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# Breeding by *In vitro* Culture to Improve Tolerance and Accumulation of Lead in *Cynodon Dactylon* L.

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## ABSTRACT

**Turfgrasses are usually important groundcover plants in many landscapes. They occupy the lowest surface of the landscape, close to pollutant particles. So, they can be an attractive option for environmental remediation. Today, high concentrations of hazardous chemicals such as lead are among the most serious environmental problems. *In vitro* selection of turfgrass accumulator or tolerant of toxic ions may lead to production of plants that have better adaption to polluted sites. This study was undertaken to investigate the tolerance or accumulation potential in Bermuda grass to high concentrations of lead under tissue culture condition and identifying differences at the molecular level among accumulators by RAPD markers. Callus that were used for *in vitro* selection were exposed to different concentrations of lead in the media. After the first mowing, tolerant plantlets were evaluated for lead accumulation potential. All plants of Bermuda grass originating mainly from regeneration pathways had undergone genetic changes. The results revealed that occurrence of somaclonal variation via somatic embryogenesis and organogenesis of Bermuda grass culture with a frequency of 33%. Although some *in vitro* derived plants showed increase in uptake potential of lead in their shoots (2 times higher Pb extraction), there were some regenerates with decreased lead accumulation in shoot, and some varieties without any changes in lead uptake properties in comparison to the control. Molecular marker could be efficient in determining the genetic changes induced by somaclonal variation. The improvement of lead accumulation in lead extraction varieties indicated a successful mutation in Bermuda grass for breeding traits such as phytoremediation purpose.**

**Keywords**: Bermuda grass, Lead accumulation, Lead-RAPD.

## INTRODUCTION

Today, high concentrations of hazardous chemicals are among the most serious environmental problems due to intensive industrial activities, (Azevedo and Azevedo, 2006; Ghosh and Singh, 2005). Among the toxic metal contaminants, lead (Pb) is one of the major elements that pollute the environment and cause serious threat for human health (Cunningham *et al.*, 1995). Most conventional clean up technologies do not provide acceptable solutions to toxic metal pollution because they are generally too costly and no feasible technology is yet available for many pollutants (Hinchman *et al.*, 1996; Kramer, 2005). Therefore, researchers have considered phytoremediation as a cost-effective and long lasting technique to remove or stabilize various pollutants (McGrath and Zhao, 2003). However, hyperaccumulators have limited ability to uptake heavy metals because of their small size, slow growth, and low amounts of biomass; and these factors

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may affect heavy metals accumulation (Nehnevajova *et al.*, 2007). Therefore, in order to have successful phytoremediation, it is important to identify suitable species that have improved capacity in this issue (Baker, *et al*. 1994). Use of plant growth regulators (such as 5-aminolevulinic acid) has become an approach to increase the plant tolerance against Pb stress conditions through enhanced biomass and uptake of nutrients in plant (Tian *et al*., 2014). In addition to biological methods, plant biotechnology can be used in the development of new plants varieties for improving agronomic performance as well as plant resistance to different biotic and abiotic stress such as heavy metals (Kaeppler *et al*., 2000; Olhoft and Philips, 1999; Skirvin *et al*., 1994). Plant tissue culture and *in vitro* selection techniques are used to increase the tolerance and accumulation of heavy metals have been reported in numerous plant species and populations (Rout *et al.,* 1999). Since some alternations are epigenetic rather than genetic via somaclonal variations in plants (Larkin and Scowcroft, 1981), analysis of regenerated plants should include specific molecular and genetic evaluations as well as morphological identifications (Sabir *et al*., 1992).

Turfgrasses are usually important groundcover plants in many landscapes, used for beauty and protection of the environment. They occupy the lowest surface of landscape, close to pollutant particles and produce high volume of biomass, so, they can be attractive option for environmental remediation. Bermuda grass is a persistent plant used as warm-season turfgrass, needs low level of maintenance requirement, and has good adaptation to drought and salty soil (Li and Qu, 2004; Beard, 1967). Turfgarss plants could uptake Pb and other heavy metals in excess of 1 mg $kg^{-1}$ when grown in non- polluted soils (Jones *et al*., 1973) and up to 100-300 mg $kg^{-1}$ of their dry matter in polluted soil (Yoon *et al*., 2006; Qu *et al*., 2003).

*In vitro* selection of turfgrass accumulator or tolerant of toxic ions may lead to production of plants that are better adapted to polluted sites and can enable better management of remediated soil. However, little research has been performed to use *in vitro* selection techniques for improving tolerance and accumulation ability to heavy metals in turfgrass genus.

Thus, this experimental study was undertaken to investigate the tolerance and accumulation potential in Bermuda grass to high concentrations of Pb during *in vitro* culture. A further objective of the study was to test whether RAPD markers could be used to identify differences at the molecular level among the Pb accumulating Bermuda grasses.

## MATERIALS AND METHODS

### Plant Material and Cultivation

The genetic homogeny of seeds of common Bermuda grass (*Cynodon dactylon* L.) used in this study was purchased from the Barunbrug Seed Co., Ltd., Denmark. These seeds were exposed to high Pb concentration in growth media for selection of elite plants during regeneration phase. The seeds were surface sterilized using 70% ethanol for 1 minute, followed by using 100% Clorox (5.25% sodium hypochlorite, active ingredient) for 20 minutes, respectively (Salehi and Khosh-khui, 2005). Callus induction medium was MS (Murashige and Skoog) supplemented with 1 mg $L^{-1}$ 2,4-D. Regeneration medium was MS medium supplemented with 1 mg $L^{-1}$ 2,4-D in combination with 0.01 mg $L^{-1}$ BA for somatic embryogenesis inducing and with 1 mg $L^{-1}$ 2,4,5-T in combination with 0.01 mg $L^{-1}$ BA for inducing organogenesis . Rooting medium was half-strength MS medium supplemented with 5 mg $L^{-1}$ NAA. All of media containing 30 g $L^{-1}$ sucrose, 7 g $L^{-1}$ agar and pH was adjusted to 5.8. All of regeneration stages from somatic embryogenesis or organogenesis to rooting and acclimatization stages were done by 100 mg $L^{-1}$ Pb, consistently. Once a substantial

root system developed, rooted explants were transferred to pots containing sterilized perlite and were grown under greenhouse conditions. After the first mowing of acclimatized plants, Pb tolerant plants were irrigated with 800 mg $L^{-1}$ $Pb(NO_3)_2$ for evaluating Pb accumulation potential. Also, some seeds were sown in pots including perlite without any treatment, as control (Figure 1).

### Plant Harvest and Metal Analysis

### Fresh and Dry Weight (FW and DW)

The shoots were harvested one month after the Pb treatment and then weighed fresh. Plant shoots were first rinsed gently in distilled water to remove particles adhered to the plants. After excess water was removed, samples were dried in an aired oven at 70ºC for 24 hours and dry weight was recorded.

### Pb Concentrations

Shoot samples were grounded and incinerated at 500°C for 5 hours. After that, the ash of each sample was digested in 20 mL of 1M solution of $HNO_3$ on a hot plate and aliquots solutions were filtered by filter paper. Pb contents were determined by using a flame atomic absorption spectrometer.

### Molecular Evaluation

All plants of Bermuda grass originating mainly from regeneration pathways together with the control plants were included in this study to investigate genetic variability in relation to Pb tolerance and accumulation. DNA was extracted from fresh leaf tissue of Bermuda grass according to modified Dellaporta protocol (Dellaporta *et al*., 1983). Ten-mer primers were used in the amplification reactions. A total of 35 primers (TIBMOLBIOL, OPG, OPN, OPD, OPE, OPR; Co., Germany) were selected to

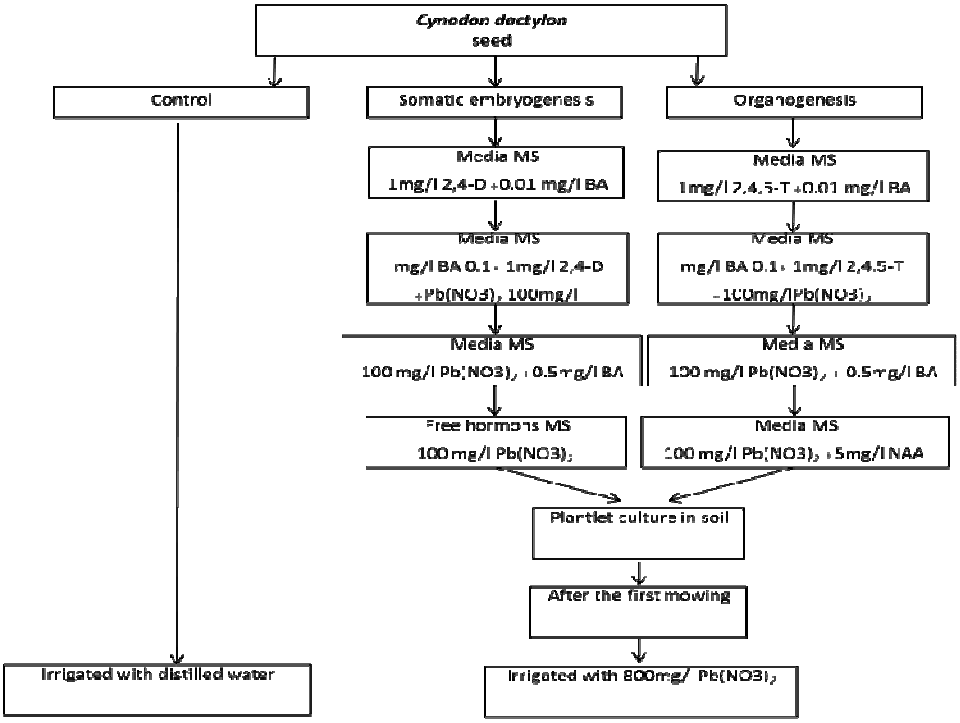


**Figure 1.** Procedure of developing new varieties of *Cynodon dactylon* tolerant or accumulator of Pb, using *in vitro* somaclonal selection.

be used in the amplification reactions using Pb tolerance Bermuda grass DNA from organogenesis and somatic embryogenesis pathways. The amplifications were performed in a DNA Thermal Cycler (iCycler, Bio Rad Co., USA). The PCR conditions were: denaturation for 4 minutes at 94°C, 35 cycles of 92°C for 1 minute, of 37°C for 1 minute, and of 72°C for 2 minutes; with a final 5 minutes extension at 72°C. Amplification products were separated by electrophoresis on 1.2% agarose gels in 1X TBE buffer at 60 volts for three hours and photographed under UV light by a Gel Doc system (UVP, Bio Rad Co., USA).

### Experimental Design and Data Analysis

A Completely Randomized Design (CRD) arrangement was used for the experiment. Data were analyzed using the ANOVA procedure of SAS statistical software (version 9.2). Each amplification product was analyzed by comparing the RAPD profiles of different plants derived *in vitro* culture in terms of presence (1) or absence (0) of each DNA fragment and Jaccard's similarity coefficient values for each pairwise comparison between accessions were estimated and a similarity coefficient matrix was constructed. Data from the similarity matrix were used for cluster analysis by the Un-weighted Pair-Group Method with Arithmetic averages (UPGMA) and the resulting cluster was represented as a dendrogram. All the calculations were performed by using the NTSYS-pc software ver. 2.02 (Roholf, 1998). The molecular somaclonal variation was estimated by the frequency of polymorphic bands in total bands scored.

## RESULTS

Results showed that 100 mg $L^{-1}$ of Pb treatment had acceptable growth and regeneration in all of the explants. Therefore, this concentration was supplemented in regeneration, proliferation, and rooting media during *in vitro* culture. In regenerated plant, results of the analysis of variance revealed that many traits such as number and length of stolon, width of leaf, fresh and dry weight and Pb concentration and uptake were affected by regeneration pathways, whereas the stolon diameter, length of internode length of leaf, and Pb concentrations were not statistically significant. Data in Table 1 show that all of regenerated plant traits, except Pb concentration, were significantly increased through organogenesis path, compared with the control. In regenerated plants of somatic embryogenic calli, traits were not significantly different than the control, except leaf width. The results indicated that the *in vitro* regeneration was effective in variation of morphological traits of Bermuda grass. Also, enhanced growth including number and length of stolon and leaf width led to increased biomass production and that was an effective factor to improve Pb uptake in regenerated varieties through organogenesis pathway.

The new varieties were evaluated for biomass production and subsequently Pb extraction (shoot dry weight×shoot Pb

**Table 1.** The morphological variations of Bermuda grass (*Cynodon dactylon* L.) plants regenerated on media containing 100 mg $L^{-1}$ Pb.[a]

| Treatment[b] | Stolon number | Stolon length (mm) | Leaf width (cm) | Fresh weight (g) | Dry weight (g) | Pb Concentration | Pb uptake (mg $Kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| 1 | 11.2[a] | 67.4[a] | 3.6[a] | 19.5[a] | 6.5[a] | 11[b] | 71.1[a] |
| 2 | 7.6[ab] | 55[ab] | 2.9[b] | 11.2[b] | 4[b] | 17.3[a] | 65.3[ab] |
| 3 | 3.8[b] | 32.8[b] | 2[c] | 9.8[b] | 3.5[b] | 12.3[ab] | 43.3[b] |

[a] Mean values followed by different letters are significantly different at $P \leq 0.05$. [b] (1) Derived organogenesis pathway; (2) derived embryogenesis pathway, and (3) Control.

**Table 2.** Comparison of Pb tolerant varieties of *Cynodon dactylon* L. (*in vitro* condition) and the control.[a]

| (%)Pb extraction | (%)DW | Regenerate varieties |
|---|---|---|
| 100 | 100 | control |
| 9/96 | 166 | Org1 |
| 4/212 | 184/5 | Org2 |
| 2/166 | 4/147 | Org3 |
| 9/193 | 4/194 | Org4 |
| 3/151 | 3/242 | Org5 |
| 8/98 | 1/77 | Emb1 |
| 6/175 | 5/92 | Emb2 |
| 3/178 | 1/173 | Emb3 |

[a] Values of dry weight and Pb extraction are given in percent (%) and represent mean of three replicates. Dry weight and metal extraction values of regenerated plants were related to control plants (100%). Org's are regenerate varieties through organogenesis and Emb's are regenerate varieties through somatic embryogenesis.

concentration). Table 2 shows dry weight and Pb extraction of *in vitro* bred varieties obtained from Pb selection lines through somatic embryogenesis (Org) and organogenesis (Emb) pathway, compared with the control plants of *Cynodon dactylon*. Among tolerant regenerated varieties, six varieties showed more Pb extraction than the control plants. The best regenerate Org2 showed a 2 times higher Pb extraction and 1.8 times higher biomass production, as compared to the controls. In contrast, excluder varieties Org1 and Emb1 produced, respectively, 1.6 and 0.7 times biomass as the control, but the Pb extraction decreased by about 2-3 times in these regenerates as compared to the control plants. Hence, Pb uptake not only was affected by Pb concentrations but also was influenced by biomass production of *in vitro* varieties. Also, improvement of biomass did not lead to higher Pb concentration in new varieties. In general, somaclonal variation occurred randomly in *in vitro* varieties: some of regenerants had enhanced and some had lower Pb accumulation properties through *in vitro* regeneration. Generally, *in vitro* organogenesis led to increased biomass production and Pb extraction in the new varieties of Bermuda grass.

We have tested the sensitivity of the RAPD technique for detecting polymorphism among new varieties from regenerated Bermuda grass selected *in vitro* condition (tolerant and accumulator of Pb) and the control. Nine of the 35 primers employed (BA-15, BB-06, BB-07, BB-08, BB-11, OPG-11, OPG-19, OPN-14) revealed scoreable polymorphisms for all of the above genotypes. The number of bands for each primer varied from 35 for primer OPN-14 to 114 for primer BA-15 for total of genotypes. Each primer (from the group of 35) generated a unique set of products ranging from 50 bp to 2,000 bp in size. A total of 1,338 bands were scored, of which 214 were variable between plants with average frequency of 15.99%. The frequencies with which RAPD band changes were observed for somaclonal varieties Bermuda grass are shown in Table 3. Frequency of band changes is regeneration pathway dependent: the frequencies for varieties derived from somatic embryogenesis pathway were more than those from organogenesis and the control.

To explore high ability of Pb extraction on DNA patterns, some unique bands were scored. The RAPD patterns obtained with BB-08, BB-07 and OPN-14 showed fragments from 100 bp to 2,100 bp (BB-08 100 , 2100, BB-07 100, 2100 and OPN-14 100, 2100) in Org2 variety (2- fold higher Pb extraction and 1.8- fold higher DW production) by organogenesis in *in vitro* culture (Figure 2 and Table 4). Also, on the basis of RAPD, profile comparing revealed two bands 2,100 and 50 bp by primer BB-08 and OPG-11, respectively, that amplified in Emb2 variety from embryogenesis pathway (Figure 2 and Table 5). These bands were not present in the other regenerated plants and the control and, probably, can be used to characterize the Pb hyperaccumulator regenerated.

**Table 4.** Presence of polymorphic RAPD bands in Bermuda grass regenerate Org2 versus other regenerates and the control.

| Band size (Bases) | Total of plant[a] | | $R^2$ (Coefficient of determination) |
|---|---|---|---|
| | Org2 | Other plant | |
| BB08-2100 | + | - | 0.84 |
| BB08-1600 | + | - | 0.89 |
| BB08-450 | + | - | 0.89 |
| BB07-350 | + | - | 0.84 |
| OPN14-700 | + | - | 0.84 |
| OPN14-100 | + | - | 0.94 |

[a] Org2 was derived Pb tolerance regenerates from organogenesis pathway that show highest Pb extraction during this study and other plants were total of embryogenesis regenerates, other organogenesis regenerates and the controls.

**Table 5.** Presence of polymorphic RAPD bands in Bermuda grass regenerate Emb2 versus other plants.

| Bands | Total of plant [a] | | $R^2$ (Coefficient of determination) |
|---|---|---|---|
| | Emb2* | Other plants | |
| BB08-2100 | + | - | 0.84 |
| OPG11-50 | + | - | 0.84 |

[a] Emb2 was derived Pb tolerance regenerates from embryogenesis pathway that show highest Pb concentration during this study and other plants were total of organogenesis regenerates, other embryogenesis regenerates and the controls.

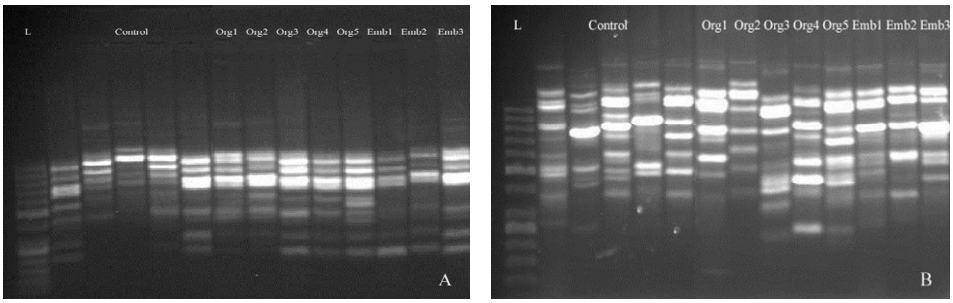


**Figure 2**. RAPD profile of primers BB-07 (A) and BB-08 (B). Abbreviations: Org1-5= Plants were derived Pb tolerance regenerates from organogenesis pathway; Emb1-3= Plants were derived Pb tolerance regenerates from embryogenesis pathway, and L= Ladder.

## DISCUSSION

Results revealed that a total of five varieties had unique morphological characters different than the control plant. In the present experiment, Org2 and Emb2 were noticeable varieties for phytoremediation purposes and were used to evaluate banding pattern of plants obtained via different inducing pathways. In this experiment, phenotypic changes and modified Pb concentration or uptake were observed in new varieties *in vitro* culture condition. Similar phenotypic variations of regenerated plants were reported by other authors. Jain *et al*. (1989) and Nehnevajova *et al.* (2007) found some alternations in leaf color, form and reduced growth in regenerates

of *Brassica juncea*. Similarly, Guadagnini (2000) obtained new varieties with morphological abnormalities in *in vitro* cultured tobacco. Mohajer and Taha (2014) reported the difference between root cells of the *in vitro* and *in vivo* in *Onobrychis sativa* based on cytological information of embryogenic and Non-embryogenic callus.

One of the challenges to enhance metal accumulation in hyperaccumulator plants is to produce large amounts of biomass as turfgrass groups. However, there are few studies dealing with turfgrasses tolerance to high levels of heavy metals and its maximum accumulation ability in their tissues (Qu., 2003; Cheng, *et al*., 2007; Taghizadeh *et al*., 2011). We found that some varieties had a Pb enhancing uptake in their shoots, ranging 1.5-2 fold compared with the controls. Also, some plants showed reduced Pb extraction in shoots. These different behaviors towards heavy metals have been reported in somaclonal varieties, for instance poplar, tobacco, and Indian mustard (Nehnevajova *et al*., 2007; Spirochova *et al.*, 2003; Guadagnini, 2000). From the eight tolerant regenerates, six varieties included four plants regenerated via organogenesis and two plants via embryogenesis (75% of the total regenerates) and showed higher shoot Pb extraction than the control plants. These findings are in agreement with Guadagnini (2000) and Nehnevajova *et al*. (2007) who used *in vitro* breeding for selected plants with enhanced metal extraction. They observed that 15 and 23% of tobacco and Indian mustard varieties, respectively, showed higher shoot metal concentration, as compared to the control. But, they obtained improved varieties in more metal extraction amount by shoots compared to our finding. The reason for such low Pb extraction in somaclonal varieties of Bermuda grass could be the use of different genus plants.

Tissue culture can successfully be used to induce variation in cultured and *in vitro* selection (Olhoft and Philips, 1999; Kaeppler *et al*., 2000). The somaclonal variation may result in qualitative and quantitative changes in regenerated varieties. As somaclonal variation might be genetic or epigenetic, it was important to carefully select the plants that could successfully transfer this variation to progeny. RAPD marker could successfully serve this purpose.

In our study, we found banding pattern polymorphism of the regenerated plants. In addition to genetic changes, phenotypic alterations also occurred in regenerated plants during tissue culture. Some authors reported RAPD polymorphisms in plants derived from tissue culture (Homhuan *et al*., 2008; Tafvizi *et al*., 2009; Al-Zahim *et al*., 1999). However, some investigators observed no differences in RAPD patterns or incidental changes in plants derived *in vitro* culture e.g. Begonia, spruce and sugar cane (Bouman and De Klerk, 2001; Fourre *et al*., 1997). There are various factors that lead to somaclonal variation at regenerant plants via *in vitro* culture; for instance, genotype, ploidy level, *in vitro* culture age, explants, exogenous hormones type and concentrations, and culture type, etc. (Skirvin *et al*., 1994). Commonly, 2,4-D as a synthetic auxin is one of the reasons for somaclonal variations induced during *in vitro* cultures. The maximum variation was reached in plants regenerated from 2,4-D embryogenic callus. Bouman and De Klerk (2001) observed that 2,4,5-T and picloram were not as potent in inducing variation as 2,4-D in begonia *in vitro* culture. RAPD analysis of Bermuda grass regenerates revealed an average frequency of 17% among plants derived via organogenesis and embryogenesis pathway. In other investigations, frequency of RAPD polymorphism has been calculated less than the frequency of polymorphic in this study. Munthali *et al*. (1996) detected an overall frequency of 0.05% in somaclones of sugar beet. Also, Al-Zahim *et al*. (1999) reported average frequency of variation in garlic regenerantes at 0.63%. However, the high variation frequency of regenerates' Bermuda grass depends on genotype, period of culture, plant hormone regulators, time of subculture, and Pb treatment.

In this study, *in vitro* bred varieties of Org2 accumulated Pb 2-fold higher than the others and their RAPD pattern revealed a number of bands that were not present in the others.

These bands may be related to Pb accumulation genes and can be used to characterize the accumulator plants. However, there is no study on Pb hyperaccumulation in levels of molecular banding pattern. Previously, Zambrano *et al*. (2003) reported a 564-bp band in Glyphosate-Tolerant Sugarcane Cellular Line which was not present in sensitive cellular line by RAPD patterns.

## CONCLUSIONS

Generally, the results of the present study show the occurrence of somaclonal variation due to different supplemented auxin in media via somatic embryogenesis and organogenesis of Bermuda grass. An interesting observation from this study is that although some *in vitro*-derived plants show the uptake potential of Pb in shoots, as evidenced by higher Pb transferred from root to shoot, there were excluder regenerates with decreased Pb accumulation in shoot, and some varieties without any changes in Pb uptake properties compared with the control. The total Pb uptake in shoots of these *in vitro* bred varieties was enhanced compared to the control. RAPD markers were shown to be efficient in determining the genetic changes induced by somaclonal variation. The RAPD banding patterns revealed bands in Pb extractor regenerates. These amplification products revealed a number of bands that can be used to characterize the accumulator regenerated Bermuda grass (Org2 and Emb2). Therefore, the improvement of Pb accumulation in accumulator and extraction varieties indicated a successful mutation in Bermuda grass for breeding traits suitable for purposes such as phytoremediation.

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## اصلاح درون شیشه ای به منظور بهبود مقاومت و تجمع سرب در برموداگرس

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## چکیده

معمولا چمن‌ها به عنوان یک گیاه پوششی مهم در اغلب فضاهای سبز می باشد. چمن ها در پایین ترین سطح فضای سبز و در ارتباط نزدیک با ذرات آلاینده هستند. بنابراین می توانند به عنوان یک گزینه جذاب به عنوان پالاینده محیط زیست به‌کار روند. امروزه، غلظت‌های بالای مواد شیمیایی خطرناک مانند سرب به عنوان یک مشکل جدی محیط زیست در آمده است. انتخاب درون شیشه ای چمن‌های مقاوم و تجمع کننده یون‌های سمی ممکن است منجر به تولید گیاهانی با سازگاری بهتر به مناطق آلوده گردد. در این پژوهش بررسی مقاومت و تجمع فلزی برموداگرس نسبت به غلظت‌های زیاد سرب در طی شرایط کشت بافت و شناسایی تفاوت در سطح مولکولی در بین تجمع کننده‌ها با استفاده از نشانگر RAPD صورت گرفت. کالوس مورد استفاده در معرض غلظت‌های مختلف سرب در محیط کشت برای انتخاب درون شیشه ای قرار گرفت. پس از اولین سربرداری، گیاهچه های مقاوم برای توان تجمع سرب مورد ارزیابی قرار گرفتند. تمامی گیاهان حاصل از مسیر باززایی دچار تغییر ژنتیکی شده بودند. نتایج وقوع تنوع سوماکلونال با فراوانی ۳۳درصد از طریق جنین‌زایی سوماتیکی و اندام‌زایی کشت درون شیشه ای برموداگرس نشان داد. اگرچه برخی گیاهان حاصل از کشت درون شیشه‌ای افزایش توان تجمع سرب در اندام هوایی (دو برابر تجمع بیشتر سرب)را نشان دادند، تعدادی از گیاهان باززایی شده نیز کاهش تجمع سرب را در اندام هوایی داشتند و برخی از واریته‌ها نیز هیچ‌گونه تغییری در صفت تجمع سرب در مقایسه با شاهد نداشتند. نشانگر مولکولی توانست به خوبی تغییرات ژنتیکی القا شده توسط تنوع سوماکلونال را شناسایی کند. بهبود تجمع سرب در واریته‌های استخراج‌گر سرب نشان دهنده یک جهش موفقیت آمیز در برموداگرس به منظور اصلاح صفاتی مانند صفات گیاه پالایندگی می‌باشد.