of sulfate in *B. juncea* L. Czern.

INTRODUCTION

The uptake of heavy metals and metalloids by plants is regulated by many soil factors, which may include the presence of competitive ions in the rhizosphere (Hopper and Parker 1999). In particular, the accumulation of metal/loids belonging to the group VI elements, such as selenium and molybdenum, is frequently associated with the level of sulfur fertilization (Mikkelsen et al., 1990; Balík et al., 2006; Mackowiak and Amacher, 2008; Shinmachi et al., 2010; Stroud et al., 2010a,b).

In many regions around the world selenium (Se) concentration in soils is generally low (<1 ppm). Increased accumulation of Se in soils can derive from industrialization, use of fertilizers and irrigation water containing selenium (Bisbjerg, 1972). Soils with 0.5–100 ppm Se are defined as "seleniferous soils" (Dhillon and Dhillon, 2003) and some plant species growing on them are able to accumulate selenium up to 0.1-1.5% dry weight (Brown and Shrift, 1981; Freeman et al., 2010).

Selenium-accumulating plants may have potential as fortified food with enhanced nutritional quality (Zhu et al., 2009) or can be used to reclaim soils in which Se is present at hazardous levels (Pilon-Smits and LeDuc, 2009). On the other hand, plants grown on seleniferous soils often contain more than the maximum allowed level for animal dietary intake (40 to 70 μg/d) and may pose a risk to livestock (Fessler et al., 2003; Hartikainen, 2005) and wildlife (Hamilton, 2004) health.

Although Se is an essential element for animals, including humans (Rayman, 2000), its essentiality for higher plants has not been unequivocally established to date. Nevertheless, the numerous beneficial effects of Se to plants are recognized (Pilon-Smits et al., 2009).

Plants take up selenium mainly as selenate (SeO_4^{2-}) (Ellis and Salt, 2003; Sors et al., 2005). Owing its close chemical similarity to sulfate, selenate is readily transported over plasma membranes by the activity of sulfate permeases (Terry et al., 2000; Shibagaki et al. 2002; Yoshimoto et al. 2002; El- Kassis et al. 2007). Once absorbed by roots, selenate is delivered via the xylem to the shoot, where it accesses the reductive sulfate assimilation pathway. Selenate is reduced to sulfide and incorporated in analogues of the sulfur amino acids, selenocysteine (Se-Cys) and selenomethionine (Se-Met) (Terry et al., 2000; Pickering et al., 2003; Sors et al., 2005; Freeman et al., 2006). The non-specific insertion of such amino acids into proteins causes the disruption of their molecular structure and loss of function, and is considered the main reason of Se toxicity to plants (Terry et al., 2000).

Several transgenic approaches have been used to improve plant Se accumulation, tolerance and volatilization (Pilon-smits and LeDuc, 2009). They include overexpression of sulfate transporters and assimilation enzymes (Pilon-Smits et al., 1999), methylation of SeCys (LeDuc et al., 2004),

and conversion of SeCys to volatile forms of Se (Van Huysen et al., 2003) or to elemental Se (Pilon-Smits et al., 2002). It has been proposed that in addition to S assimilation, other metabolic pathways may be implied in a plant's natural capacity to accumulate and tolerate Se (White et al., 2004), as for instance those involved in ethylene and jasmonic acid biosynthesis (Tamaoki et al., 2008; Freeman et al., 2010). Furthermore, a Se binding protein, SBP1, has been recently found to enhance the tolerance of *A. thaliana* plants to selenate (Agalou et al. 2005; Hugouvieux et al., 2009), and a number of genes responsible for Se tolerance have been identified via transcriptome analysis (Van Hoewyk et al., 2008).

Unlike Se, molybdenum (Mo) is an indispensable trace element for plants, being incorporated into molybdopterin, an essential cofactor for enzymes involved in sulfite detoxification, purine catabolism, nitrate assimilation, and abscisic acid biosynthesis (Mendel and Hänsch 2002; Schwarz and Mendel 2006; Hänsch and Mendel, 2009; Schwarz et al., 2009; Kruse et al., 2010). The hexavalent form (molybdate) is the most common in agricultural soils (Kaiser et al 2005), and its bioavailability to plants and microorganisms tightly relies on the soil pH and organic matter, concentration of adsorbing oxides, and rate of water drainage (Reddy et al., 1997). In acidic soils, for instance, Mo tends to be unavailable to plants. This is why most Mo deficiencies occur on acid, rather than on neutral or alkaline soils (Kaiser et al., 2005).

The concentration of Mo in soils can increase as a result of industrial, mining and agricultural activities (Gupta, 1997), and excess Mo is known to reduce plant growth and thus productivity (Kevresan et al. 2001; Liu and Yang 2003; Nautiyal and Chatterjee 2004).

Although Mo transport and homeostasis have been well characterized in bacteria and some eukaryotes (Mendel and Häensch, 2002), in plants still relatively little is known about the mechanisms of Mo transport, distribution and accumulation inside cells, and only in recent years a high-affinity Mo transporter (MOT1) has been isolated and characterized in *A. thaliana* (Tomatsu et al., 2007). Although MOT1 belongs to the sulfate transporter superfamily, it seems not to work in sulfate transport (Tomatsu et al. 2007). MOT1 is localized to mitochondria and, in part, to plasma membranes and to vesicles (Tomatsu et al., 2007; Baxter et al., 2008). It is mainly expressed in roots, especially levels in endodermis and stele cells. However, its expression can also be detected in the shoot, and for this reason MOT1 is believed to function as a regulator of total plant Mo accumulation and homeostasis (Baxter et al., 2008; Tejada-Jiménez et al., 2009).

Plants have long been assumed to take up molybdate through sulfate transporters, as the two anions are chemical analogues and may compete for the binding site of the same transporters (Dudev and Lim 2004; Alhendawi et al 2005). Indeed, expression of the sulfate transporter SHST1 from *Stylosanthes hamata* in a *Saccharomyces cerevisiae* mutant defective in sulfate transport, YSD1, increased its capacity to take up molybdate when grown in the presence of low Mo concentrations (Fitzpatrick et al., 2008). While sulfate did not inhibit the transport of Mo through this transporter, molybdate reduced sulfate transport via SHST1. In addition to sulfate transporters, phosphate transporters have been suggested as potential transporters of molybdate across the plasma membrane (Heuwinkel et al., 1992).

Once inside cells, Mo can interfere with S metabolism being a substrate for the reaction catalyzed by the enzyme ATP sulfurylase (APS). The same is true for selenate, but in contrast to selenate, no stable Mo-containing products are formed in the reaction (Reuveny, 1977). Overexpression of APS in *B. juncea* led to increased Mo accumulation, but lower tolerance. The reduced tolerance to Mo was perhaps because APS couples ATP to Mo to form a complex that is unstable, resulting in a loss of ATP (Wangeline et al., 2004).

The relationship between Se and S, or between Mo and S, has practical significance for crop production as it allows the manipulation of Se and Mo accumulation and distribution in plant tissues through S application (MacLeod et al, 1997; Balìk et al., 2006; Shinmachi et al., 2010). The use of sulfur-containing fertilizers to correct soil S deficiency, for instance, may reduce Se accumulation in crops (Stroud et al., 2010a,b; Shinmachi et al., 2010) and cause Mo-deficiency (MacLeod et al., 1997). Furthermore, whether plants are used for Se and Mo phytoremediation purposes, high S content in soil might interfere with their capacity to remove inorganic forms of the two elements.

On account of this, the present research was intended to deeply study the interactions of S nutrition with Mo and Se in *B. juncea*, sulfur's lover species frequently employed in phytoremediation.

To this aim, plants were supplied with different combinations of S and Se, or S and Mo (*see Materials and Methods*). Experiments were carried out over a short time period (24 h) because selenate and molybdate were given to plants at a very high concentration (200 μM), the same as that of sulfate in the S-sufficient condition. Physiological and biochemical analyses were performed, including measurement of plant growth and quantification of elements (Mo, Se and S), sulfate and thiols (cysteine and glutathione) in plant tissues. In addition, the sulfate uptake capacity of plants was assayed, as well as the transcript levels of a putative low affinity sulfate transporter involved in sulfate xylem loading (*BjSultr2;1*), *SBP1* and *MOT1* in roots.

MATERIALS AND METHODS

Plant material

Seeds of *B. juncea* (L.) Czern. (Cv. PI 426314) were surface-sterilized by rinsing in 70% (v/v) ethanol for 30 – 60 s, then in 5% (v/v) sodium hypochlorite (NaClO) for 30 min while rocking on a platform, and washed in distilled water for 5 x10 min. The seeds were allowed to germinate and grow for 8 d in half-strength MS agar medium (Murashige and Skoog, 1962) inside a chamber with a 13 h light/11 h dark cycle, air temperature of 20/15 °C, relative humidity of 70/85% and at a photon flux density (PFD) of 280 mol $m^{-2}s^{-1}$. Subsequently, germinated seedlings were transferred and cultivated for 4 d to 3 L plastic beakers (density = 16 plants per beaker) containing a thoroughly aerated Hoagland modified nutrient solution with the following composition (μM): KH_2PO_4 40, $Ca(NO_3)_2$ 200, KNO_3 200, FeNaEDTA 10, B 4.6, Cl 1.1, Mn

0.9, Zn 0.09, Mo 0.01. Part of the plants was supplemented with 200 μM $MgSO_4$, and part was grown in the absence of S. In the latter case, $MgCl_2$ (200 μM) replaced $MgSO_4$. The nutrient solution in each beaker was renewed every two days.

Seedlings were then divided into 6 groups and grown for 24 h under the following conditions: with 200 μM sulfate (+S), without sulfate (-S), with 200 μM sulfate plus 200 μM selenate (+S+Se), no sulfate plus 200 μM selenate (-S+Se), with 200 μM sulfate plus 200 μM molybdate (+S+Mo), no sulfate plus 200 μM molybdate (-S+Mo). In the solutions lacking sulfur, $MgSO_4$ was replaced by 200 μM $MgCl_2$. Selenium and molybdenum were supplied in the form of natrium selenate (Na_2O_4Se) and ammonium molybdate (($NH_4)_6Mo_7O_{24}$), respectively.

B. juncea seedlings were harvested at different time points (0, 10 min, 1 h, 6 h and 24 h), carefully washed with distilled water to remove any Se or Mo bound to the root surface, and dried with blotting paper. Part of the plant material was immediately frozen with liquid nitrogen and kept at −80°C for further molecular and physiological analyses. For fresh weight measurements, ten plants per treatment were used and weighed separately. Plant tolerance to Se or Mo was expressed as relative fresh weight (also known as tolerance index) calculated as fresh weight observed in the presence of the metal/loid divided by fresh weight under the control condition (+S). The experiment was replicated three times.

Elemental analysis

Foliar and root tissues were dried for 48 h at 80° C and then digested in nitric acid as described by Zarcinas et al. (1987). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used as described by Fassel (1978) to determine each digest's elemental concentrations.

Sulfate content

Fresh foliar and root tissues (500 mg) were ground in liquid nitrogen and extracted with 10 ml of distilled water. The samples were incubated for 2 h in a heating block at 85°C. The extracts obtained were filtered and analysed by HPLC using a Dionex IonPac AS11 4 mm column, coupled to guard column AG 14. The column was eluted over a period of 18 min with 3.5 mM Na_2CO_3/1 mM $NaHCO_3$ in H_2O, at a flow rate of 0.9 ml/min and at 1400 PSI pressure. Sulfate content was expressed in mg kg^{-1} f. wt. and data were the means of five replicates per treatment with fifty plants in each replicate.

Sulfate uptake

Sulfate uptake experiments were carried out as described by Quaggiotti et al. (2003), using the radioisotope $^{35}S–(SO_4^{2-})$ furnished by Perkin Elmer Life Sciences (Boston, MA, USA). The sulfate uptake capacity was assayed in plants grown under the conditions +S, -S, +S+Se, -S+Se, +S+Mo, -S+Mo. Also, to compare the effects of Se and Mo to the ones induced by S, the uptake of sulfate was evaluated in +S and –S plants added with 200 mM sulfate (+S+S and -S+S, respectively).

Sulfate uptake rates were measured at times 10 min, 30 min, 1 h, 6 h, or 24 h after the addition of S, Se or Mo to sulfur-sufficient and sulfur-starved plants. Groups of eight plants per each treatment were transferred for 5 min to vessels containing a complete nutrient solution buffered with 15 mM Tris–MES, pH 5.6. Sulfate in the solutions (200 μM $MgSO_4$) was labelled with $^{35}SO_4^{2-}$ (5 MBq $mmol^{-1}$). The temperature of the solutions was maintained at 23°C and a lamp (OSRAM 400 W – HR) with a PFD of 280 mmol $m^{-2} s^{-1}$ provided an adequate light supply. After the uptake period the plants were removed and placed with their roots in ice–cold unlabelled solution for 2 min to remove sulfate that passively entered the apoplastic root space. Shoots and roots were harvested separately, blotted dry, weighed and digested for 24 hours with 10 ml of 0.1 N HCl at room temperature. Extract (1 ml) was added to 4 ml of liquid scintillation fluid (Hionic-Fluor 6013319, Packard Bioscience, The Netherlands), and the radioactivity was determined using a liquid scintillation counter (Packard Instruments, Downers grove, IL). The experiment was replicated three times.

Semi-quantitative RT-PCR

Extraction of total RNA from frozen roots was performed using the Nucleon Phytopure kit (Amersham-Pharmacia, UK). The RNA was analyzed spectrophotometrically (A280/A260), and electrophoresis (1% agarose gel) was used to verify intact nucleic acid. RNA was then treated with 10 U of Dnase RQ1 (Promega, Milano, Italy) per 50 μg RNA at 37°C for 30 min. Five μg of RNA from each treatment were used in the reverse transcriptase reaction, using 200 U of ImProm-II™ Reverse Transcriptase (Promega, Milano, Italy) and oligodT as primers in 20 μl reaction mixtures. Mixtures were incubated at 37°C for 60 min, 70°C for 5 min, and 4°C for 5 min to stop the RT reaction. PCR experiments were performed using primers designed on conserved sequences of *PP2A* (phosphatase 2A), *MOT1* and *SBP1* genes from *A. thaliana*, and primers specific for *BjSultr2;1* from *B. juncea* (Table 1). For all PCR reactions, 1 μl of the cDNA was used in a 20 μl reaction mixture with 2 μl of a 0.025 U/μl Taq polymerase (Amersham-Pharmacia-Biotech, Piscataway, NJ, USA). Between 14 and 30 cycles were tested to determine the optimal number of cycles, corresponding to the exponential phase in the amplification for each gene. The annealing temperature varied from 54°C to 61°C depending on the gene transcript. The constitutively expressed *PP2A* gene was used as internal control to normalize the obtained gene expression results. PCR products were separated by electrophoresis in a 2% agarose gel, stained with SYBR® Safe (Invitrogen, San Giuliano Milanese, MI, Italy) and analyzed with the ImageJ program (ImageJ 1.23 J, Wayne Rasband, National Institute of Health). To confirm the expression analysis results, PCR reactions were carried out on cDNAs obtained from two different RNA extractions from two independent experiments, and were repeated at least 4 times for each cDNA.

DNA was eluted from the agarose gel with the QIAquick Gel Extraction-Kit Protocol (QIAGEN, Valencia, CA, USA) kit. Gene sequencing was carried out at the BMC Genomics (Padova, Italy), using the ABIPRISM original Rhodamine Terminator kit (PE Biosystems, Branchburg, NJ,

USA) and specific and universal primers. Gene sequences were further compared with Blastx and Blastn (NCBI, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) (Table 2).

Statistical Analysis

Analysis of variance (ANOVA) was performed followed by pairwise post-hoc analyses to determine which means differed significantly. Statistically significant differences ($P<0.05$) are reported in the text and shown in the figures.

RESULTS

Plant growth

The effect of selenate and molybdate on plant growth (tolerance index) was evaluated in terms of fresh weight production relative to the control treatment (+S = 100%) at the 24 h time point (Table 2). The fresh weight of –S plants slightly decreased compared to the control, and was more reduced in plants supplied with Se or Mo, especially in the absence of S (-14.6% and -21.3% for –S+Se and –S+Mo, respectively). The root-to-shoot ratio was unchanged over the 24 h in +S and -S plants, but diminished in plants supplied with Se or Mo (Table 3).

Accumulation of Se, Mo, S and sulfate in plant tissues

Selenium and Mo accumulated in plants in a similar way, as a function of time and S availability. Both Se and Mo accumulated to a greater extent in –S plants, with the maximum values measured after 24 h (Figure 1). While both elements were offered at the same concentration, Mo accumulated to a higher level than Se. Specifically, after 24 h the Mo level was roughly 5- and 10-fold the concentration of Se in leaves and roots, respectively.

The S concentration in plants grown in the presence of S was almost 2-fold higher than in S-starved plants (Figure 2). Sulfur levels decreased only in +S+Se (-25%) and +S+Mo (-31%) plants after 24 h. At this time, the ratio S/Se was about 76 in leaves and 19 in roots of +S plants, while in − S plants it was 22 in leaves and 12 in roots. In leaves of +S+Mo and –S+Mo plants, the ratio S/Mo was 14 and 7, respectively. In roots the S/Mo ratio was lower, being 4 in +S plants and 0.9 in –S plants.

The sulfate pool was steady in the +S control, whereas it slightly decreased over 24 h in leaves of -S plants (Figure 3). When selenate was added to the nutrient solution, the sulfate content was reduced in leaves of +S plants after 6 h (Fig 3A), while it increased in roots after 24 h (Figure 3 C). Conversely, in –S+Se plants sulfate accumulated in leaves (Figure 3 A) and concomitantly diminished in roots (Figure 3 C). In plants supplied with molybdate, the amount of sulfate

diminished in both roots and leaves of S-sufficient and S-starved plants already after 1 h (Figures 3 B,D).

Effects of Se and Mo on sulfate uptake

In order to evaluate whether selenate or molybdate at 200 μM concentration could alter sulfate uptake activity in *B. juncea*, the influx of sulfate was measured using $^{35}SO_4^{2-}$ as a tracer (Figure 4). In +S and -S plants the rates of sulfate uptake were steady over 24 h. Specifically, plants of the -S condition exhibited sulfate uptake rates 4-fold higher than S-replete plants (3079 ± 95 vs. 754 ± 66 nmol SO_4^{2-} g^{-1} h^{-1} f.wt.).

Treating S-sufficient plants either with Se or Mo led to a considerable decrease of the sulfate uptake capacity after 24 h (Figure 4 A). The reduction of sulfate uptake was more pronounced in +S+Se plants (- 55%) compared to +S+Mo plants (- 30%). In +S+S plants sulfate influx was almost linearly reduced with increasing time of exposure, and after 24 h the reduction was approximately 75% of the +S control (Figure 4 A).

The supply of Se or Mo to –S plants resulted in a substantial decrease in sulfate uptake rate after 10 min, especially in –S+Mo plants (- 47%). At the 24 h time point the sulfate uptake rate was drastically repressed (~ - 95%) in both –S+Se and –S+Mo plants (Figure 4 B).

Given that sulfate uptake is known to be reduced when plants grow in the presence of S, the sulfate influx was also measured in S-deprived plants to which sulfate was restored (-S+S) (Figure 4 B). In this way it was possible to compare the effects of sulfate addition on sulfate uptake with the effects of the analogues selenate and molybdate. As observed for –S+Se and –S+Mo plants, the rates of sulfate uptake were reduced after 10 min of sulfate restoration to –S plants (- 40%), and the maximum repression was recorded at 24 h (- 87%).

Effect of selenate, molybdate and sulfate on the transcript levels of BjSultr2;1, SBP1 and MOT1.

The effects of selenate and molybdate on sulfate transport in *B. juncea* were also evaluated at the transcriptional level. The transcript level of the low affinity sulfate transporter *BjSultr2;1* was higher in –S plants compared to the +S control (Figures 5 A, B). In –S plants the *BjSultr2;1* transcript abundance significantly decreased after 6 h of Se exposure, while in +S+Se plants a reduction was observed after 24 h (Figure 5 A). When plants were grown in the presence of molybdate, the transcript level of *BjSultr2;1* did not vary in S-sufficient plants (+S+Mo), but was strongly repressed in –S+Mo plants after 6 h (Figure 5 B).

The transcript level of the selenium binding protein, *SBP1*, was higher in –S plants than in the +S control (Figures 5 A, B). Treating +S plants with selenate promptly enhanced *SBP1* expression, whereas in –S plants the *SBP1* transcript level was reduced after 6 h (Figure 5 A). Up-regulation of *SBP1* by molybdate was evident in +S and –S plants only at the 24 h time point (Figure 5 B).

With respect to the molybdate transporter *MOT1*, transcript abundance was comparable in +S and –S plants (Fig 5 A, B). After 10 min of molybdate treatment, *MOT1* transcript was induced to a similar extent in +S+Mo and –S+Mo plants, and remained unchanged within 24 h. On the contrary, treating plants with Se had no effect on *MOT1* gene expression.

The effect of sulfate restoration to S-deprived plants (-S+S) on *BjSultr2;1*, *SBP1* and *MOT1* gene expression was further evaluated (Figure 5 C). The transcript level of *BjSultr2;1* and *SBP1* was appreciably reduced by sulfate resupply, while the *MOT1* transcript level was unchanged.

In +S+S plants, the transcript levels of *BjSultr2;1* and *SBP1* were weakly down-regulated after 24 h of extra S addition, and *MOT1* transcript was unchanged (data not shown).

Effects of Se and Mo on S-containing compounds

Selenium is known to interfere with S metabolism and, like Mo, may influence the synthesis of non-protein thiols involved in plant responses to metal stress. Therefore, the effects of selenate and molybdate on cysteine (Cys) and glutathione (GSH) contents were evaluated. In +S and -S plants the levels of Cys (Table 4) and GSH (Table 5) did not vary over the period of study. The supply of selenate to S-sufficient plants significantly lowered the Cys level in roots after 10 min, while 1 h of Mo exposure reduced Cys in both foliar and root tissues. In +S+Se plants GSH levels decreased in roots after 1 h and in leaves after 6 h. When +S plants were grown in the presence of Mo, the reduction of GSH was observed already after 10 min in roots and only after 24 h in leaves of +S+Mo.

In S-deficient plants treated with Se, the Cys concentration increased in roots (after 6 h) and in leaves (after 24 h). In –S+Mo plants Cys accumulated more after 24 h of treatment in roots. The synthesis of GSH was enhanced in roots and leaves of –S+Se plants after 6 h. In –S+Mo plants, increased levels of GSH were observed at 1 h and 6 h after Mo supply in leaves, while no variations was recorded in roots.

DISCUSSION

Accumulation of high levels of molybdenum and selenium in plant tissues may be desirable for phytoremediation purposes. However, the presence of sulfur in soil may significantly influence the distribution and accumulation of molybdenum and selenium in plants (Balìk et al., 2006;; Shinmachi et al., 2010).

To provide new insights for the efficient use of plants in Se or Mo phytoremediation, the present research was aimed at studying the interaction of selenate and molybdate with the transport and

assimilation of sulfate in *B. juncea*. Selenate and molybdate were given to plants at high concentration (200 μM), which was the same of sulfate in the S-sufficient condition, to compare the effects of Se and Mo with the ones of sulfate on sulfur assimilation. The use of equimolar concentrations of Se, Mo and S was also helpful to study the effects of sulfur on the accumulation of Se and Mo in plant tissues.

The exposure of plants to 200 μM Se or Mo concentration for 24 h caused retardation of plant growth. The toxic effect of Se or Mo was more pronounced in S-deficient plants, although it was significantly different from the +S condition only in plants treated with molybdate. The presence of Se or Mo affected more root growth, as proved by the decrease of the root/shoot ratio in plants treated either with Se or Mo. This is in accordance with Shibagaki et al. (2002) and El-Kassis et al. (2007), who reported that root growth is the potential target of Se toxicity, and with Kevresan et al. (2001) who observed a 50% root growth reduction in pea plants treated with Mo.

From the literature it is known that [S]/[Se] ratio in plants is more important than the Se content alone for determining Se toxicity (White et al., 2004; El-Kassis et al., 2007; Barberon et al., 2008). Plants that manifest toxicity symptoms in response to Se application usually have a low [S]/[Se] ratio (White et al., 2004). In contrast, in our study the level of Se toxicity in +S and − S plants was similar after 24 h, as no differences were measured in the tolerance index, although the ratio S/Se was much lower in sulfur deficiency. It seems that the Se content rather than the S/Se ratio was mainly responsible for toxicity, since a similar amount of Se was recorded in roots of S-sufficient and S-starved plants.

The importance of [S]/[Mo] ratio as an indicator of Mo toxicity in plants has not been proven yet. However, Mo toxicity was found to decrease in different plant species when S was added to soil, as a result of reduced Mo uptake (McGrath et al., 2010). The ratio S/Mo resulted lower in –S plants, as in the case of Se. However, the toxicity effect was more pronounced in –S plants than in +S plants, as confirmed by the significant reduction of tolerance, and could be related to the higher amount of Mo measured in the roots.

Selenate and molybdate are known to influence the assimilation of sulfate in plants by interfering with the S transport system (White et al., 2004; Fitzpatrick et al., 2008).

The reduction of the sulfate uptake capacity in S-sufficient *Brassica* plants exposed to selenate or molybdate appeared after 24 h of treatment. On the contrary, the addition of 200 μM sulfate (+S+S plants) reduced the rates of sulfate uptake already after 30 min. Sulfur has been previously reported to decrease the sulfate uptake capacity when measured at S concentration lower than the one at which plants were cultivated (Koralewka et al., 2007). The same effect was observed in *B. juncea* plants after short-term exposure to the sulfate analogous chromate (Malagoli, unpublished results).

Our findings indicated that molybdate and selenate do not act as sulfate or chromate in down-regulating sulfate uptake in sulfur-sufficient plants. However, the observed Se and Mo accumulation in the first 6 h of treatment indicate that Se and Mo are taken up by plants, likely through transport systems different from the sulfate one. For instance, with respect to Mo, the up-regulation of the *MOT1* transporter-coding gene was observed in *B.juncea* plants after short

exposure to molybdate. For Se, specific root transporters have not been identified yet, although their existence has been hypothesized in Se accumulators (Sors et al., 2005).

After 24 h of treatment, the sulfate uptake and the *BjSultr2.1* transcript level were remarkably reduced in +S+Se plants, whereas in +S+Mo only the sulfate uptake was partially reduced and no variation in *BjSultr2.1* transcript accumulation was observed. The stronger effect of Se on S transport may ascribe to the replacement of S by Se in the sulfur metabolic pathways leading to the disruption of structure and function of proteins (Pilon-Smits and LeDuc, 2009).

In -S plants sulfate uptake is repressed similarly by the three anions already after 10 min. However, the regulation of sulfate uptake appears to be different in plants treated with Se or Mo compared to –S+S plants. Indeed, the repression of *BjSultr2.1* transcript accumulation is consistent with the sulfate uptake reduction in -S+S plants, while in –S+Mo and –S+Se plants the *BjSultr2.1* transcript level decreased only after 6 h. It is well known that sulfate repletion down-regulates sulfate transporters at the transcriptional level (Maruyama-Nakashita et al., 2003; Rouached et al., 2008; Koralewska et al., 2009). To our knowledge, a similar effect on regulating sulfate transporter genes by Se or Mo has not been reported, It may be assumed that the immediate reduction in sulfate uptake in -S+Se and -S+Mo plants could be due to selenate or molybdate binding to the active sites of the root plasma membrane sulfate transporters, which was favoured by the lack of competition between sulfate and the analogues oxianions for the transport across plasmalemma. As a consequence, the transport of molybdate and selenate through the sulfate carriers could occur and may justify the higher Se and Mo accumulation in S-starved plants than in +S plants, as reported in previous studies (Pezzarossa et al. 1999, Alhendawi et al. 2005, Balìk et al. 2006, Fitzpatrick et al. 2008). In addition, Shimanchi et al (2010) showed that enhanced expression of sulfate transporters in roots of wheat plants grown at low S-fertilization caused a remarkable accumulation of Se and Mo.

The observation that *MOT1* expression did not vary in relation to S availability indicate that this transporter is not responsible for the higher accumulation of Mo in S-starved plants and supports the evidence that molybdate can be taken up by other transporters, including the sulfate ones (Fitzpatrick et al., 2008). *MOT1* only partially contributes to Mo accumulation and can be considered a transporter specific for molybdate as its transcript did not change in +Se plants.

Selenium and molybdenum influenced not only sulfate transport but also sulfur assimilation. In +S plants, Se and Mo treatments affected the synthesis of S-compounds (Cys and GSH) likely due to the competition of selenate and molybdate with sulfate for their access to the sulfur metabolic pathway. This competition is known to cause the disruption of sulfur metabolism and the reduced synthesis of sulfur-containing compounds, such as Cys and glutathione (El-Kassis et al., 2007). In S-starved plants supplied with either Se or Mo the level of Cys and GSH increased in most respects, although the S content did not change substantially. It may be hypothesized that the S pool was directed towards the synthesis of S-containing stress molecules to cope the toxicity effects caused by high Se and Mo concentration, as shown for other metals (Heiss et al., 2001).

B. juncea plants could also synthesize the selenium binding protein 1 at high level in response to Se and Mo toxocity. The expression analysis of *SBP1*, confirmed that its transcript is more accumulated under S-starvation (Hugouvieux et al, 2009). However, treating –S plants with selenate decreased the level of *SBP1* transcript since 6 h treatment, likely because of higher GSH accumulation. Indeed, glutathione has been proved to act as a negative regulator of *SBP1* gene expression when exogenously supplied to plants (Hugouvieux et al, 2009). The addition of selenate to +S plants promptly induced the accumulation of *SBP1* transcript, indicating that *in B. juncea* the protein is involved in Se tolerance. A similar result was previously reported in *A. thaliana* (Hugouvieux et al, 2009).

SBP1 could be also involved in Mo tolerance since *SBP1* transcript significantly increased in both +S+Mo and –S+Mo plants. However, as the increase was observed only after 24 h treatment, it may be hypothesized that very high concentration of Mo in plants can induce *SBP1* gene expression.

In conclusion, the present study provides evidence that selenate and molybdate interfere with sulfate transport, mainly when plants grown under S-deficiency. The way through which Se and Mo can affect S uptake and sulfate transporter gene expression is different form that one reported for sulfur, indicating that selenate, molybdate and sulfate trigger different signals for the regulation of S transport. Given the high values of Se and Mo accumulation in plants and the trend of sulfate uptake in –S and +S plants, it may be evinced that Se and Mo could be taken up by plants by more than one transport system.

Se and Mo influenced the S-assimilatory pathway by changing the content of S-metabolites and induced the transcription of proteins involved in metal tolerance and regulated by S availability to plants.

FIGURES
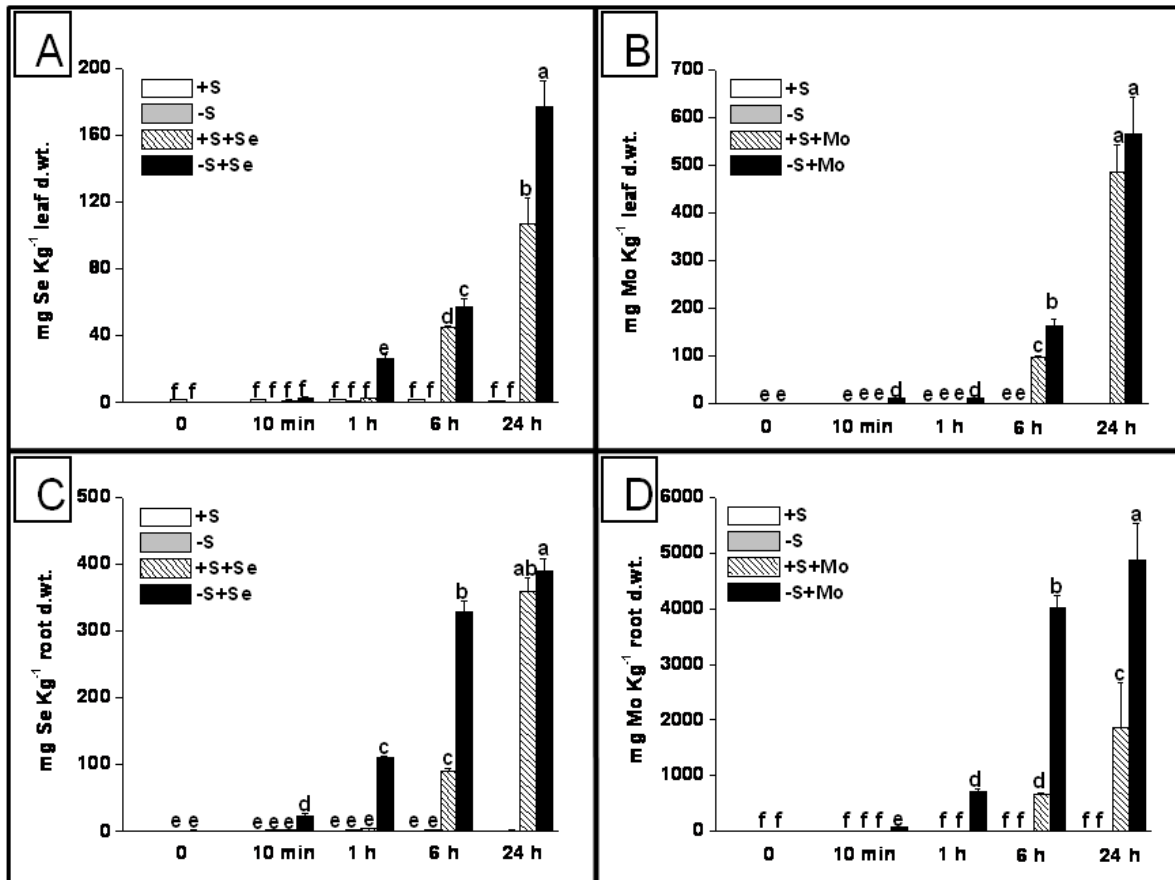
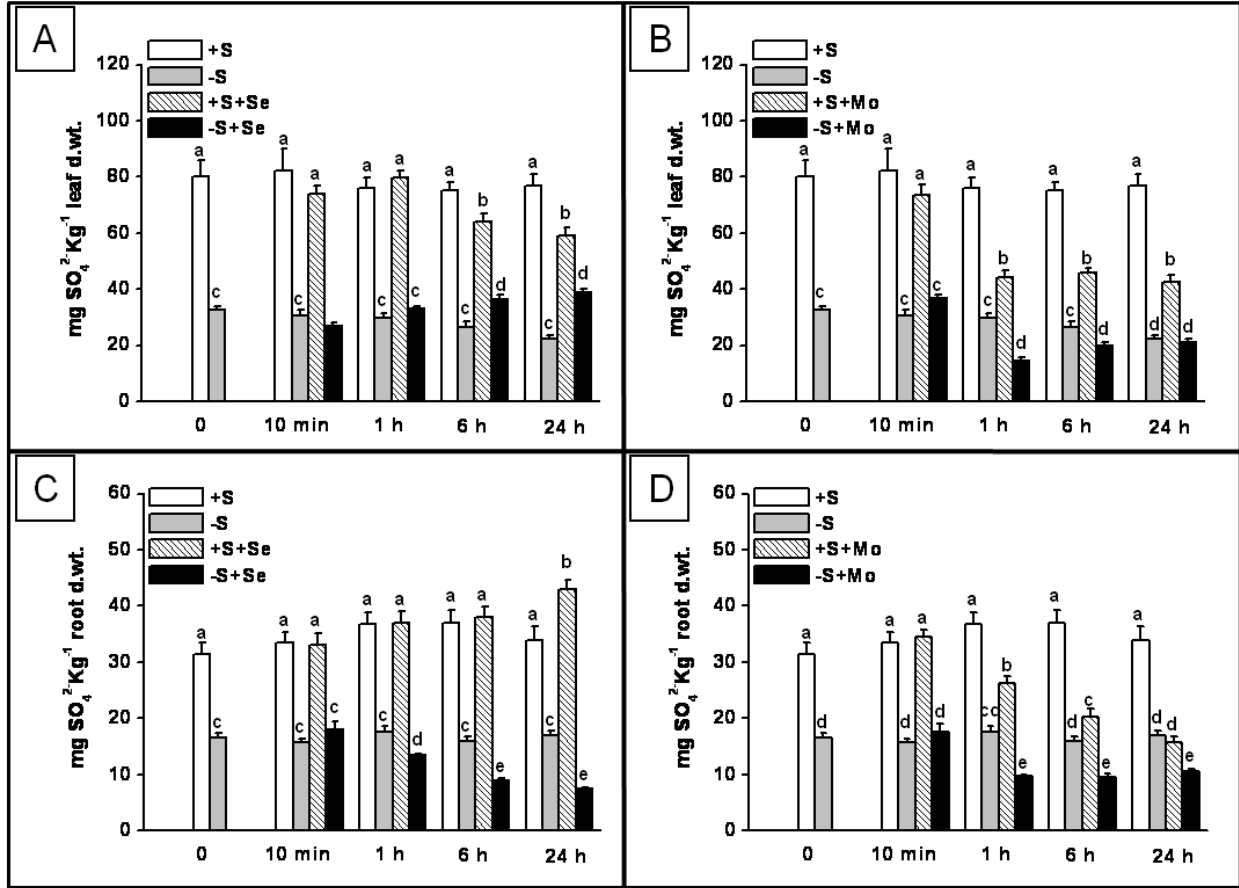**Figure 1.** Concentration of selenium (Se) and molybdenum (Mo) in leaves (A and B, respectively) and roots (C and D, respectively) of *B. juncea* plants grown in the presence of different combinations of S, Se and Mo. The measurement of Se and Mo was realized in three replicates ($\pm$ *std*) with fifty plants in each per treatment. Letters above bars indicate significant differences ($P < 0.05$).
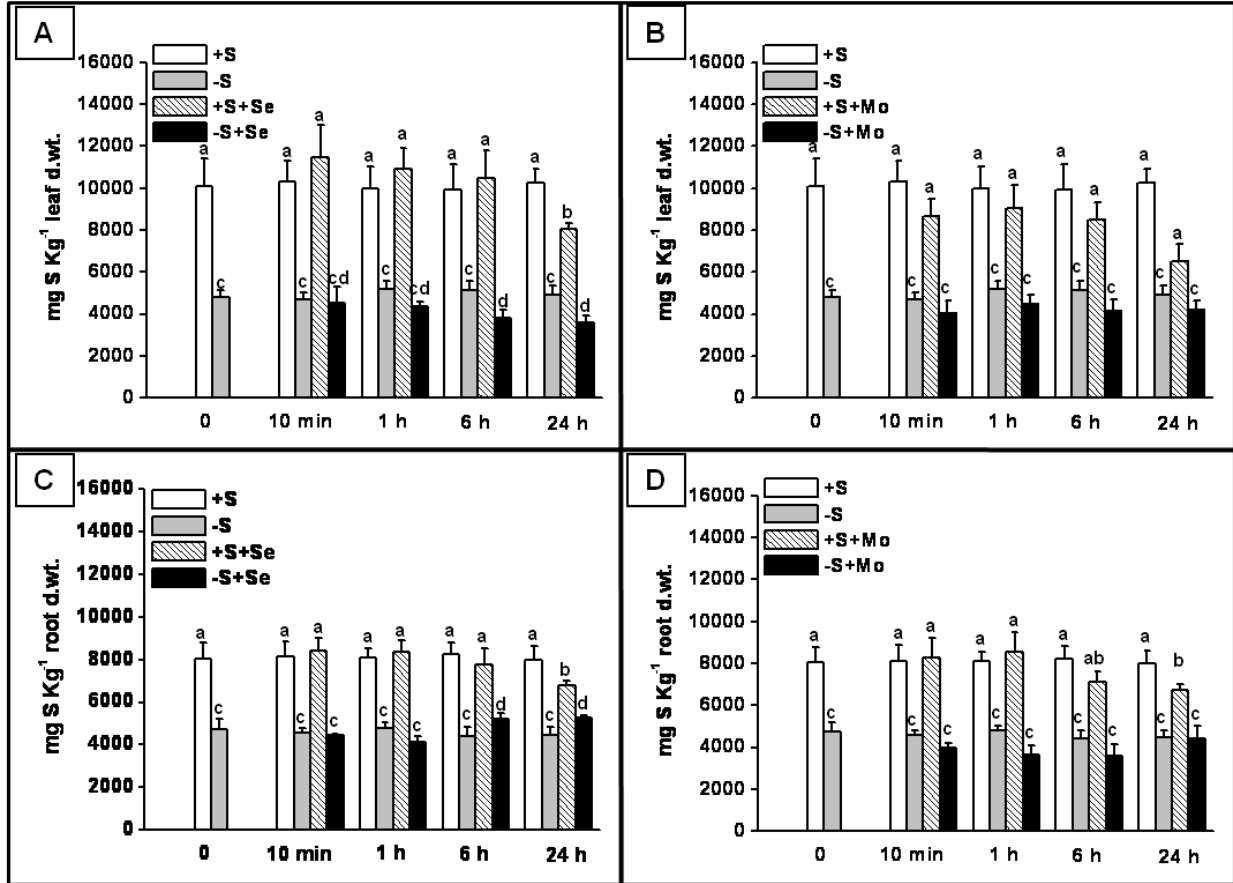
Figure 2. Concentration of sulfur (S) in leaves and roots of *B. juncea* plants grown in the presence of different combinations of S and Se (**A** and **C**, respectively) or S and Mo (**B** and **D**, respectively). The measurement of S was realized in three replicates ($\pm$ *std*) with fifty plants in each per treatment. Letters above bars indicate significant differences ($P < 0.05$).

Figure 3. Concentration of sulfate (SO_4^{2-}) in leaves and roots of *B. juncea* plants grown in the presence of different combinations of S and Se (**A** and **C**, respectively) or S and Mo (**B** and **D**, respectively). The measurement of S was realized in three replicates ($\pm$ *std*) with fifty plants in each per treatment. Letters above bars indicate significant differences ($P < 0.05$).
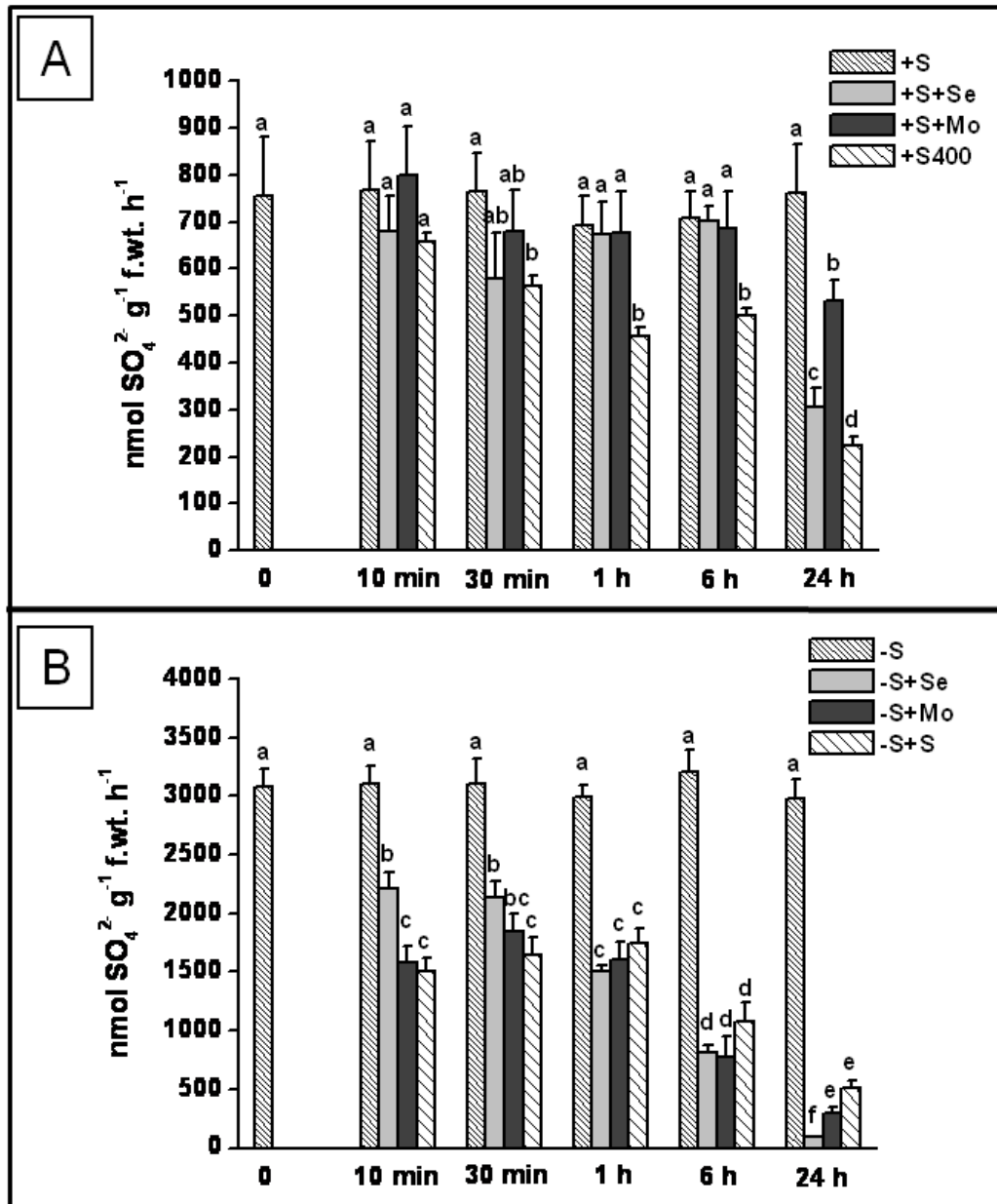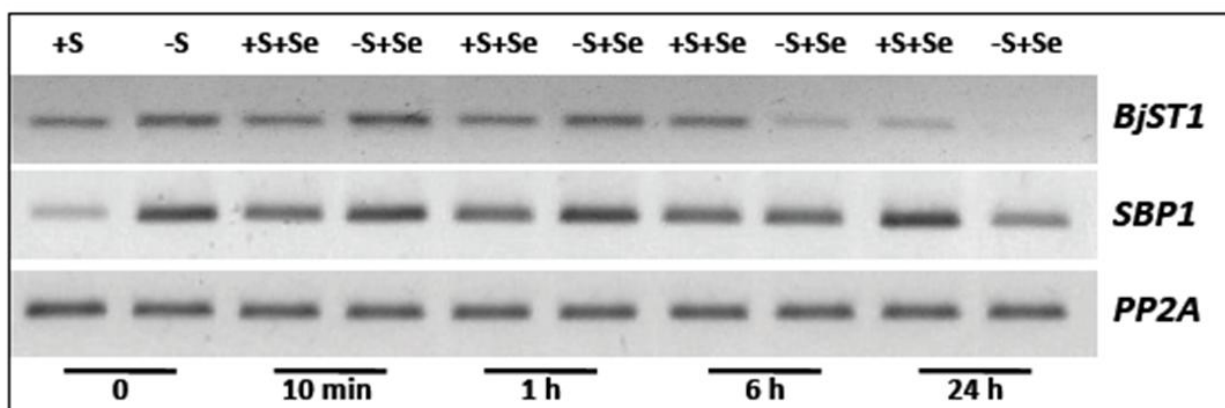
Figure 4. Sulfate uptake in *B. juncea* plants grown for 4 days with 200 mM SO_4^2 and then added with selenium (+S+Se), or molybdenum (+S+Mo), or 200 mM SO_4^2 (+S+S) for varying times (0 - 24 h) (**A**). Sulfate uptake in *B. juncea* plants grown in minus sulfur (-S) for 4 days and then treated with selenium (-S+Se), or molybdenum (-S+Mo), or replete with S (-S+S) for varying times (0 - 24 h) (**B**). Sulfate uptake was evaluated by measuring the rate of $^{35}SO_4^{2-}$ absorption over a 10 min pulse period. Data are the means of three replicates ($\pm$ *std*) with 8 plants in each per treatment. Letters above bars indicate significant differences ($P < 0.05$).

Figure 5. Relative gene expression of root transcript accumulation of sulfate low affinity sulfate transporter, *BjSultr2;1*, selenium binding protein, *SBP1*, and molybdenum transporter, MOT1in *B. juncea* plants grown in the presence of different combination of S and Se (A), or of S and Mo (B), or grown under sulfur starvation (-S) and further given with sulfur (-S+S) (C). Quantification of *BjSultr2;1*, SBP1, MOT1 was performed using the semi-quantitative PCR method in the linear range. Total RNA was reverse transcribed and PCR amplified at different cycles using specific primers. The constitutively expressed phosphatase 2A (*PP2A*) gene was used as an internal control. Analyses were repeated at least four times for each cDNAs obtained from two different RNA extractions.

TABLES

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
PP2A	TGCTATTCAGCACATTCTCCCTTGCG	GCGCCAAGCTTATCGTCAAAGAAGC
BjSultr2;1	TACTTCACAAATTGAAACGAG	GGACAATATATCATTTATTCTG
SBP1	TGCCGGGGCTAATCACCGACT	CCGTCTTCTCCAACCGCCTTAACA
MOT1	TCTGTTGTGGCTGTGTGTAAGCTG	ACCCACCACTCCTCCCACCAA

Table 1. Oligonucleotide primer name and sequence for primers of *B. juncea* used in RT-PCR reactions. *PP2A:* protein phosphatase 2A; *BjSultr2;1*: sulfate transporter; *SBP1*: selenium binding protein; *MOT1*: molybdenum transporter.

	Ratio root/shoot		Tolerance index (%)
	0	24 h	24 h
+S	0.432 ± 0.075 a	0.444 ± 0.092 a	100.00 ± 1.12 a
-S	0.420 ± 0.025 a	0.406 ± 0.036 a	94.05 ± 1.98 b
+S+Se	—	0.290 ± 0.031 b	88.43 ± 1.77 c
-S+Se	—	0.262 ± 0.015 b	85.15 ± 1.95 c
+S+Mo	—	0.269 ± 0.013 b	87.72 ± 2.54 c
-S+Mo	—	0.224 ± 0.016 c	78.20 ± 2.23 d

Table 2. Effect of different combinations of S, Se and Mo on root/shoot ratio and tolerance index (TI) of *B. juncea* plants within 24 h. The ratio and TI were calculated on the basis of the fresh weight measurement. For TI, the +S control was posed = 100%. Different letters indicate significant differences among treatments ($P < 0.05$, ± *std*).

	Cys ($\mu mol\ Kg^{-1}$ f.wt.)					
	Leaves					
Time	+S	-S	+S+Se	-S+Se	+S+Mo	-S+Mo
0	25.48 ± 4.11a	11.00 ± 1.78b	—	—	—	—
10 min	26.87 ± 3.02a	12.29 ± 1.32b	24.78 ± 2.26a	15.32 ± 1.96b	25.06 ± 1.21a	11.32 ± 1.34b
1 h	27.60 ± 2.32a	15.21 ± 1.24b	23.51 ± 4.55a	13.03 ± 2.14b	17.39 ± 3.12b	11.59 ± 1.67b
6 h	21.09 ± 3.31a	14.00 ± 2.23b	22.92 ± 6.35a	15.24 ± 2.24b	18.59 ± 2.24b	16.15 ± 3.43b
24 h	27.09 ± 4.21a	12.46 ± 1.13b	23.61 ± 1.32a	25.80 ± 2.21a	11.81 ± 4.11b	13.46 ± 2.78b
	Roots					
0	31.00 ± 5.23c	19.00 ± 1.22d	—	—	—	—
10 min	30.20 ± 3.22c	16.23 ± 2.54d	18.25 ± 2.16d	19.32 ± 3.24d	30.1 ± 1.44c	20.99 ± 1.11d
1 h	28.31 ± 3.14c	17.06 ± 1.83d	18.07 ± 1.45d	16.95 ± 2.63d	15.5 ± 2.32d	14.59 ± 2.31d
6 h	27.90 ± 1.78c	16.41 ± 2.11d	18.62 ± 5.67d	29.30 ± 4.35c	15.8 ± 4.11d	20.05 ± 3.25d
24 h	30.05 ± 2.21c	15.70 ± 1.18d	18.71 ± 4.89d	43.81 ± 4.25b	16.0 ± 2.38d	63.02 ± 4.76a

Table 3. Effect of different combinations of S, Se and Mo on cysteine (Cys) content in *B. juncea* leaves and roots. The measurement of Cys was realized in three replicates (± *std*) with three plants in each per treatment. Values marked with different letters for leaves or roots are significantly different ($P < 0.05$).

	GSH (μmol Kg^{-1} $f.wt.$)					
			Leaves			
Time	+S	-S	+S+Se	-S+Se	+S+Mo	-S+Mo
0	174.42 ± 27.65a	74.00 ± 8.44c	—	—	—	—
10 min	167.53 ± 23.33a	76.01 ± 8.72c	177.98 ± 3.02a	78.67 ± 4.66c	179.89 ± 1.21a	86.01 ± 14.63c
1 h	164.93 ± 31.78a	76.91 ± 7.46c	191.00 ± 2.32a	90.09 ± 13.51c	185.03 ± 3.12a	114.05 ± 24.82b
6 h	177.96 ± 18.91a	73.37 ± 7.54c	104.72 ± 3.31b	102.22 ± 14.89b	167.35 ± 2.24a	106.84 ± 3.56b
24 h	156.44 ± 14.80a	65.28 ± 8.19c	71.55 ± 4.21c	141.16 ± 33.32ab	100.43 ± 4.11b	88.31 ± 22.62bc
			Roots			
0	175.69 ± 21.98a	91.14 ± 4.31c	—	—	—	—
10 min	204.70 ± 22.12a	98.91 ± 3.66c	201.10 ± 10.12a	98.00 ± 5.65c	120.47 ± 18.88b	83.53 ± 6.44c
1 h	220.21 ± 14.97a	108.51 ± 6.76bc	82.62 ± 11.34c	70.72 ± 13.47cd	81.44 ± 7.62c	74.49 ± 13.08c
6 h	200.84 ± 15.11a	96.33 ± 4.81c	72.10 ± 1.38d	129.05 ± 12.38b	93.15 ± 21.09bc	79.55 ± 14.68c
24 h	208.08 ± 23.23a	93.25 ± 6.44c	78.03 ± 5.15cd	128.04 ± 16.43b	106.73 ± 15.12bc	105.34 ± 13.42bc

Table 4. Effect of different combinations of S, Se and Mo on total glutathione (GSH) content in *B. juncea* leaves and roots. The measurement of GSH was realized in three replicates (± *std*) with three plants in each per treatment. Values marked with different letters for leaves or roots are significantly different ($P < 0.05$).

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COPPER HOMEOSTASIS IN Populus trichocarpa

INTRODUCTION

Role of Copper in plants.

In plants copper plays critical roles in photosynthetic and respiratory electron transport, oxidative stress protection, cell wall metabolism, and ethylene perception (Marschner, H. 1995). Copper deficiency results in changes in root, stem and leaf architecture and visible chlorosis (Marschner, H. 1995). At the cellular level copper deficiency results in reduced photosynthetic
electron transport (Henriques F. S. 1989, Shikanai T. 2003), a reduction of plastoquinone and pigment synthesis (Baro´n, M 1992), and a disintegration of the thylakoid membrane (Henriques, F. S. 1989, Baszynski, T 1978). On the other hand, when copper is present in excess, plants develop toxicity symptoms such as an inhibition of root elongation (Murphy, A., and Taiz, L. 1995). Toxicity symptoms in the shoot include decreased photosystem II function and an inhibition of chlorophyll synthesis that is accompanied by a reduction of the thylakoid membrane surface and generation of excess reactive oxygen species in the chloroplast (Pa¨tsikka¨et al., 2002).

Copper proteins

 The copper protein plastocyanin (PC) is one of the most abundant proteins in the thylakoid lumen (Kieselbach, T et al. 1998, Schubert, M et al. 2002) and in plants is essential for electron transfer between the cytochrome *b6f* complex and photosystem I. A class of abundant intracellular copper proteins is formed by the Cu,Zn superoxide dismutase (SOD) enzymes, which function to catalyze the dismutation of superoxide radicals (O_2 .) into H_2O_2 (Asada, K. (1999)). Three genes encode for Cu,Zn-SOD in the *Arabidopsis* genome (Kliebenstein, D. J. et al., 1998); CSD1 is active in the cytosol, CSD2 is active in the chloroplast stroma, and CSD3 is active in the peroxisome. CSD1 and CSD2 are the major isoforms in green tissue.
Much of the machinery involved in cellular copper uptake and intracellular distribution in plant cells has now been described. Particularly, the delivery of copper to the ethylene receptors and the photosynthetic machinery is well characterized (Pilon, M. et al., 2006; Puig, S. et al., 2007). Copper enters plant cells via the Cu- transporters and is subsequently delivered to plastocyanin in the thylakoid lumen and to CSD2 in the stroma by a machinery that includes two chloroplast-localized coppertransporting P-type ATPases and a metallo-chaperone for Cu,Zn-SOD (Pilon, M. et al 2006). In chloroplasts, another SOD called FSD1 utilizes iron as the cofactor. Copper availability was found to regulate the expression of CCS, CSD1, CSD2, and FSD1 in *Arabidopsis* and other dicot plant species (Abdel-Ghany et al. 2005 ; Abdel-Ghany et al. 2005;

Cohu, C. M et al., 2007). When copper levels are sufficient, the Cu,Zn-SOD proteins are expressed, and FSD1 expression is shut-off. Conversely, when copper is limiting, CSD1 and CSD2 expression is down-regulated, and FSD1 becomes abundant. The down-regulation of CSD1 and CSD2 under copper limitation involves a microRNA, *miR398*, which is itself up-regulated under copper limitation (Yamasaki, H. et al., 2007).

miRNA

MicroRNAs (miRNAs) are a class of gene products of ;21 nucleotides in length that are derived from primary miRNAs transcribed from miRNA loci (Lee et al., 2002). In plants, the primary miRNAs are cleaved by Dicer-like 1 (DCL1) (Xie et al.,2004) and possibly other proteins in the nucleus to produce an ;60- to 300-nucleotide hairpin structure known as the miRNA precursor. The miRNA precursor may be transported out of the nucleus by HASTY or retained in the nucleus and further processed by DCL1 to release the stem portion of the hairpin as an miRNA:miRNA* duplex (for reviews, see Bartel, 2004; Kidner and Martienssen, 2005). The duplex, which comprises a mature miRNA of ;21 nucleotides and a similarly sized miRNA* fragment on the opposing arm of the miRNA precursor, is then presumably unwound by a helicase, releasing the singlestranded mature miRNA. The miRNA then enters the RNAinduced silencing complex (for review, see Bartel, 2004) and guides the complex to identify target messages for posttranscriptional gene silencing through direct target cleavage (Llave et al., 2002a; Rhoades et al., 2002; Emery et al., 2003; Kasschau et al., 2003; Palatnik et al., 2003; Tang et al., 2003; Achard et al., 2004; Juarez et al., 2004; Kidner and Martienssen, 2004; Laufs et al., 2004; Mallory et al., 2004a, 2004b; McHale and Koning, 2004; Baker et al., 2005) or, in a few cases, for translational repression (Aukerman and Sakai, 2003; Chen, 2004).

So far, a large number of miRNAs have been found in various plant species (Llave et al., 2002b; Park et al., 2002; Reinhart et al., 2002; Palatnik et al., 2003; Bonnet et al., 2004; Floyd and Bowman, 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Wang et al., 2004a, 2004b; Adai et al., 2005; Bedell et al., 2005). In flowering plants, miRNAs are major regulators of developmental processes such as flower, leaf, and root development; miRNAs have also been predicted or confirmed to regulate other processes such as pathogen invasion, mineral nutrient homeostasis and responses to stress (Jones-Rhoades M. W. et al., 2006).

Laccase

Laccase is another Cu-enzyme that is certainly down regulated by miRNA in Arabidopsis (Pilon et al., 2007). Laccase (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2), ascorbate oxidase (AAO), and ceruloplasmin are members of a family of copper-containing metalloenzymes, commonly referred to as the blue copper oxidases. These enzymes are unique in that they, along with cytochrome *c* oxidase and the alternative oxidase found in plant mitochondria (Siedow et al., 1995), are the only enzymes known to catalyze the four-electron reduction of O_2 to H_2O. Laccase, in particular, appears to be very widely distributed, having been identified in bacteria, fungi, plants, and insects (Dean et al., 1994). Laccases are somewhat promiscuous with regard to

substrate specificity, oxidizing a variety of diphenol, dinaphthol, phenylenediamine and anthranilate-derived metabolites.

The oxidized products of these reactions often polymerize with each other or with molecules in the surrounding extracellular matrix to form chemically resilient structures that serve to protect the organism from various environmental stresses. Recently, five divergent laccase cDNAs (*lac1*, *lac2*, *lac3*, *lac90*, and *lac110*) were characterized from *Populus trichocarpa* (Ranocha et al., 1999, 2000). These genes were obtained from a xylem-enriched cDNA library and were most highly expressed in stem tissue. At least two of the five genes, *lac90* and *lac110*, encode proteins that are capable of oxidizing lignin precursors in vitro.

Targets

Given that the importance of biomass production in short rotation coppice, possibily coupled with a remediating action on polluted soils, becomes important to study the behavior of a model high biomass producing species like *Populus trichocarpa* in Cu balance : in Arabidopsis , as reported above, we can see a down regulation of Cu proteins in situation of Cu deficiency or closest to deficiency. Probably the response in Poplar leaves is the same , but in stems it should be different. Once investigated the Cu managing in regards to Cu chaperones and SOD, we want to see the behavior of Laccase that is involved in cell wall construction and, for some group of these enzymes, also in lignification (Ranocha et al., 2002).

MATERIALS AND METHODS

Poplar's propagation

the propagation started from 10 original tissue culture nodal cuttings of the Nisqually-1 poplar clone (*Populus trichocarpa*) sent in vials from Department of Forest Ecosystems and Society of Oregon State University (original cutting length : about 1 cm).They were transferred in agar medium plus MS salts (M5519 by SIGMA containing 0.1 μM $CuSO_4$) and IBA (indole butyric acid) 0.1 mg/L in sterile magenta boxes. The transfer was done under sterile hood using sterile forceps. After transfer, magenta boxes were put in grow chamber settled with following parameters : 70% of humidity, 16 h of day light, 24 C.

Poplar cuttings grew for 6 weeks in growth chamber, then plants grown enough (to touch the top of magenta box) were cut in 3 parts with sterile scissors under sterile hood : the top with merystem and other two pieces of stem including a leaf bud ; for each part one or two leaves,

depending on their size, were left to allow cutting to survive during the first period before root system development. About 50% of magenta boxes prepared for the 2nd generation were added with 5µ M of $CuSO_4$. Propagation was carried on in the same way for four generations in order to collect a good amount of plant tissue; this plant tissue (leaves and stems) were stored in 15 ml tubes in -80 °C freezer.

Poplar proteins extraction

 We used 2 ml microfuges Eppendorf tubes put on ice. For each sample we prepared 3 tubes : 2 of them empty and one filled with 100 µL 2 x sample buffer (with DTT). Sampling : we have grinded very well tissue in a cold mortar with a cold pestle and liquid nitrogen 0.3-0.6 g of leaves tissue, ~ 0.5 g of stem tissue to obtain a powder. The powder was transferred in a empty cold tube and 400 µL of extraction buffer (1M K_2HPO_4,1M KH_2PO_4, 50 mg of ascorbate , 25 µL of beta mercaptoethanol, 100 µL of Triton X-100) were added for 10 minutes vortexing each 3 minutes. After that, we have centrifuged one minute at 13200 rpm in microcentrifuge. The supernatant was removed and 100 µL of this were added to the tube filled with the same amount of 2 x sample buffer with DTT . The remained amount was used to determine protein concentration with Bradford assay. Sample plus buffer was warmed up at 95 °C for 5 minutes.

Bradford protein assay

 we made a standard curve with different concentrantions of BSA (bovin serum albumin) protein red at 595 nm with spectrophotometer to determine protein concentration in poplar extracts. We used Bradford reactive solution 1:5 diluted.

Western blot

we have run denaturated proteins in a 15% SDS page gel with Biorad electrophoresis machine at 120 V for 1 h. After that, protein bands were transferred in nitrocellulose by Biorad western blot machine, running at 50mA for 16 h. Immunodetection procedure was developed as follows : nitrocellulose was rinsed one minute in TBS + T, 30 minutes in 5% milk solution, then washed in TBS + T 2 minutes.
Incubation in first antibody (antimouse and antichicken for our proteins) solution (diluted 1:1000) was 2 hours long. After that nitrocelluloses were rinsed five times in TBS+T, then incubated 45 minutes in second antibody (antirabbit diluted 1:10000). After second incubation, nitrocell were rinsed four times in TBS+T, one time in TBS and one time in AP buffer. Each step was done on a rocker. Staining solution was composed as follows : 40 µL of NBT + 75 µL in 10 ml of AP buffer. Staining was carried on in dark (because all reagents are light sensitive) until protein bands begun to be colored. At this point reaction was immediately stopped by deionized water rinsing. After that, keeping nitrocelluloses in the dark, a picture was kept with scanner.

Native gel

 crude proteins run in a 15% acrylamide gel without SDS to avoid their denaturation. Run was carried on in +4 °C fridge at 100 V for 3-4 hours. After run gel was soaked 20 min in NBT/d-water (1mg/ml) in the dark on a rocker. After that gel was rinsed off and put in untreated or treated staining solution on the rocker for 20 minutes. Then gel was put in a tray filled with deionized water under a strong light source (as a common lamp) and left there for 10-30 minutes to allow gel becoming darker in the background (bands remain uncolored). Gel and reagents must kept in the dark until end of staining.

 Untreated staining solution -to resolve the activities of all SODs- was prepared as follows : 1M K_2HPO_4 (1.47 ml), 1M KH_2PO_4 (148 µL), Temed (145 µL). All brought up to 45 ml.

Poplar RNA extraction

 Leaf and stem tissues (50-200 mg) were grinded with pre cold and sterilized mortar and pestle, using liquid nitrogen. The fine powder obtained was put in 2 ml Eppendorf tubes with 1 ml of Trizol. Mix was left at R.T. for 5 minutes and shacked every minute. Then tubes were centrifuged at 12000 rpm for 10 minutes at 4 °C. Supernatant was transferred in a new tube and 200 µL of chloroform were added. Then tube was shacked for 15 seconds and left at R.T. 2-3 minutes. Then it was centrifuged 15 minutes at 12000 rpm at 4 °C.
The supernatant was transferred in a new tube, in which 0.5 ml of isopropanol was added. Mix was left to incubate 10 minutes at R.T, then it was centrifuged for 10 minutes at 4 °C at 12000 rpm and supernatant was discarded. Pellet was washed two times (3 minutes per wash) with ethanol 70% (1 ml) and pellet was finally dried and stored in -80 °C freezer.

DNAse treatment

 pellet was dissolved in 18 µL of dd-water plus 2 µL of DNAse buffer and 1 µL of DNAse. The mix was left to incubate at 37 °C for 30 minutes, then it was addicted with 2 µL of 25mM EDTA and left to incubate again for 10 minutes. The reaction was stopped adding180 µL of dd-water. After that a new RNA extraction was done following the same procedure described above, but now using just 0.5 ml of Trizol and adjusting all following solutions. Finally DNA pellet was resuspended in 20 µL of dd-water and left to incubate for 10 minutes at R.T.

RNA quantification
  Thermo scientific Nanodrop 2000 spectrophotometer was used to quantify RNA. Measurement was done at 260nm using 2 µL of my 20 µL RNA diluted in 198 µL of dd-water. RNA run in a 1% agarose gel . Ethidium bromide to liquid agarose was added to stain bands (1 µL / 10 ml of gel). 1.358 µg of RNA were loaded in each well.

RT PCR of Laccases DNA

 master mix for each sample was composed as follows : Reaction buffer 4 µL, Ribolock RNAse inhibitor (20u/ µL) 1 µL, 10 mM dNTP mix 2 µL, MMuLV Reverse Transcriptase(20u/ µL) 2 µL , 1 µL of esameric primers and 2.5 µg of RNA. (Reagents from Fermentas First strand cDNA synthesis). Mix was brought up to final volume (20 µL) with DEPC. Each tube was left in a thermostatic bath at 25 °C for 5 minutes, then at 37 °C for 60 minutes. Finally reaction was stopped warming up to 70 °C for 5 minutes.

PCR of Laccases DNA

 master mix for each sample was composed as follows : buffer 2µl, dNTPs 2µl, Primer FW 0.5µl, Primer RW 0.5 µl , Taq 0.2 µl , 1µl of cDNA and 13.8 µl of DNAse free dd-water. PCR incubator was settled as follows : 38 cycles , pre-denaturation 96 ℃ for 5 minutes, denaturation 96 ℃ for 30 s , annealing 55 ℃ for 30 s , extention 72 ℃ for 5 minutes.

Primer

 poplar laccases sequences were found in NCBI gene database (http://www.ncbi.nlm.nih.gov/). Alignment of sequences was made by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and primes design was made using PRIMER BLAST by NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=NcbiHomeAd). Poplar laccases miRNA were found on miR Base (http://microrna.sanger.ac.uk/sequences/). Primers were designed to retrotranscript the mRNA sequence containing *miRNA 397a* target sequence. The specific probes for *miRNA 397a* target sequences were FW: 5'-ATCATCGCTGAAGGATTTGG-3' RW 5'-CAGTGCATTAGCCACACACC-3' for *lac90a*, FW: 5'-GGGGATCTCTACCCTTGCTC-3' RW 5'-TCGTCGTGTTATCAAATGGC-3' for *lac110a,* FW:5'-CGGACTTCGCAGCGGCCATA-3' RW 5'-GCTCGTCGTTGAGTGCAGCG-3' for *lac110b,* FW: 5'-ACCAACTCTGTATGCCAGGG-3' RW 5'-GCCTCTCTGGCCTGTGATAG-3' for *lac 1a*, FW: 5'-AGTACGCTTGCTGGCTCCGA-3' RW 5'-TTGGTGGTCTGGCCTGGGGC-3' for *lac 1c*, FW 5'-CTGTCTGCCTCCTCCCCGCT-3' RW 5'-GTGGTCTGGCCTGGGGCAAT-3' for *lac 1d*, FW: 5'-TGCGCACGGGCTGGTCCGACG-3' RW 5'-ACCCGGAGAAGGGCAGCCAGGA-3' for *lac 2*, FW 5'-GAATGGTGGAAGGCTGATGT-3' RW 5'- GCCCTGCAATCTTGAAGAAG-3' for *lac 3*.
As constitutive Actin was chosen : FW 5'-GGAGCTGAGAGATTCCGTTG-3' RW 5'-GGTGCAACCACCTTGATCTT-3'.
ptc-**miR397**a MIMAT0002038: 5'- UCAUUGAGUGCAGCGUUGAUG-3', Reverse Complement 5'- CATCAACGCAGCACTCAATGA-3'.

RESULTS AND DISCUSSION

At 0.1 µM CuSO₄ (in Murashige & Skooge standard) *CSD1*and *CSD2* seems clearly down regulated both in leaves and in stems compared with 5 µM CuSO₄ added MS. The differences between treatments is stronger in stems than in leaves. Particularly *CSD1* in 5 µM CuSO₄ is expressed 4 times more than in 0.1 µM CuSO₄ . Because poplar cuttings grew in magenta boxes, didn't develop secondary wood, maintaining a green stem; This light photosintetic activity should have downregulated Cu proteins stronger than in a woody stem. To maintain a minimal SOD activity is expected, like in *Arabidopsis thaliana,* an upregulation of others SOD using different cofactors like FSD1 and MnSOD (Yamasaki H. et al., 2007). The first one resulted undetectable in poplar using same antibody thought for *Arabidopsis* probably because they aren't able to recognize poplar FSD1. MnSOD expression seems not only not affected by down regulation of Cu/Zn SODs, but it seems to follow the same behavior of CSD in condition closest to Cu deficiency, although differences in this case aren't so clear as for CSD. Plastocyanin, as easily predicted, maintained same level in both treatments and in both tissues confirming the previous hypothesis of an affection of Cu/Zn SOD in stems because tissue was still photosynthetizing. Two copper chaperones considered did not give clear results : CCS showed to much weak bands (data not showed) ,maybe depending on antibodies like for FSD1, and COX II did not showed differences between treatments (Figure 1)

Once seen that Cu homeostasis in poplar is pretty similar to Arabidopsis, Laccases expression was investigated. *Lac1*, *lac2*, *lac3*, *lac90*, and *lac110* ,characterized from *Populus trichocarpa* resulted strongly expressed in stem (Ranocha et al., 1999, 2000), but differently from Ranocha et al., we found that only lac90a is active exclusively in stem, while the other ones are more or less strongly expressed also in leaves. Lac 90a according to Cu homeostasis in Arabidopsis (Abdel-Ghani and Pilon 2007) is more strongly expressed in Cu replete condition.

Lac 110b in Cu replete conditions is three times more relatively expressed in stems than in 0.1µM Cu conditions, and two times in leaves. Comparing leaves and stems enzyme is more active in stem in both conditions. Lac110a doesn't show a clear trend in stems were enzyme is expressed equally in two treatments, while in leaves it appears to be downregulated in 0.1µM Cu conditions. Lac 1a, 1d and lac 3 shows same behavior : no differences between treatments in leaves and a clear downregulation at 0.1µM Cu in stems. An opposite behavior is shown by the last group composed by Lac 2 and Lac 1c in which laccases is more expressed in leaves at Cu concentration of 0.1µM Cu ; this happens particularly for lac 1c ; in stems these proteins seems expressed very weakly in both treatments, totally in opposition with data of Ranocha et al. This squared suggests us the need of more investigations and the comparison of these treatments in different growth conditions, like agargel vs hydroponics for instance and at different age because our cuttings were harvested before formation of secondary wood.

FIGURES
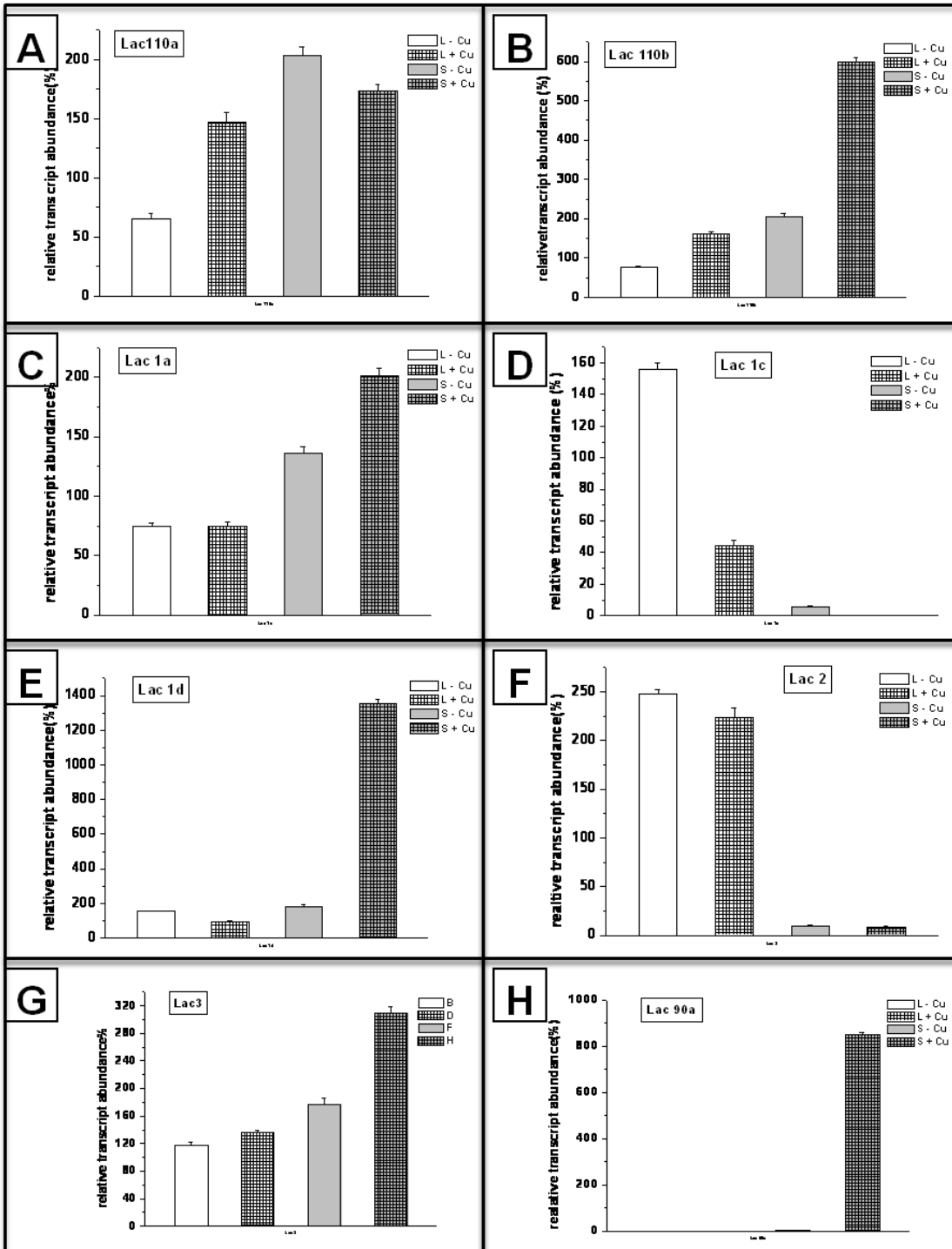
Figure 1 : Analisis of laccase transcript accumulation in leaves and stems of *P. trichocarpa* grown in Cu-sufficient(+Cu) or Cu-deficient (-Cu) condition. L –Cu = leaves at 0.1 μM of $CuSO_4$; L + Cu = leaves at 5 μM of $CuSO_4$; S - Cu = stems at 0.1 μM of $CuSO_4$; S + Cu = stems at 5 μM of $CuSO_4$. Y axis is the relative expression based on actin expression.
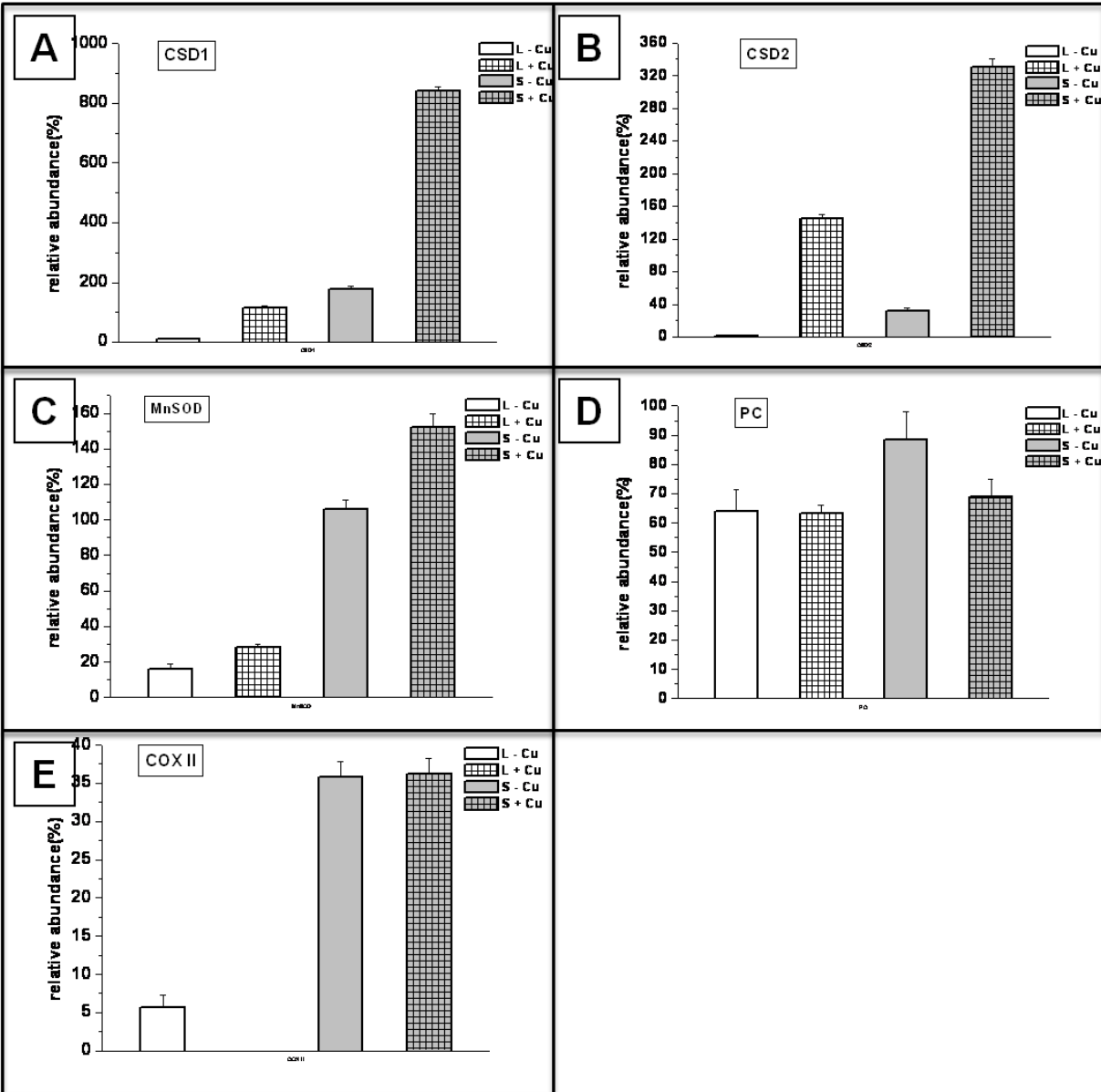
Figure 2 : Cu proteins relative expression in leaves and stems of *P. trichocarpa*. L –Cu = leaves at 0.1 µM of CuSO4 ; L + Cu = leaves at 5 µM of CuSO4 ; S - Cu = stems at 0.1 µM of CuSO4 ; S + Cu = stems at 5 µM of CuSO4 . Y axis is the relative expression based on RUBISCO expression

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