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Enhancing the lead phytostabilization in wetland plant *Juncus effusus* L. through somaclonal manipulation and EDTA enrichment

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Abstract We investigated the role of ethylene diamine tetra-acetic acid (EDTA) and somaclonal manipulation on improving lead (Pb) phytostabilization in mat rush (*Juncus effusus* L.). Seedlings were raised from seeds and callus to study variations in Pb uptake and tolerance. The seedlings were treated with 0.5 and 1.0 mM Pb as alone, and each with 2.5 and 5.0 mM of EDTA. Plants grown from both sources accumulated relatively larger Pb contents in their root tissues that were further enhanced by EDTA supplementation in the hydroponics medium. The tendency of storing higher Pb contents in roots compared to shoots in *J. effusus* was also evident from lower translocation factor (TF) value that facilitated the plants to avoid from Pb-induced shoot injury. Callus grown plants were more responsive to EDTA amendment showing improved growth, Pb uptake and chlorophyll contents under Pb stress. Both kinds of *J. effusus* plants tolerated Pb toxicity by

modifying antioxidative enzyme activities. Enrichment of Pb-treated media with EDTA further elevated enzymic activities of plant roots contributing to defy Pb-induced oxidative burst. Thus, plant improvement through somaclonal manipulation in *J. effusus* along with EDTA enrichment could be an appropriate technique for phytostabilization of Pb-contaminated environments.

1. Introduction

Environmental dilemmas largely upshot from the enrichment of soil and water with heavy metals emerged through mining, burning of fossil fuels, sewage disposal and industrial operations (Krzesowska, 2011). Unlike certain metals such as copper, zinc and manganese, which are essential for various physiological processes of plants, lead (Pb) is a highly toxic metal pollutant, which interferes with the plant metabolic processes (Pourrut et al., 2011). Higher Pb concentrations in plant tissues accelerate reactive oxygen species (ROS) production responsible for lipid membrane damage that subsequently impairs chlorophyll, phtosynthetic process and suppresses overall plant growth (Doncheva et al., 2013).

For combating the harmful effects of oxidative stress, plants have evolved an antioxidative defense mechanism consisting of enzymatic and non-enzymatic components (Shahid et al., 2013), such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). The SOD is a major scavenger that catalyzes the dismutation of superoxide to O_2 and H_2O_2. The H_2O_2 is further detoxified by CAT and/or POD to H_2O and O_2 (Doncheva et al., 2013; Pourrut et al., 2011). Also, APX reduces H_2O_2 using ascorbate as an electron donor in the ascorbate–glutathione cycle (Shahid et al., 2012). Since physiological alteration in plants after a certain threshold level triggers the induction of antioxidative enzymes, measurement of enzymatic activity could be used as a good stress indicator in plants.

Recently, phytoremediation is increasingly being proposed as a significant technology for reclaiming the metal polluted soils. However, low solubility and formation of stable complexes in soil limit the phytoextraction of Pb from the polluted environments (Lim et al. 2004; Shahid et al., 2012). Nevertheless, use of synthetic chelators, e.g. EDTA has been used for enhancing metal solubility, uptake, and translocation to aboveground parts (Saifullah et al., 2009). These chelators could ameliorate Pb toxicity in plants via detoxifying metal ions (Zhang et al., 2013). In addition to chemcial applications, various other techniques including breeding, mutagenesis or callus culturing are commonly used to get plants with desirable traits, for example genetically stable somaclonal variants with improved metal tolerance have been developed through in vitro culturing (Akhtar et al., 2012; Guadagnini et al., 1999; Nehnevajova et al., 2007).

Constructed wetlands are increasingly applied as low energy based green technologies for phytoremediation of heavy metals from polluted environments (Sun and Saeed, 2009). The selection of plant species for phytoremediation purpose is based either on their potential to accumulate higher metal contents per unit dry biomass or on growth rates (McGrath et al., 2002). Exceptionally fast growth rate and the potential to tolerate range of metals make *Juncus effusus* an ideal candidate for use of phytoremediation purpose (Grube et al., 2008; Najeeb et al., 2009, 2011). Earlier studies with *J. effusus* were primarily focused on Pb uptake and translocation mechanism (Yanqun et al., 2004), and less attention was paid on enhancing phytostabilization through genetic manipulation. In this perspective, we used somaclonal variations and EDTA as a tool to improve phytoremediation potential of *J. effusus*. We hypothesized that (1) somatic variants would have higher Pb-phytoremediation potential (2) EDTA would enhance the Pb-tolerance in *J. effusus* plants via detoxifying free Pb ions in their tissues.

2. Materials and methods

2.1. Plant material and cultural conditions

Seeds of *J. effusus* cultivar Nonglin-4 were obtained from the University of Nottingham Ningbo, Ningbo, China. Upon receipt at the Zhejiang University, Hangzhou, China, these seeds were used for germinating plants and culturing callus. For the plant growth, glass-house facilities of Institute of Crop Science Zhejinag University were used, while laboratory-based experiments/analyses were performed at the College of Agriculture and Biotechnology, Zhejinag University. Healthy seeds were surface sterilized with 1% sodium hypochlorite (NaOCl) for 20 min, and then washed three times with distilled water (Xu et al., 2009). Nursery was developed on substrate by mixing perlite and vermiculite in 3:1 ratio (v/v). The uniform size (about 15 cm) seedlings were selected and shifted to plastic trays having half strength basal nutrient solution, and were allowed to grow in glass-house at $19 \pm 2\,°C$ temperature under 16/8 h light/dark regime.

For comparison, plantlets were raised from callus at the College of Agriculture and Biotechnology, Zhejiang University by the following method. Mat rush seeds were first surface-sterilized and placed in growth vessels containing hormone free MS media (Murashige and Skoog, 1962). Vessels were sealed and placed under fluorescent light ($100\,\mu mol\,m^{-2}\,s^{-1}$) with light/dark regime of 16/8 h and $19 \pm 2\,°C$ temperature to develop seedlings. These seedlings were used for callus induction and subsequent plant regeneration through the protocols developed by Xu et al. (2009). The basal culm segments (0.5–1.0 cm) of seedling explants were cultured in Petri dishes containing solidified MS callus induction medium supplemented with $0.5\,mg\,L^{-1}$ 6-benzylaminopurine (BA), and $2\,mg\,L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D). From the callus, plant regeneration was achieved by shifting embryogenic calli on MS regeneration medium-I and II (containing $0.5–1.0\,mg\,L^{-1}$ BA, $1.0\,mg\,L^{-1}$ kinetin and $3.0\,mg\,L^{-1}$ indoleacetic acid). Plantlets, induced from the regenerative calli were transferred to half strength MS plant multiplication medium. At a certain height (approximately 15 cm), these plants were shifted to Hoagland's modified nutrient solution for hardening (2 weeks), and then treated with Pb and EDTA.

2.2. Sample preparation and treatments (Pb and EDTA)

Two week old uniform size seedlings were transferred to the pots each containing 3.2 L Hoagland's modified nutrient solution. Hydroponics solution had micro-molar (μM) concentration of nutrients as: 2000 KNO_3, 50 KCl, 500 $Ca(NO_3)_2.4H_2O$, 200 $MgSO_4.7H_2O$, 100 NH_4NO_3, 10 KH_2PO_4, 12 H_3BO_3, 2.0 $MnSO_4.H_2O$, 0.5 $ZnSO_4.7H_2O$, 0.2 $CuSO_4.5H_2O$, 0.1 Na_2MoO_4, 0.1 $NiSO_4$, 20 Fe-EDTA. Concentration of KH_2PO_4 was adjusted regularly at 10 μM to prevent Pb precipitation in the nutrient solution.

After establishment of the seedlings to Hoagland's solution, two set of *J. effusus* seedlings, obtained from seed and regenerated from callus, were exposed to various concentrations of Pb [applied as $Pb(NO_3)_2$] and EDTA. There were seven treatments of Pb/EDTA in triplicates both for seed and callus grown plants. These were: control (without Pb and EDTA), Pb 0.5, Pb 1.0, Pb 0.5 + EDTA 2.5, Pb 1.0 + EDTA 2.5, Pb 0.5 + EDTA 5.0, Pb 1.0 + EDTA 5.0 (all in mM concentration). The two Pb concentrations as 0.5 and 1.0 mM were selected for treatment on the basis of previous results of Verma and Dubey (2003) who suggested these concentrations as moderate to high Pb respectively in the heavy metal polluted soils. EDTA application rate was selected on the basis of our previous studies, where 2.5 and 5.0 mM EDTA concentrations significantly enhanced the metal accumulation by *J. effusus* (Najeeb et al., 2009, 2011). Treatment pots were arranged with two factor factorial randomized complete design under glasshouse conditions with 30/25 °C temperature and 70%/90% relative humidity (day/night). The pH of nutrient solution was maintained at 5.8 by adding 0.1 M NaOH or 0.1 M HCl, and it was continuously aerated. Since our previous results showed that *J. effusus* performs well under lower pH level, we used relatively low pH (5.8) for the growth media (Najeeb et al., 2009; Xu et al., 2009). The nutrient solution was regularly changed after every 4 days, followed by the repetition of respective Pb/EDTA treatments. On the 15th day of treatments, plants were collected from each pot, cleaned, weighed and then stored at −80 °C for further analysis.

2.3. Pb and chlorophyll determination

The Pb concentrations in plant tissues (root and shoot) were measured following the procedure of Hsu and Kao (2003). Topmost fully expended leaf samples were used for analyzing Pb contents, while for roots, whole root mass was dried at 65 °C for 24 h and ground, and 0.2 g of the prepared sample was used for elemental analysis. A sub-sample of the dried plant mass (root and shoot) was converted into ash in Muffle furnace at 550 °C for 12 h. The ash residues were digested with 31% HNO_3 and 17.5% H_2O_2 at 72 °C for 2 h and transferred to 50 mL flask to make the volume up to the mark by de-ionized water. The Pb contents were determined through atomic absorption spectrophotometer (AA-6800, Shimadzu, Japan). Standard reference material GBW10010 from the Institute of Geophysical and Geochemical of Earth, China, and internal reference materials were used for quality assurance and measuring Pb contents in plants. The analysis showed high Pb recovery rates (>90%), where Pb concentration in the standard reference material was very close to the given value, confirming the accuracy of the method. We extracted chlorophyll from fresh leaves at the top 3rd position using 800 mL L^{-1} acetone, and leaf chlorophyll contents were measured by the method described by Lichtenthaler (1987).

2.4. Biochemical assays

Fresh plant root and shoot samples were ground in phosphate buffer (pH 7.8) using pre-chilled mortar and pestle. The extract was used for measuring activities of SOD, POD and malondialdehyde (MDA) contents following the method reported by Leu and Zhou (1999).

Catalase (CAT) activity of *J. effusus* roots and shoots was determined by the reduction rate of H_2O_2 in a specific period of time (Cakmak et al., 1993). Plant extracts were treated with 5 mL of 0.1 N H_2O_2 and kept at 20 °C for 5 min. Then 1 mL of a 20% KI solution was added with 3 drops of 10% $(NH_4)_6Mo_7O_{24}$ and 5 drops of 1% starch solution. A 0.02 N $Na_2S_2O_3$ reagent was used for titration of reaction solution until the disappearance of blue color.

2.5. Statistical analysis

Two-way analysis of variance (ANOVA) was used to analyze the data with SAS statistical package (version 9.0) for ANOVA, followed by Fisher's protected LSD test to identify homogenous groups within the treatment means. If the *F*-test showed significant differences among the means, multiple range tests were performed at the level of 5% probability.

3. Results

3.1. Plant growth attributes

Application of higher Pb (1.0 mM) concentrations in hydroponics culture inhibited the growth of *J. effusus* plants, while, control plants appeared healthy containing greater biomass and greenish in color. Despite no significant change in root and shoot lengths of the plants originating from different sources, the callus grown seedlings attained significantly greater dry biomass than seed grown seedlings under all the treatments. Development of relatively thicker roots and shoots not only helped callus grown plants to survive under metal toxicity but also to sequester higher Pb contents in their tissues. Lower Pb concentration (0.5 mM) had no effect on dry biomass of seed grown plants, however, it significantly reduced the shoot and non-significantly the root dry biomass of callus grown plants (Table 1). Higher dose of Pb (1.0 mM) significantly decreased the plant dry biomass (root and shoot) of the seedlings originating from both sources. EDTA amendments appreciably improved the shoot dry biomass of Pb-treated callus grown plant, while, no significant change was recorded in dry biomass of Pb-treated seed grown plants under EDTA amendments. The positive impact of EDTA on shoot growth was probably due to the restricted movement of Pb in roots and lower translocation.

Increasing Pb concentrations in the growth media inhibited the root and shoot length of two kinds of *J. effusus* plants (Table 2). No significant improvement in plant height was noticeable under lower EDTA (2.5 mM) concentration but the higher EDTA concentration (5.0 mM) recovered root

Table 1 Biomass of *Juncus effusus* L. as influenced by Pb and EDTA amendments.

Concentrations (mM)	Root dry weight (g plant^{-1})		Shoot dry weight (g plant^{-1})	
	Seed grown	Callus grown	Seed grown	Callus grown
Control	0.13 ± 0.01[a]	0.23 ± 0.02[a]	0.16 ± 0.01[a]	0.27 ± 0.04[a]
Pb 0.5	0.12 ± 0.01[a]	0.20 ± 0.02[ab]	0.15 ± 0.01[a]	0.20 ± 0.02[b]
Pb 1.0	0.07 ± 0.01[b]	0.17 ± 0.01[bc]	0.11 ± 0.01[b]	0.17 ± 0.01[c]
Pb 0.5 + EDTA 2.5	0.11 ± 0.02[a]	0.19 ± 0.01[b]	0.13 ± 0.02[ab]	0.28 ± 0.02[a]
Pb 1.0 + EDTA 2.5	0.08 ± 0.01[b]	0.17 ± 0.01[bc]	0.13 ± 0.01[ab]	0.25 ± 0.03[a]
Pb 0.5 + EDTA 5.0	0.11 ± 0.02[a]	0.20 ± 0.03[ab]	0.16 ± 0.02[a]	0.28 ± 0.02[a]
Pb 1.0 + EDTA 5.0	0.09 ± 0.02[b]	0.18 ± 0.01[bc]	0.12 ± 0.02[b]	0.26 ± 0.02[a]
Mean	0.10 ± 0.01[B]	0.19 ± 0.02[A]	0.14 ± 0.02[B]	0.24 ± 0.02[A]

Values are the mean of three individual replicates. Means followed by the same letter within each column are not significantly different at $P < 0.05$.
Means sharing the same capital letters (A) show non-significant difference between callus and seed grown plants.

Table 2 Growth attributes of *Juncus effusus* L. as affected by Pb and EDTA amendments.

Concentrations (mM)	Root length (cm)		Shoot length (cm)	
	Seed grown	Callus grown	Seed grown	Callus grown
Control	26.4 ± 1.8[a]	23.1 ± 1.9[a]	37.7 ± 2.2[a]	38.7 ± 2.3[a]
Pb 0.5	23.1 ± 1.5[ab]	22.4 ± 2.1[a]	33.9 ± 2.5[b]	35.8 ± 2.1[ab]
Pb 1.0	21.4 ± 1.9[b]	19.5 ± 0.9[b]	32.1 ± 2.6[b]	32.1 ± 2.7[b]
Pb 0.5 + EDTA 2.5	23.8 ± 1.5[ab]	21.6 ± 1.6[ab]	34.1 ± 2.9[ab]	34.8 ± 2.4[ab]
Pb 1.0 + EDTA 2.5	20.9 ± 1.6[b]	20.6 ± 1.2[b]	31.6 ± 2.6[b]	32.9 ± 3.0 b
Pb 0.5 + EDTA 5.0	25.0 ± 1.3[a]	24.1 ± 1.7[a]	32.9 ± 2.8[b]	35.0 ± 3.3[ab]
Pb 1.0 + EDTA 5.0	22.6 ± 1.2[b]	20.3 ± 0.7[b]	31.8 ± 0.5[b]	32.6 ± 1.2[b]
Mean	23.3 ± 0.9[A]	21.7 ± 0.9[A]	33.4 ± 0.7[A]	34.6 ± 0.8[A]

Values are the mean of three individual replicates. Means followed by the same letter within each column are not significantly different at $P < 0.05$.
Means sharing the same capital letters (A) show non-significant difference between callus and seed grown plants.

length of Pb-stressed plants. In general, seed grown plants had longer roots than callus grown seedlings, while, callus grown plants possessed relatively longer shoots, although the difference was statistically non significant.

3.2. Lead accumulation

J. effusus plants experienced a consistent rise in Pb contents in their tissues (shoots and roots) under the growing Pb concentrations in nutrient media that was further enhanced by EDTA amendments (Table 3). The plants tended to store greater Pb contents in their roots compared to their aboveground parts as was evident from the low translocation factor (TF). Furthermore, the callus grown plants deposited more Pb contents in root tissues than seed grown plants under all the treatments, and the difference was significant. Contrastingly, seed grown plants contained significantly greater Pb contents in their shoots as compared to the callus grown plants. Increasing EDTA concentrations significantly enhanced the uptake and translocation of Pb in *J. effusus* plants.

Table 3 Lead uptake and translocation by *Juncus effusus* L. under Pb and EDTA application.

Concentration (mM)	Pb concentrations mg 'kg^{-1} in roots		Pb concentrations mg kg^{-1} in shoots		TF value	
	Seed grown	Callus grown	Seed grown	Callus grown	Seed grown	Callus grown
Control	1.3 ± 0.2[e]	1.5 ± 0.3[f]	1.1 ± 0.1[g]	1.2 ± 0.1[g]	0.84	0.80
Pb 0.5	2548 ± 60[d]	4543 ± 105[e]	79 ± 5.1[f]	54 ± 5.4[f]	0.03	0.01
Pb 1.0	3160 ± 89[c]	5057 ± 82[c]	207 ± 8.6[e]	142 ± 7.8[e]	0.06	0.02
Pb 0.5 + EDTA 2.5	2643 ± 87[d]	4736 ± 118[d]	320 ± 7.6[d]	241 ± 6.5[d]	0.12	0.05
Pb 1.0 + EDTA 2.5	3873 ± 81[b]	5855 ± 110[b]	638 ± 10[b]	554 ± 7.7[b]	0.16	0.09
Pb 0.5 + EDTA 5.0	3253 ± 92[c]	4847 ± 116[d]	561 ± 7.4[c]	381 ± 6.4[c]	0.17	0.07
Pb 1.0 + EDTA 5.0	4243 ± 93[a]	6042 ± 97[a]	953 ± 14[a]	782 ± 9.5[a]	0.22	0.12
Mean	2818 ± 71[B]	4441 ± 90[B]	394 ± 7.6[A]	308 ± 6.2[B]	0.22[A]	0.16[B]

Values are the mean of three individual replicates. Means followed by the same letter within each column are not significantly different at $P < 0.05$.
Means sharing the same capital letters (A) show non-significant difference between callus and seed grown plants.

3.3. Variations in chlorophyll contents

Incremental Pb concentrations in growth media significantly suppressed the chlorophyll (chlorophyll *a* and *b*) contents of seed and callus grown plants (Table 4). EDTA application improved chlorophyll contents of Pb-stressed plants. The plants developed from callus exhibited relatively better response toward EDTA application in terms of chlorophyll recovery, while in seed grown plants, improvement was significant in chlorophyll *b* contents only. Overall, treatment Pb 0.5 + EDTA 5.0 mM proved the best dose for chlorophyll contents of both type of plants. The Pb-induced stress variably affected the chlorophyll *a* and *b* contents of seed and callus grown plants. In seed grown plant, increasing Pb concentration in the growth media gradually increased the chlorophyll *a/b* value that was reduced by EDTA amendments. No significant change in chlorophyll *a/b* value of callus grown plants was observed under application of Pb alone, however, chlorophyll *a/b* value significantly increased under treatment of Pb 1.0 + EDTA 2.5 mM. On an average, the seed grown plants contained greater chlorophyll *a* contents, while relatively higher chlorophyll *b* contents were observed in callus grown seedling but the difference was non-significant.

3.4. Biochemical attributes

Application of Pb and ETDA considerably altered the activities of various biochemical attributes of *J. effusus*. Lipid peroxidation in Pb-treated plants of *J. effusus* was estimated in terms of MDA contents. Compared to the control plants, Pb (1.0 mM) treatment significantly raised the root and non-significantly the shoot MDA contents of seed and callus grown plants (Fig. 1a). EDTA (2.5, 5.0 mM) amendments non-significantly reduced root MDA contents of Pb-stressed callus and seed grown plants, while, shoots MDA contents increased under higher EDTA (5.0 mM) concentrations.

Modification in antioxidative enzyme activities of *J. effusus* was noted under different levels of Pb alone or in combination with EDTA (Fig. 1b–d). Under increasing Pb concentrations, both seed and callus grown plants experienced a consistent rise in the activity of tested antioxidative enzymes i.e. POD, CAT and SOD in roots as well as in shoots. *J. effusus* roots experienced a further increase in the activity of these enzymes under increasing EDTA levels. However, changes in antioxidative enzyme activities were variable in plant shoots under

Pb + EDTA application. The SOD activity progressively reduced in shoots of Pb-stressed plants under increasing EDTA concentrations. Compared to Pb treatment alone, EDTA application significantly elevated POD activity in the shoots of Pb-stressed plants. The CAT activity of Pb-treated shoots showed no significant increase under EDTA application.

4. Discussions

Shoot dry biomass of *J. effusus* is very sensitive to heavy metal induced stress and, therefore can be used as a stress indicator (Grube et al., 2008). In the current study, although there was no visible phyto-toxic symptoms of Pb stress in the fronds of *J. effusus* even treated with 1.0 mM Pb, the plant biomass was inhibited under higher Pb concentrations. Higher Pb concentrations in the growth medium hindered plant metabolic processes (Liu et al., 2008), damage cell membrane integrity (Huang et al., 2008) and consequently reduced plant growth. Survival of *J. effusus* under heavy metal toxicity could be the result of increased level of antioxidative enzymes, metal exclusion from shoots, cellular sequestration (Weis and Weis, 2004) and detoxification into roots (Yanqun et al., 2004; Deng et al., 2004).

Application of EDTA improved plant growth Pb-treated seed and callus grown plants. Positive effects of EDTA applications were evident on Pb-stressed (0.5 mM) callus grown plants, which showed a substantial increment in shoot dry biomass. Pb toxicity in plants is associated with the presence of free divalent ions (Pb^{2+}), which influence leaf biochemistry. In addition, the presence of free Pb^{+2} ions in nutrient media inhibits enzymatic activity by reacting with the sulphydryl group (Seregin and Ivanov, 2001). On the other hand, EDTA reduces Pb toxicity possibly by forming complexes with Pb ions and reducing the availability of free Pb ions in plant tissues (Shahid et al., 2013; Xu et al., 2007). Role of proper EDTA concentrations has already been suggested in reducing Pb toxicity in crop plants (Doncheva et al., 2013; Liu et al., 2008; Shahid et al., 2013). Huang et al. (2008) found that exogenously applied EDTA (200 mM) protected *Sedum alfredii* plants from root cell death.

J. effusus has been found as one of the seventeen terrestrial plant species growing and accumulating exceptionally large concentrations of Pb, Cd, Cu and Zn at Lanping Pb–Zn mine area in China (Yanqun et al., 2004). In this study, both seed and callus grown plants accumulated large quantities of Pb

Table 4 Chlorophyll contents (mg g^{-1} dry weight) in *Juncus effusus* L. leaves under Pb and EDTA amendments.

Concentration (mM)	Chlorophyll *a*		Chlorophyll *b*		Total chlorophyll (*a* + *b*)		Chlorophyll (*a/b*)	
	Seed grown	Callus grown	Seed grown	Callus grown	Seed grown	Callus grown	Seed grown	Callus grown
Control	4.05[a]	3.94[a]	3.36[bc]	3.71[b]	7.41[b]	7.65[b]	1.20[ab]	1.06[b]
Pb 0.5	3.92[ab]	3.73[b]	3.13[c]	3.69[b]	7.06[c]	7.42[bc]	1.25[ab]	1.01[b]
Pb 1.0	3.83[b]	3.53[c]	2.84[d]	3.41[c]	6.67[d]	6.94[c]	1.35[a]	1.04[b]
Pb 0.5 + EDTA 2.5	3.95[ab]	3.91[a]	3.72[ab]	3.81[b]	7.67[ab]	7.72[b]	1.06[b]	1.03[b]
Pb 1.0 + EDTA 2.5	3.67[b]	3.89[ab]	3.81[a]	2.94[d]	7.48[b]	6.83[c]	0.96[bc]	1.33[a]
Pb 0.5 + EDTA 5.0	4.13[a]	3.95[a]	3.72[ab]	4.32[a]	7.84[a]	8.26[a]	1.11[b]	0.91[c]
Pb 1.0 + EDTA 5.0	3.88[b]	3.85[ab]	3.82[a]	3.54[c]	7.70[ab]	7.38[bc]	1.02[bc]	1.09[b]
Mean	3.92[A]	3.83[A]	3.48[A]	3.63[A]	7.40[A]	7.46[A]	1.13[A]	1.07

Values are the mean of three individual replicates. Means followed by the same letter (a–d) within each column are not significantly different at $P < 0.05$.
Means sharing the same capital letters (A) show non-significant difference between callus and seed grown plants.
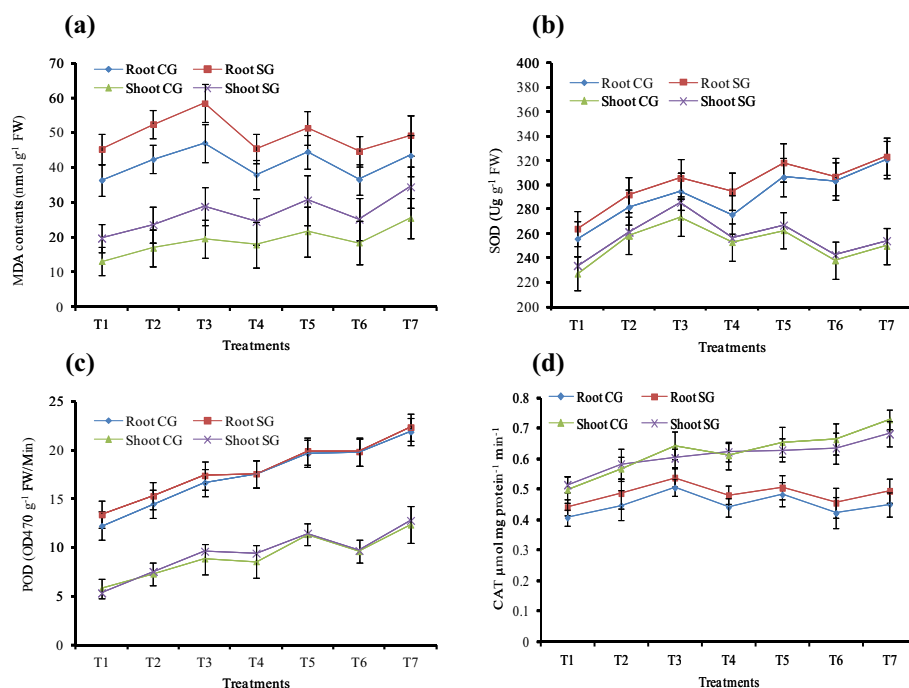
Figure 1   (a) Alterations in malondialdehyde (MDA) contents (nmol g^{-1} fresh weight) in the roots and shoots (*Juncus effusus* L.) as influenced by Pb and EDTA levels. (b) Changes in superoxide dismutase (SOD) activity (Ug g^{-1} fresh weight) in the roots and shoots (*Juncus effusus* L.) under different levels of Pb and EDTA. (c) Peroxidase (POD) activity (OD470 g^{-1} fresh weight min^{-1}) in the roots and shoots (*Juncus effusus* L.) as varied by Pb and EDTA levels. (d) Catalase (CAT) activity (μmol mg^{-1} protein min^{-1}) in the roots and shoots (*Juncus effusus* L.) as influenced by Pb and EDTA additions. T1: control (without Pb and EDTA), T2: Pb 0.5 mM was added, T3: Pb concentration was increased to 1.0 mM, T4: Pb 0.5 + 2.5 mM EDTA, T5: Pb 0.5 + 5.0 mM EDTA, T6: Pb 1.0 + 2.5 mM EDTA and T7: Pb 1.0 + 5.0 mM EDTA. Fresh weights (FW) of both seed-grown (SG) and callus-grown (CG) plant parts were used for measuring the enzymatic activities.

in their tissues with relatively more deposition in their roots. *J. effusus* plants possess a natural potency to tolerate higher metal (Zn, Cu, Cd, Mn, Cr and Pb) concentrations by restricting them in their underground parts (Deng et al., 2004; Najeeb et al., 2009, 2011). Compared to seed grown plants, callus grown plants were more responsive to Pb accumulation in roots. It shows potential of callus grown plants to translocate lower Pb contents to the aboveground parts by immobilizing them into underground parts. It was also evident from improved Pb tolerance and growth recovery of callus grown plants in response to EDTA amendments as compared to seed grown plants. Similar results have been reported by Nehnevajova et al. (2007) and Sibov et al. (1999) who used tissue culture-induced somaclonal variations for selecting plants with improved metal tolerance.

Presence of Pb^{+2} ions inside plant cells interferes the normal functioning of photosystem II of chlorophyll, and consequently reduce chlorophyll and carotenoids (Kumar et al., 2011). In the present study, reduced chlorophyll contents of Pb-stressed *J. effusus* shoots could be the result of Pb-induced inhibited activity of δ-ALA-dehydratase (Sharma et al., 2005), chloroplast impairment and interruption in photosynthetic activity (Islam et al., 2008). Change in chlorophyll *a* to *b* ratio is an important parameter for estimating physiological responses of plants to metal toxicity stress (Li et al., 2009). In plants, Pb accumulation not only reduces total chlorophyll contents but also the ratio of chlorophyll *a* to *b*, due to comparatively rapid hydrolysis of chlorophyll *a* (Abdel-Basset

et al., 1995). Relatively stable chlorophyll *a/b* value of callus grown plants under Pb stress suggested that these plants had higher capability to endure metal induced stress. EDTA application to growth media improved the chlorophyll contents of Pb-stressed plants, while, the chlorophyll recovery was better in callus grown plants.

Mode of Pb-induced toxicity in plants is different from other transition metals i.e. it produces OH^{-1} radicals via Fenton reactions and disturbs photosynthetic electron transport leading to production of ROS and chlorophyll degradation (Islam et al., 2008). We observed increased lipid peroxidation and altered antioxidative enzyme activity of *J. effusus* seedlings under Pb toxicity stress. Since lipid peroxidation is a biochemical marker for stress-induced damage in plants (Zhou and Leul, 1999), elevated MDA level under increasing Pb concentration indicated that Pb toxicity induced oxidative damage to *J. effusus* plants. Lead induced lipid peroxidation has already been reported in plants including *S. alfredii* (Liu et al., 2008) and in *Najas indica* (2010).

J. effusus endured Pb toxicity through up-regulation of antioxidative enzymes viz. SOD catalyzed the dismutation of ROS such as O^{-2} and OH^{-1} to O$_2$ and H$_2$O$_2$, while CAT and POD decompose H$_2$O$_2$ to water. Tolerance to heavy metal (Cu, Cd, Pb, Zn and As) stress in *J. effusus* is found to be the function of up-regulation of antioxidative enzyme activities (Najeeb et al., 2009; Sun et al., 2010). Similarly elevated levels of antioxidant enzymes SOD, APX and GR have been reported to induce tolerance to Pb toxicity in *N. indica* (Singh

et al., 2010) and in pea (Malecka et al., 2009). It was obvious from metal uptake analysis that EDTA amendments caused relatively higher Pb accumulation in plant roots. Elevated activities of antioxidative enzymes in root tissues contributed to defy Pb-induced oxidative injury in the plant roots. However, increased Pb translocation damaged plant shoots and possibly was responsible for diminution of the activities of some antioxidative enzymes.

5. Conclusions

This study reflected that Pb-tolerant variants of *J. effusus* with higher phytostabilization potential could be developed through somaclonal manipulation. Application of EDTA alleviated the Pb-induced toxicity by improving plant growth, biomass and antioxidative enzyme activities. Under Pb stress, callus grown plants were more responsive to EDTA application for growth and chlorophyll recovery. Thus, the plants developed through somaclonal manipulation along with the addition of appropriate EDTA concentration could be suitable for cultivation in Pb-polluted wetlands.

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