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Effects of Metal Toxicity on Growth and Pigment Contents of Annual Halophyte (*A. hortensis* and *A. rosea*)

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ABSTRACT: The toxicity of four potentially toxic trace elements (Cu, Ni, Pb and Zn) to Annual Atriplex (*A. hortensis* and *A. rosea*) was examined to determine if this plant showed sufficient tolerance to be used to phytoremediate soils polluted with these heavy metals. The soils, which contained up to (per kilogram) 501 mg Cu, 1674 mg Ni, 1334 mg Pb and 3588 mg of Zn were sampled around metal-contaminated site in southwest of France. We submit therefore that it could be that the presence of some heavy metals accumulated in the plants may have reached toxic levels thereby causing inhibition to their growth and pigment contents. The plant growth expressed as shoot and root dry weight of Atriplex plant was adversely inhibited when exposed to high concentrations of polluted soil. Significant increases in chlorophyll content were observed in leaves for three Atriplex varieties after the plants were exposed to stress treatments. The carotenoid and anthocyanin content also decreased. Red variety of Atriplex accumulated more anthocyanins in leaves than green and rosea ones. The lipid peroxidation increased, considerably at 100% polluted soil, which is a typical plant reaction to the oxidative stress. We proposed for the reduction state of photosynthetic parameters to be a useful tool in bioassay toxicity testing of metal polluted soil. These results demonstrate that heavy metal contamination of soil has adversely affected the photosynthetic parameters of annual Atriplex. The present study shows that exposure to heavy metals induced oxidative stress which was accompanied by growth inhibition, enhanced lipid peroxidation levels, increase content of chlorophyll, decrease content of carotenoids and anthocyanins. Finally, it was concluded that annual Atriplex has a high ability to tolerate Cu, Ni, Pb and Zn, so it might be a promising plant to be used for phytostabilization of metal contaminated soil.

Key words: Heavy metals, Annual Atriplex, Chlorophyll, Carotenoid, Anthocyanin, MDA

INTRODUCTION

Metals in terrestrial ecosystems are important for their influence on development and growth of plants (Hall & Williams 2003). Heavy metals are naturally occurring in the earth's crust but anthropogenic and industrial activities have led to drastic environmental pollutions in distinct areas. The retention of high concentrations of heavy metals in the environment exerts toxic effects on fauna and flora (Mishra and Tripathi 2008). Exposure to heavy metal toxicity has become a major limiting factor in the growth of crop plants, affecting the sustainability of agricultural production (Lin *et al.*, 2005; Molas 2002). Large parts of agricultural soil are contaminated with heavymetal**s** by natural and anthropogenic activities. The use of phytoremediation is now accepted as a commercial alternative to conventional techniques for diffuse or moderately contaminated soils (Li 2006; Pilon-Smits 2005). The use of halophyte species for phytoextraction purposes has been recommended because these plants are naturally present in environments characterized by an excess of toxic ions (Schwartz *et al*., 2003; Zhao *et al*., 2003). Among the halophyte flora, species belonging to the genus *Atriplex* may be of special interest because of their high biomass production associated with a deep root system able to cope with the poor structure and xeric characteristics of several polluted substrates. *Atriplex* spp., are highly salt tolerant and serve as pioneer species on mine tailings in semiarid western Australia and are used in

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revegetation of mine tailings in the Western United States (Glenn *et al.*, 2001; Jefferson 2004; Rosario *et al.*, 2007).

To minimize the adverse effects of reactive oxygen species (ROS), plants have evolved numerous non-enzymatic and enzymatic antioxidant defenses. Photosynthesis is arguably the most important of plant processes, and is essential for production of biomass. Heavy metal phytotoxicity may result from alterations of numerous physiological processes (Rascio and Navarri-Izzo 2011). Heavy metal has deleterious effects on the content and functionality of the photosynthetic pigments (Broadley *et al.*, 2007). This can be caused by the inhibition of pigment synthesis (Gangwar *et al*., 2011; Huang *et al*., 2013), or direct oxidative damage to the pigments (Oláh *et al*., 2010).They comprise impairments of chlorophyll synthesis resulting in chlorotic leaves, changed ratios of chlorophyll a and b (Viehweger and Geipel 2010, Mysliwa-Kurdziel *et al*., 2004) and photosynthetic activity (Küpper *et al*., 2007), dwarfism of plants or effects on root ultrastructure (Barcelo *et al*., 2004). Inhibition of photosynthesis induces oxidative stress (Romero-Puertas *et al.*, 2004; Benavides *et al.*, 2005), which can contribute to the degradation of photosynthetic structures and induction of senescence (McCarthy *et al.*, 2001), and to stress acclimation of plants through signal-transduction processes (Maksymiec 2007). Carotenoids are plant pigments that function as non-enzymatic antioxidants (Strzalka *et al*., 2003). Carotenoids may undergo a high turnover under photo-oxidative stress due to chemical quenching of singlet oxygen (Edge & Truscott, 1999). More recent studies have found that anthocyanins are produced in response to various abiotic stresses, including metal stress (Chalker-Scott 1999; Hale *et al*., 2001). Carotenoids and anthocyanins are the main pigments known to be involved in protecting plant organs from stresses. Heavy metals inhibit chlorophyll and carotenoid biosynthesis and retard the incorporation of these pigments in photosystems. The decrease in pigment photosynthesis as a consequence of reduced absorption of essential mineral nutrients is an indirect reason for plant chlorosis (Gajewska *et al*., 2006). Moreover, anthocyanins are considered light attenuators and antioxidants, their main function is the quenching of the reactive oxygen species generated by stress (Neill and Gould, 2003). The release of lipid peroxidation products such as malondialdehyde (MDA) is one of the principal causes of toxicity of heavy metals (Osuala, 2012; King *et al*. 2012). MDA is produced as the decomposition product of polyunsaturated fatty acids of biomembranes. The oxidative stress leads to significant increase in the free MDA pool (Weber *et al.*, 2004). The concentration of malondialdehyde MDA increased in leaves of sunflower under heavy metal stress condition (Yadav, 2010).This study was aimed to be a first step toward the understanding the effect of different concentrations of heavy metals in the responsiveness of growth and different photosynthetic parameters under the mentioned conditions in Annual Atriplex, which may ultimately lead to phytostabilization applications.

MATERIALS & METHODS

Seeds of two varieties of *A. hortensis* were obtained from CN Seeds Ltd. (Ely,UK). The varieties used were *A. hortensis* var. *purpurea* (green) and *A. hortensis* var. *rubra* (red). The seeds of *A. rosea* were collected from the site of Usinor and, the plants were then grown in sand cultures at the "Conservatoire Botanique" Laboratory of the "Tête d'Or", in Lyon. Seeds of *A. hortensis* and *A. rosea* were germinated for 3 days on a Petri dish filled with water-soaked sponge. Seedlings were transplanted to the pots and filled with soils having different concentrations of metals, and incubated one month in a greenhouse; five seedlings were planted per pot. The treatments with polluted soil consisted:

- Control, 100% sand (S) and 0% polluted soil (PS)
- 25% PS and 75% S
- 50% PS and 50% S
- 75% PS and 25% S
- 100% PS and 0% S.

The soil used in this study were collected from a known metal-contaminated site located near Saint Etienne in southwest of France. Past industrial activities have contributed to elevated metal concentrations in this site. Contamination of heavy metals was mainly concentrated in the top 15cm at the site. The polluted soil was dried at 60 °C for 48 h, sieved to 2 mm and ground to pass a 250-µm mesh sieve. The pH of the mine soil was 8.1, which is suitable for plant growth. Selected characteristics of the soil collected from this study are shown in Table 1. Soil was maintained as 70% in field capacity, and weighted with water every day. Plants were grown in 10 cm diameter pots in a growth chamber at a thermoperiod and a photoperiod of 22 °C/16 h the day, and 20 °C/8 h the night (150 µmol photons m^{-2} s^{-1}).

The tolerance index which is a measure of the tolerance of the plant to heavy metals, was determined by comparing the dry biomass of plants subjected to metal treatment with the control using the relationship outlined by Wilkins (1978):

$$\text{Tolerance index} = \frac{\text{Biomass of treated plants}}{\text{Biomass of control plant}} \times 100$$

Table 1. Soil characteristics from the contaminated site near Saint Etienne, France

Composition	Soil nature	
	Sand	Polluted soil
Mineral elementals	Concentration (mg kg^{-1})	
CaO	6,76	3,74
Fe_2O_3	1,33	43,62
K_2O	2,32	0,66
MgO	0,79	1,69
MnO	0,02	0,55
Na_2O	1,23	0,63
P_2O_5	0,06	0,19
SiO_2	76,56	32,22
Heavy metals		
Ni	3,3	1673,7
Pb	14,2	1333,5
Cu	2,3	501
Zn	22,4	3587,9

The tolerance index was determined at the end of the treatment. Changes in this parameter were used to evaluate metal toxicity.

For measurements of anthocyanin content (three replicates), leaf discs were powdered in liquid nitrogen and extracted with methanol containing 1 % HCl. Anthocyanins were extracted as previously described (Close *et al*., 2000) before absorbance measurements at 530 and 657 nm. The corrected value of absorbance was calculated (A530-0.25A657) to remove the absorbance of chlorophyll and degradation products (Mancinelli *et al*., 1975). The results are expressed as anthocyanin content per gram of fresh weight.

At the end of the experiment period, chlorophyll and carotenoid pigments in fully expanded leaves (a randomly selected mixture of old and young leaves) from each treatment were extracted using 0.05 g of fresh material in 10 mL of 80 % aqueous acetone. After filtering, 1 mL of the solution was diluted with a further 2 mL of 80 % aqueous acetone and the extracts were measured for chlorophylls (a, b) at 645 and 663 nm and for carotenoids at 470 nm. Concentrations of pigments [µg g fresh weight (f. wt)$^{-1}$] were obtained by calculation, using the method of Lichtenthaler (1987).

After metal stress application, the fully expanded leaf material (200 mg) was homogenized in 2 cm3 of 0.1% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 15,000xg for 10 min, and 0.5 cm3 of the supernatant obtained was added to 1.5 cm3 thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 90°C in a shaking water bath for 20 min, and the reaction was stopped by placing the reaction tubes in an ice water bath. Then the samples were centrifuged at 10,000xg for 5 min, and the absorbance of the supernatant was read at 532 nm (Hernandez and Almansa 2002). The value for non-specific absorption at 600 nm was subtracted. The amount of MDA was calculated from the coefficient of extinction of 155 $mmol^{-1}$ cm^{-1} (Cakmak and Horst 1991).

All treatments were repeated five times. Data in the text and figures indicate mean values ± SD. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to LSD de Fisher test.

RESULTS & DISCUSSION

Tolerance index of shoots and roots were affected by the polluted soil treatments (Fig. 1). Exogenous addition of different concentrations of heavy metals (nickel, lead, copper, and zinc) showed varying toxicity to *Atriplex* plant. The extent of toxicity increased with increasing concentration of the metals. High polluted

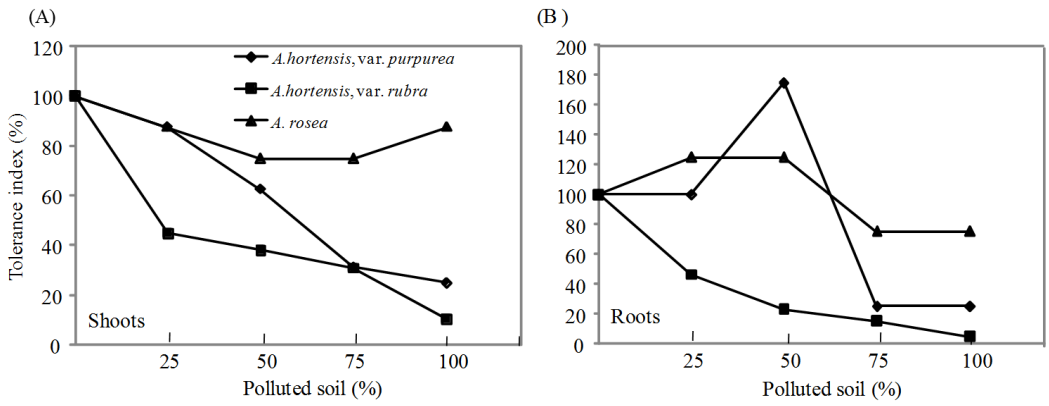

Fig.1. Tolerance index of shoot (A) and root (B) measured in response to different concentrations of polluted soil in the culture medium are reported for *A. hortensis*, var *purpurea*, *A. hortensis*, var. *rubra* and *A. rosea*. Measurements were performed on samples coming from individual plants. Mean ± SD; n=5.

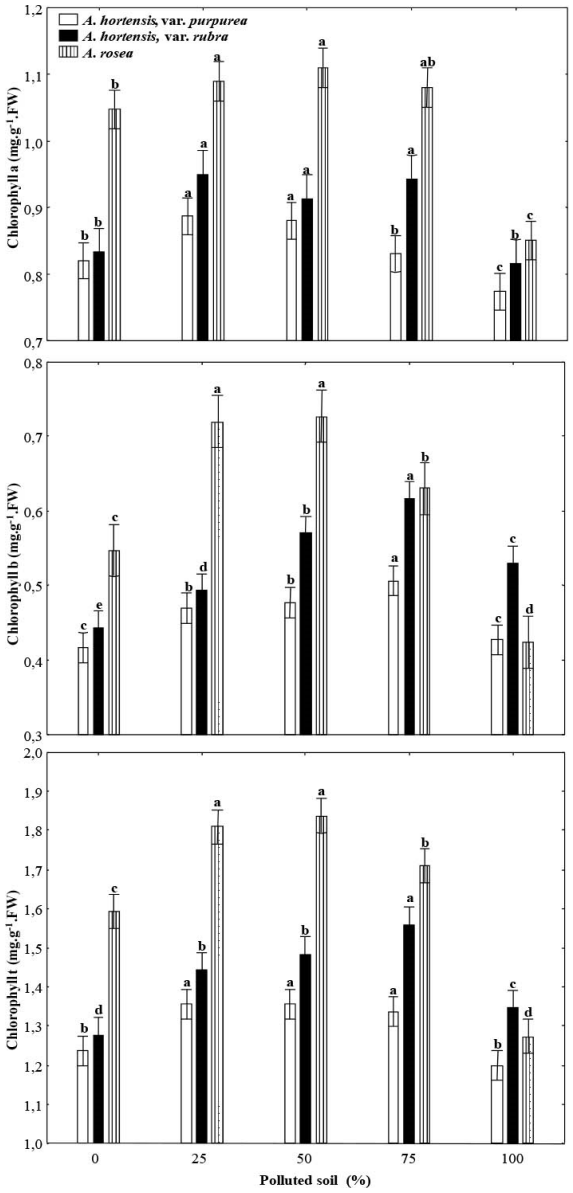

Fig. 2. Changes in the levels of chlorophylls a, b and total in *A. hortensis* and *A. rosea* shoots treated with metal polluted soil; mean of five determinations, pigment content in mg/g dry weight. Mean ± SD; n=5.

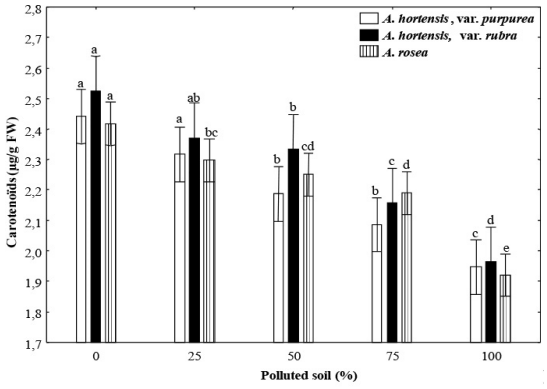

Fig. 3. Carotenoids content in the leaves of *A. hortensis* and *A. rosea* subjected to different treatment with metal polluted soil. Data are mean ± SD (n = 5). Different letters indicate that the mean value is significantly different (P < 0.05).

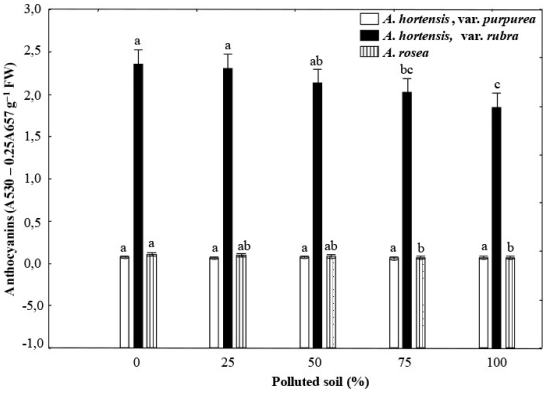

Fig.4. Anthocyanins content in the leaves of *A. hortensis* and *A. rosea* subjected to different treatment with metal polluted soil. Data are mean ± SD (n = 5). Different letters indicate that the mean value is significantly different (P < 0.05).

soil concentration had more severe inhibiting effect on roots than on shoots. In *A. rosea*, the shoot tolerance index was unaffected by metal treatments probably because the seeds of *A. rosea* were collected from the site of Usinor. At 50% level of polluted soil tolerance index of roots were higher than non-metal addition, and then decreased at high concentrations of polluted soil, especially for *A. rosea*.

Photosynthetic pigments were compared in *Atriplex* plants treated with metal polluted soil, both of Atriplex resulted in a substantial decrease in pigment levels. The chlorophyll content in the plants was significantly affected by heavy metal treatment (Fig. 2). Increasing the polluted concentration in the growth medium was responsible for an increase in the total chlorophyll content in the leaves of *Atriplex* plants, the total chlorophyll content strongly decreased only in the variant with 100% polluted soil (Fig. 2). By contrast, the carotenoid content of the leaves decreased with increasing metal concentration (Fig. 3). We observed that the presence of polluted soil in the medium caused the decrease of anthocyanin content in shoots of *A. hortensis*, var. *rubra*. We can claim that metal stress has no effect in the anthocyanin contents of *A. hortensis*, var. *purpurea* and *A. rosea* (Fig. 4).

Our results showed an increase in MDA content with gradual increase in soil polluted, especially in A.

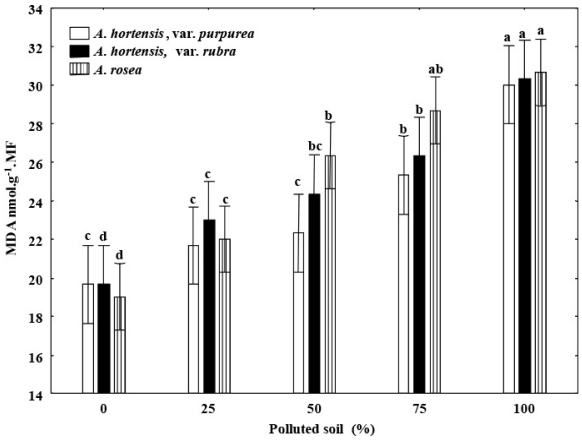

Fig. 5. Malondialdehyde (MDA) contents in shoots of *A. hortensis* and *A. rosea* under four metal polluted soil regimes. Means ± SD of five replications. Different letters indicate that the mean value is significantly different (P < 0.05).

rosea, suggesting that severe heavy metal stress may accentuate the lipid peroxidation of Atriplex plant. MDA production was highest for *A. rosea* followed by red orach and then green orach at each concentration tested (Fig. 5). As compared with control, *A. hortensis* and *A. rosea* significantly increased MDA contents by 50 % in *A. hortensis* and by 60 % in *A. rosea*, respectively. The difference in MDA content was significant between *Atriplex* at control and polluted soil treatments. Heavy metal contamination of soils is one of the major environmental stresses for plants and there is increased interest in the use of plants to decontaminate soils polluted by heavy metals. Heavy metals are known to cause oxidative damage to plants through the formation of reactive oxygen species (ROS) which cause damage to biomolecules such as membrane lipids, proteins, etc. Damage of the cellular membranes, especially for the plasma membrane, is one of the primary events in heavy metal toxic effects in plants (Janicka *et al.*, 2008).

The most common effect of metal toxicity in plants is stunted growth, leaf chlorosis and alteration in the activity of many key enzymes of various metabolic pathways (Arduini *et al.*, 1996). In our study, with varied concentrations of polluted soil, tolerance index of shoots and roots of *Atriplex* got affected (Fig. 1). The greater impact of heavy metal was observed on shoot growth as compared to the root. The plant growth expressed as shoot and root dry weight of Atriplex plant (Fig. 1) was adversely inhibited when exposed to high concentrations of polluted soil. The reduction in the growth of *Atriplex* could be also due to the suppression of the elongation growth rate of cells (Aidid and Okamote, 1993).

Our results indicate that detoxification mechanisms contribute to different extent towards the protective strategies of Atriplex plants. Changes in the levels of photosynthetic pigments in plants grown in polluted soil with different metals are introduced in Fig. 2. Oxidative stress due to the existence of the toxic metals can be demonstrated by chlorophyll content. Significant increases in chlorophyll content were observed in leaves for three Atriplex varieties after the plants were exposed to stress treatments (Fig. 2). There is an inhibition of chlorophyll biosynthesis except in high level of polluted soil (100%). It was suggested that heavy metals could interfere with chlorophyll biosynthesis either through the direct inhibition of enzymatic steps or through the substitution of the central Mg ion (Cenkci *et al.* 2010; Pourraut *et al.*, 2011). Carotenoids production was decreased in comparison to control (Fig.3). Carotenoids serve as antioxidants against free radicals and photochemical damage (Sengar *et al.*, 2008). Thus less effect on carotenoids might represent its supportive role against oxidative stress. Carotenoids are known to quench the oxidizing species and triplet state of the chlorophyll and other excited molecules in the pigment bed, which are seriously involved in disrupting metabolism through oxidative damage to cellular components (Candan and Tarhan 2003). The carotenoids (Car) content decreased less than chlorophyll (Chl) content, as describes Singh *et al*. (2003), metals affect generally chlorophylls more than carotenoids, and this not agrees with our results obtained for all concentrations of polluted soil tested (Fig. 3). Reduction in the levels of photosynthetic pigments, including carotenoids, on exposure to biotic or abiotic stressors have been observed in many species (Macfarlane and Burchett 2001; Thao and Yanyun 2005; Lau *et al.*, 2006). Metals can enhance or reduce carotenoid production depending on metal types (Fargasova 1998; Singh *et al.*, 2003). We observed that the presence of polluted soil in the medium caused the decrease of anthocyanin in leaves of the *Atriplex* var. *rubra* (Fig.4). Anthocyanins have been proposed as having a function in metal accumulation or tolerance, although metal complexation has long been known to have a role in determining anthocyanin color (Elhabiri *et al.*, 1997). Anthocyanins are produced in response to metal stresses (Hale *et al*., 2001) and believed to increase the antioxidant response of plants in order to uphold the regular physiological status against biotic or abiotic stresses (Neill *et al*., 2002).

Our results showed an increase in MDA content with gradual increase in soil polluted, especially in *A. rosea*, suggesting that severe heavy metal stress may accentuate the lipid peroxidation of Atriplex plant (Fig.5). Moreover, heavy metals stimulated lipid

peroxidation and damaged the chloroplast membranes. It is widely accepted that lipid peroxidation is an important and probably primary event in the progress of several diseases as well as degenerative processes associated with aging (Kohen and Nyska 2002, Szweda *et al.,* 2003). The products of lipid peroxidation initiate further free radical chain reactions and can cause oxidative damage to proteins and DNA (Stocker and Keaney 2004; Vassalle *et al.,* 2004). The measurement of MDA, a product of lipid peroxidation, is routinely used as an index of lipid peroxidation under stress conditions. MDA is the decomposition product of polyunsaturated fatty acids of biomembranes and its increase shows that plants are under oxidative stress. Thus increased MDA content shows the generality of oxidative stress and this may be one of the potential mechanisms by which toxicity due to heavy metals is manifested in plant tissues (Gupta *et al.,* 2009).

CONCLUSIONS

In summary, we identified two different trends linking important characters such as metal tolerance and pigment contents. These trends, which should still be validated through the analysis of larger *Atriplex* varieties, represent a good basis to better understand mechanisms involved in the plant response to metals and, extent, to develop breeding strategies aimed at limiting accumulation in *Atriplex* leaves. In the present study, exposure to heavy metals affected different parameters of *Atriplex*: plant growth, chlorophyll, anthocyanin, carotenoid content and MDA. Exposure of *A. hortensis* and *A. rosea* to polluted soil induced inhibition to the growth, which was accompanied by membrane damage, increased Chlorophyll content and MDA. Anthocyanin and carotenoid contents decreased with heavy metal treatment in comparison to the control. Among the plants, *A. rosea* was the most efficient in response to heavy metals while it is was most suitable for phytostabilization of sites contaminated with polluted metals.

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