GENETIC ASPECTS OF HEAVY METALS PHYTOREMEDIATION ABILITIES OF SUNFLOWER PLANTS

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Phytoremediation is generally defined as the use of plants to remediate contaminated soils or waters; it can be applied to the remediation of inorganics as well as organics primarily in the rhizosphere. Phytoremediation is still in its initial stages of research and development and the number of field tests conducted to date is still small (Dietz and Schooner, 2001). The basic knowledge that plants can be used for environmental remediation has been around for a long time, however, only recently has the value of metal-accumulating terrestrial plants for environmental remediation been fully realized (Salt *et al*., 1996). There are different types of phytoremediation including phytostabalization, phytoextraction and biovolatilization (Lytle and Lytle, 2001).

The feasibility of using vegetation as a viable, cost-effective alternative to clean up metal-contaminated soils depends largely on the identification and selection of plant species that possess the ability to accumulate metals, while producing high biomass using current crop production and management practices. Metal-hyperaccumulator plants have long been of interest to biologists because of their distinctive ecology, physiology, genetics and their high degree of metal tolerance. Over 400 species of hyperaccumulator plants are now known, most of which are of limited distribution in special ecosystems, restricted to metalliforous soils, and mostly trees and small grasses with very limited economical values (Boominathan and Doran, 2003).

Sunflower (*Helianthus annuus* L.) plants is believed to be domesticated from wild sunflower around 1000 B.C. in the western US, wild sunflower is highly branched with small heads and small fruits, in contrast to the unbranched stems, large head of domesticated sunflower. The crop is cultivated now worldwide for oil-seed production, and to lesser extent as animal and human food (Datta, 1988).

The present study was undertaken to select heavy metal tolerant sunflower plants, after chemical mutagenesis and to try to understand the genetic nature of heavy metal tolerance. Genetic characterization of materials using DNA molecular markers based on RAPD analysis was carried out. The role of superoxide dis-

mutase (SOD) and glatathione S-transferase (GST) enzymes activities, in the ability of sunflower plants to tolerate soil heavy metals.

MATERIALS AND METHODS

Materials

An annual sunflower (*Helianthus annuus* L.) land race obtained from Burg-el-Arab region was used as the basic genetic material in the present study. Collected seed samples were grown under greenhouse conditions at the Faculty of Agric. (El-Shatby), Alexandria Univ., for several generations.

Methods

Achenes (seeds) of this local variety were treated with 0.2% for 3.5 hours ethyl methane sulphonate-EMS (Sigma, USA).

Exposure to heavy metals

Samples were allowed to continue germination in petri dishes at room temperature in the presence of concentrations of the following heavy metal salts, dissolved in distilled water: Zinc sulfate, $ZnSO_4$. $7H_2O$, 0-1000 ppm solutions, Copper sulfate, $CuSO_4$. $5H_2O$, 0-1000 ppm solutions, Cadmium chloride, $CdCl_2$, $2H_2O$, 0-500 ppm solutions and Nickel chloride, $NiCl_2$. $6H_2O$, 0-500 ppm solutions.

Then the $L.D_{50}$ doses for each heavy metal were calculated from the resulting growth curves according to El-Metainy *et al.* (1971).

Original (EMS-untreated) achenes were not included, since it turned to be highly susceptible to heavy metals.

Selection of heavy metal tolerant mutants

EMS treated achenes were germinated in petri dishes at laboratory conditions, in the presence of the LD_{50} doses of either one of zinc sulfate (575 ppm), copper sulfate (465 ppm), cadmium chloride (165 ppm),or nickel chloride (195 ppm). At maturation, individual capatum were harvested and viable achene setting for each treatment was calculated. The total numbers of viable selected achenes for each treatment were grouped for each treatment, since the numbers of viable achenes were very limited; especially for cadmium stressed lines.

Enzyme assay

Super oxide dismutase (SOD) activity was determined according to a modified procedure from those suggested by Giannopolitis *et al.* (1977). Glutathione S-transferase (GST) activity in the present materials was measured according to Habig and Jakoby (1981).

Polymerase chain reaction (PCR) procedures

DNA purification was performed on 10-30 mg leaves fresh/frozen (liquid nitrogen frozen), using the procedures

suggested by Sambrook *et al*. (1989) and RAPD Polymerase Chain Reaction (PCR) was conducted to detect random amplified polymorphic DNA (RAPD) markers using 10-mer random primers showed in Table (1) according to (Williams *et al*., 1990).

Statistical analysis

Analysis of variance (ANOVA) tests were done according to the methods suggested by Snedecor (1956). The statistical parameter, known as probability exact test that was developed by Fisher (1935). According to this test the probability estimates are calculated according to the 2 x 2 contingency tables, using a BASIC computer program published by Forbes (1984).

RESULTS

In the present study, EMS (0.2%, 3.5 h) treated plants tolerant to the four heavy metals, i.e., Zn, Cu, Ni and Cd, were selected. Only five seedlings (the most vigorous) out of 100 treated seedlings for each treatment were selected and transferred to soil to continue their growth till maturity at green-house conditions.

The numbers of viable and aborted achenes collected from each plant, in addition to the percentage viability for each heavy metal group at maturation showed in Table (2).

All selected groups turned to be more tolerant than their corresponding control. The zinc group was significantly tolerant than the control, it showed about 1.5 folds increase in tolerance to zinc sulfate. The copper group was highly significant tolerant than the control, it showed about 2 folds increase in tolerance to copper sulfate. The cadmium group was also highly significant than the control, it showed about 3.5 folds increase in tolerance to cadmium chloride. Moreover, the nickel tolerant group was highly significant tolerant than the control, it showed about 3 folds increase in nickel chloride tolerance.

Enzyme colorimetric assay

Table (3) summarizes the mean of superoxide dismutase (SOD). It can be seen from these results that all groups of sunflower plants under study responded almost equally to heavy metal stresses. The zinc, copper, nickel or cadmium tolerant selected groups showed about two fold increases in SOD activities in stressed conditions when compared with the non-stressed condition. However, the response of the non-selected control group was much smaller than those of the selected groups.

In the present study, the activities of SOD in selected and unselected lines, under stressed or non-stressed conditions were assayed (Table 3). Although significant differences were noticed between stressed and non-stressed conditions for all lines, no significant differences were detected between selected lines, i.e., zinc tolerant, copper tolerant, nickel tolerant, and cadmium tolerant lines. These findings suggest that induction of SOD is

non-specific, but it is produced in response to any biotic or abiotic stresses.

Table (4) summarizes the mean of glutathione S-transeferase activities of selected and non-selected materials under normal and stress conditions. It can be seen from these results, that a significant increases in GST activities were induced in response to stress in all materials tested. In addition, significant differences between non-selected and zinc tolerant, copper tolerant, nickel tolerant, and cadmium tolerant selected groups were also encountered.

DNA fingerprinting

Genetic characterization of control and heavy metal tolerant plants, based on DNA molecular markers. Ten random 10-mer primers were used for RAPD-PCR amplification of genomic DNA samples from control (non-tolerant) in addition to cadmium, nickel, copper, and zinc tolerant plants. Only five primers succeeded in amplifying the present sunflower DNA samples and generated various fragments differed in molecular size (bp), while the others failed.

Figure (1A) shows agarose-gel electrophoretic separations of DNA fragments amplified using primer (A04). The control (non-tolerant) plants generated two adjacent fragments with molecular size of about 400 bp, copper and zinc tolerant plants showed only one fragment with molecular size of about 400 bp. Cadmium tolerant plants showed two fragments, small one with less than 100 bp in size and a large one, more than 1000 bp in size. Nickel tolerant plants showed five fragments in total, one less than 100 bp, two about 400 bp, and two larger than 1000 bp.

Figure (1B) presents the fragments amplified using primer (A06), where all samples showed three fragments, but the four heavy metal tolerant groups generated fragments different from those of the control. The non-tolerant control plants revealed three fragments, the first is > 100 bp, the second is about 200 bp, and the third is about 800 bp. On the other hand, all tolerant groups showed three similar fragments, the first is about 250 bp, the second is about 600 bp, and the third is more than 1000 bp.

Figure (1C) shows the fragments generated by primer (O10), polymorphic patterns can be noticed. The control revealed three small adjacent fragments around 100 bp in size, while the copper and zinc tolerant plants showed only one fragment, more than 1000 bp in size and seem to be similar to each other. Cadmium tolerant plants showed two fragments, one about 800 bp and the other is more than 1000 bp. Nickel tolerant plants showed the highest number of fragments, three around 100 bp as those of the control; two about 800 bp, and one more 1000 bp.

Figure (1D) presents the fragments amplified using primer (A07), where the control plants failed in generating any fragment. Copper tolerant plants generated two fragments ranging in size

between 800 and 900 bp. While zinc tolerant plants showed the two fragments as those of copper tolerant plants. An additional fragment with more than 1000 bp in size was detected. Cadmium tolerant plants showed in all three fragments ranging in size between 600 to 900 bp. Nickel tolerant plants showed two fragments only, one more than 1000 bp and the other less than 1000 bp.

Figure (1E) shows the fragments amplified using primer (A03). All heavy metal tolerant plant groups were monomorphic, showing only one fragment with about 300 bp in size. On the other hand, the control plants showed six different fragments, ranging in size between 300 and > 1000 bp.

Form the results of DNA fingerprinting, summarized in table (6), it can suggested that the selected tolerant plants are genetically different from each other and and different from the original non-selected control plants.

In the present study, it was possible to associate a specific polymorphic DNA fragment (about 600 pb in size) generated by the random primer "O10" to nickel tolerance (Fig. 1C). Such a marker can be of great importance in future programs of sunflower plants evaluation and selection for nickel tolerance.

DISCUSSION

Judged from the available literature, it seems that conventional plant breeding for heavy metal tolerance is rather rare, since phytoremediation studies depend mainly on wild hyper-accumulator species. Selection for heavy metal tolerant or sensitive plants was reported by Yang *et al*. (2000) for lead tolerance in *Oryza sativa*, by Van-Hoof *et al.* (2001) for copper tolerance in *Silene vulgaris*, by Shu *et al*. (2002) for lead, zinc and copper tolerance in Paspalum distichum and Cynodon dactome and by Lopez Errasquin and Vazquez (2003) for copper, zinc, and cadmium in a strain of *Trichoderma atroviride*.

On the other hand, most of the recent studies in this field concentrate on tolerant gene transfer from microrganisms or wild hyperaccumulators to higher plants using genetic engineering methodology (Bennett *et al*., 2003; Gisbert *et al*., 2003).

In the present study, the activities of SOD in selected and unselected lines, under stressed or non-stressed conditions. Although significant differences were noticed between stressed and non-stressed conditions for all lines, no significant differences were detected between selected lines, i.e., zinc tolerant, copper tolerant, nickel tolerant, and cadmium tolerant lines. These findings suggest that induction of SOD is non-specific, but it can be produced in response to any biotic or abiotic stresses. Similar results were reported by Reichheld *et al.* (1999).

Glatathione S-transferase (GST) activities in selected and non-selected sunflower lines, under normal and stressed conditions were estimated, in an

attempt to explain the observed sunflower lines differences in tolerance to heavy metals. The importance of phytochellatins in plant response to stresses was reported by Clemens (2001). The present results revealed also significant differences between selected and unselected lines, and between stressed and non-stressed materials. It was also observed that the cadmium tolerant line produced highly significant increases in GST activity when compared with the unselected control or the other selected lines. It seems that the over expression of GST in cadmium (Z = 48) tolerant line than the nickel (Z = 28) tolerant line, copper (Z = 29) tolerant line and zinc (Z = 30) tolerant line, is due mainly to its heaver atomic weight (Zenk, 1996).

Similar results were reported by Reichheld *et al.* (1999).Another enzyme, glutathione S-transferase (GST), plays a major role in phytochelatines synthesis (a family of proteins involved in heavy metal detoxification).

Molecular markers as DNA non-specific (random) primers RAPD analysis were used in the present study to characterize genetically the four selected sunflower lines in comparison to the nonselected control plants.

From the results of DNA finger printing, it can be suggested that the selected tolerant plants are genetically different from each other and different from the original non-selected control plants. Furthermore, primer O10 amplified a unique polymorphic DNA fragment, about 600 bp, that can be considered as specific to nickel tolerant line and not present the other lines tested.

On the other hand, amplified DNA fragments patterns revealed clear differences between the selected lines themselves and between these lines and the nonselected control plants. Similar results, concerning the significance of DNA molecular markers utilization in sunflower and other related Helianthus spp were reported by (Yu *et al*., 2002; Bert *et al*., 2002).

SUMMARY

A land race of sunflower (*Helianthus annuus* L.) collected from Burg-el-Arab region; 70 km west of Alexandria city, was used in the present study. Achene samples were treated with EMS (0.2% for 3.5 hours), then tolerant genotypes were selected (intensity of selection was 5%) after zinc sulfate, copper sulfate, cadmium chloride, and nickel chloride stress treatments. Tolerant lines and their corresponding non-selected controls were characterized using DNA molecular markers (PCR based RAPD analysis, with 10 random primers). Differences in superoxide dismutase (SOD) and glutathione S-transferase (GST) activities were also monitored in Zn-tolerant, Cu-tolerant, Cd-tolerant, Ni-tolerant lines, and their corresponding controls.

Selected lines proved to be significantly tolerant, to their specific heavy metal, when compared with the controls under greenhouse conditions. A

600 bp fragment amplified by primer "O10" seems also to be specific to Ni-tolerant line. SOD activity seems also to be nonspecific to the studied heavy metals, but it increases in response to abiotic stresses in general. On the other hand, GST activity increased significantly in Cd-tolerant line compared with the other selected lines or the non-selected controls. These findings suggest that proper selection programs can produce genotypes tolerant to heavy metals in sunflower plants.

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Table (1): Code numbers and sequences of the 10-mer random primers used in the present study.

Primer	Sequence
A01	5`CAGGCCCTTC3`
A03*	5`GGCTGCAGAA3
A04*	5`TTGGTAGCCC3`
A06*	5`TGCTGCAGGT3`
A07*	5`GGCTGCAGAA3`
A11	5`AGGGGTCTTG3`
B14	5`TCCGCTCTGG3`
O03	5`CTGTTGCTAC3`
O10*	5`TCAGAGCGCC3`
Z14	5`TCGGAGGTTC3`

* Primers annealed with the present DNA materials.

Table (2): Numbers of normal and aborted achenes and percentages of viable settings of treated sunflower plants.

Treatment	Plant No.		
	No. of aborted achenes	No. of normal achenes	% Normal settings
Control (EMS)	67	468	87.47
EMS+Zn	141	344	71.98*
EMS+Cu	216	173	44.34*
EMS+Ni	216	90	29.41**
EMS+Ni	201	34	14.47**

* Significant, $P < 0.05$ ** highly significant, $P < 0.01$

Table (3): Means and stander errors analysis of variance of measured enzyme of super oxide dismutase (SOD) activity for stressed and control sunflower plants for heavy metals tolerance.

Plant group	Mean enzyme	
	Non-stressed	Stressed
Control	0.41±0.01	0.53±0.01
Zn-tolerant	0.47±0.01	0.91±0.02
Cu-tolerant	0.44±0.01	0.82±0.02
Ni-tolerant	0.45±0.02	0.89±0.03
Cd-tolerant	0.46±0.02	0.99±0.03

ANOVA

S.O.V.	df	SS	MS	F
4 Stressed	1	0.313998	0.31399	17.021 *
Plant group	4	0.120566	0.03141	1.634 ^{ns}
Error	4	0.073792	0.01845	
Total	9	0.508356		

* Significant $P < 0.05$ ns = not significant

Table (4): Means and analysis of variance of enzyme of glutathione S-transferase (GST) activity for stressed and control sunflower plants for heavy metals tolerance.

Plant group	Mean enzyme	
	Non-stressed	Stressed
Non-selected	2.65±0.21	4.21±0.36
Zn-tolerant	2.67±0.24	10.87±0.94
Cu-tolerant	2.12±0.18	9.87±0.72
Ni-tolerant	2.88±0.52	10.65±0.63
Cd-tolerant	2.76±0.43	22.34±0.94

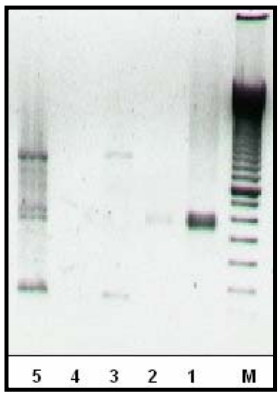
ANOVA

S.O.V.	df	SS	MS	F
4 Stressed	1	201.1523	201.1523	9.42223 *
Plant group	4	819.1181	204.7795	9.76224 *
Error	4	85.3947	21.3487	
Total	9	1105.6651		

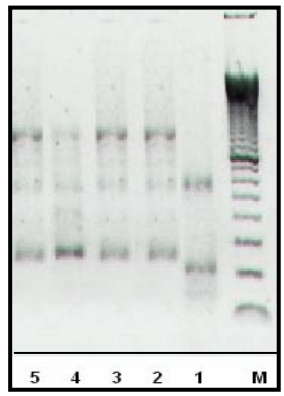
* Significant at $P < 0.05$

Table (5): DNA fragment numbers and sizes, PCR amplified using random primers of tolerant and non-tolerant sunflower plants.

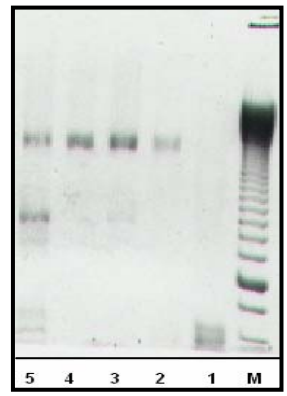
Primer Groups		Numbers of fragment of different sizes (bp)				
		100-300	400-600	700-900	>1000	Total
A	C	-	2	-	-	2
	Cu	-	1	-	-	1
	Cd	1	-	-	1	2
	Zn	-	1	-	-	1
	Ni	1	2	-	2	5
B	C	2	1	-	-	3
	Cu	1	1	-	1	3
	Cd	1	1	-	1	3
	Zn	1	1	-	1	3
	Ni	1	1	-	1	3
C	C	3	-	-	-	3
	Cu	-	-	-	1	1
	Cd	-	-	1	1	2
	Zn	-	-	-	1	1
	Ni	3	-	2	1	6
D	C	-	-	-	-	-
	Cu	-	-	2	-	2
	Cd	-	1	2	-	3
	Zn	-	-	2	1	3
	Ni	-	-	1	1	2
E	C	-	2	2	2	6
	Cu	1	-	-	-	1
	Cd	1	-	-	-	1
	Zn	1	-	-	-	1
	Ni	1	-	-	-	1



(A) Primer A04

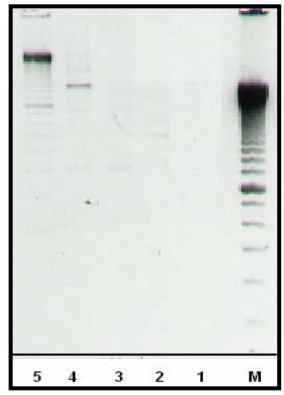


(B) Primer A06

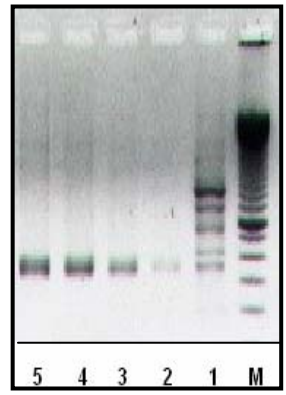


(C) Primer O10

Fig. (1): Photographs showing agarose gel electrophoretic separation of PCR amplified DNA fragments treated and control groups using several random primers. (M) Molecular marker, 100 - 2000 bp, (1) Control, (2) Copper tolerant, (3) Cadmium tolerant, (4) Zinc tolerant and (5) Nickel tolerant plants.



(D) Primer A07



(E) Primer A03