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GENETIC CHARACTERIZATION OF INDIAN MUSTARD (*Brassica juncea*) GENOTYPES TOLERANT TO HEAVY METALS

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ABSTRACT

In the present research work, ethyl methanesulfonate (EMS) agent was applied to select highly tolerant Indian mustard (*Brassica juncea*) plants to some heavy metals (zinc, cadmium, nickel, and molybdenum). Four hyper-accumulator lines were selected, that proved to be more tolerant to the corresponding heavy metal when compared to the wild-type control, for genetic analysis in an attempt to elucidate the genetic bases of heavy metal tolerance in higher plants. Total soluble protein electrophoretic profiles indicated that metallothioneins (5-8 kDa) were expressed in Cd, Mo, Ni, and Zn hyperaccumulator lines when compared with their corresponding wild-type plants. Peroxidase and super oxide dismutase isozyme patterns were not conclusive. The expression of glutathione synthetase (GSH) gene(s) was monitored in the four genotypes lines and the wild-type control, using RT-PCR technologies. Increases ranging between 5 to 2 folds were encountered in the genotypes lines relative to the controls. According to the present findings, it seems that *B. juncea* hyperaccumulator genotypes have evolved a number of mechanisms to cope with heavy metal stress such as the expression of several metallothioneins and the over expression of GSH gene(s). However, the results of oxygen radical scavengers were not conclusive, and need further justifications.

INTODUCTION

Human activities, such as mining, heavy industries, and modern agri-culture practices have lead to the accelerated release of metal pollutants into the environment. Accordingly, soil pollution has become recently a serious problem attracting considerable public attention that calls for immediate action **(**Garbisu and Alkosta, 2003; Pauwels *et al.,* 2006; Vij and Tyagi, 2007).

Phytoremediation, is an emerging cost effective, non-intrusive, and aesthetically pleasing technology that uses the remarkable ability of plants to concentrate elements compounds from the environment, and metabolize various molecules in their tissues, thus it appears to be a very promising solution for the removal of pollutants from the environment. In the field of environ-mental remediation, the utilization of plants to transport and concentrated metals from the soil into the harvestable parts of roots and above ground shoots (phytoextraction) may be a promising approach among others (Hall, 2002 and Alkorta *et al.,* 2004**).**

Brassicaceace is a family containing many metal accumulating species**.** Ni hyperaccumulation has been reported in 7 genera and 72 species and Zn in 3 genera and 20 species **(**Prasad and Freitas 2003**).** *Brassica juncea* (L) Czern has the capacity to take up and accumulate to very high levels heavy metals such as Cd, Cu, Ni, Zn, Pb and Se **(**Blaylock and Huang 2000**).** Lines of *B. juncea* have been used for chelate–assisted phytoextraction of heavy metal from contaminated soils **(**Blaylock and Huang 2000). Genetic manipulation of plant metal metabolism, especially in *Brassica spp.*, has enhanced metal tolerance and accumulation by over producing metal chelating molecules (citrate, phytochelating, metalo-thioneins, hytosiderophores, ferritin) or by the over expression of metal transporter proteins (Hall, 2002).

The present study was undertaken to select heavy metal tolerant plants from *B. juncea*, using chemical agent. Genetic characterization of obtained tolerant plants will be considered, using glutathione GSH gene expression based on RT-PCR analysis. The role of super oxide dismutase (SOD) and other biochemical markers (isozyme patterns and total soluble proteins) will also be used to assist the ability of Indian mustard plants to tolerate some soil heavy metal contaminants.

MATERIALS AND METHODS

A. Materials: Indian mustard (*Brassica juncea*) seeds were which obtained from the Agriculture Research center, Giza, Egypt, was used as basic genetic material in the present study. Seed samples were grown under greenhouse conditions at the Genetic Engineering and Biotechnology Research Institute, GEBRI,Sadat city,Menofiya University, for several generations.

B. Methods:

EMS agent: EMS agent was used according to the procedure suggested by Hoffmann (1980). 2.0g of well dried seeds of *Brassica juncea* plants were soaked in 50mL plastic tube with 40mL of 100m*M* phosphate buffer (pH 6.8) at 4°C overnight and transferred to 0.3% solution of ethyl methansufonate- EMS (Sigma, USA). The mixture was incubated for 4.5 h at room temperature with gentle agitation and then seeds were thoroughly washed 20 times with water (40mL per wash). Than, seeds were planted immediately in suitable pots at greenhouse conditions to obtain the M1 seeds.

Heavy metal treatments: EMS-M1 and untreated (control) seeds were germinated in Petri dishes (12 cm in diameter) in the presence of either one of the LD50 concentrations of the following metal salts; Zinc sulfate (0.8 g/l), Disodium molybdate (0.9g/l), Cadmium chloride (0.5g/l) and Nickel chloride (1 g/l). The most vigorous (2-week old) seedlings were then selected for each metal salt and were transferred to soil with continuous irrigation with the specific heavy metal solution when needed to continue their growth until maturity at green-house conditions (3 replicates were used for each treatment). For each selected genotypes, growth rate was monitored by measuring plant height weekly and percent of germination.

SDS-PAGE and isozyme electrophoresis Esterase (Est.) and peroxidase (Prox.) isozymes were studied using agar-starch-polyvinyl pyrolidine (P.V.P.) gel electrophoresis according to the procedure described by Sabrah and EL-Metainy (1985). Detected Super oxide dismutase (SOD) isozymes and water soluble

proteins were detected with 7.5% SDS-polyacrylamide gel electrophoresis according to Davis (1964) and Studier (1973).

RNA extraction: Total RNA isolation kit (Gentra system, USA) was used for the extraction of total RNA from all samples. The Oligotex buffer set mRNA protocol (Quiagen system, USA) was used for mRNA extraction in the present study.

Primer Design: Based on Gene bank data for *Brassica juncea*, glutathione synthesis (GSH) gene sequences (obtained from the NCBI database), and the following primers were used:

F= 5'- CCC GGA TCC ATG GAG TCT TCA AGT CCC - 3'

R= 5'- CCC CTC GAG TTA GCG CTC GAT TTG TAT - 3'

RT-PCR: RNA Isolation was carried out from 100mg leaf tissues according to the manufacture information (Ready to Go RT-PCR Beads, Amersham Pharmacia Biotech. USA). The PCR conditions were: 42°C for 30 min for one cycle. Denaturation at 95°C for 5 min, annealing 55°C for 1 min, polymerization at 72°C for 1 min, for 32 cycles then 72°C for 10 min, then hold at 4 °C.

Statistical analysis: Means, the standard errors and Student t-test was carried out using the JMP IN5 software (Lehman *et al.,* SAS institute Inc, 2005). Level of gene expression was estimated in comparison to the wild-type level (analyzed using Phortix v.6 gel documentation software).

RESULTS AND DISCUSSION

Although Indian mustard plants are considered as one of the best heavy metal accumulators among the flowering plants (Muthukumar *et al.,* 2007), EMS was used, in the present study, to search for superior genotypes or hyper accumulators. As a result of EMS treatment, superior accumulators were chosen according to the data presented in Table-1, shown percentage of seed germination, and means of plant heights (4- week old) for the Cd, Mo, Ni and Zn hyper accumulator genotypes and their untreated wild-type controls, under heavy metal stress. These four genotypes lines were thereafter used for detailed genetic profiling, for the better understanding of metal accumulation in Indian mustard plants. Heslot *et al.,* (1959) applied EMS for the first time in higher plants breeding programs to increase genetic variability. On many latter occasions, it was reported that EMS not only produced higher mutation rates than irradiation treatments, but produced more mutations relative to sterility. Zimmer (1961) indicated that chemical mutagens are characterized by the existence of a specific threshold-value, after which the survival rate shows a quick decline, whereas for radiation a more gradual decline in the number of survivals is expected. Chemically induced genotypes with increased better performances were isolated from various plant species, including tomato, Arabidopsis and barley (Raskin and Ladyman 1988).

Table-1: Percentage of seed germination, and means of plant heights (4 week old) for the Cd, Mo, Ni and Zn hyperaccumulator genotypes and their untreated wild-type controls, under heavy metal stress.

Genotypes	% germination	Plant height (cm)
Cd hyperaccumulator genotypes	60.1	10.1 ± 0.8*
Cd-wild-type control	48.2	9.3 ± 0.6
Mo hyperaccumulator genotypes	63.4	10.9 ± 0.9*
Mo-wild-type control	53.5	9.1 ± 0.5
Ni hyperaccumulator genotypes	58.8	10.4 ± 0.8*
Ni-wild-type control	43.6	8.7 ± 0.4
Zn hyperaccumulator genotypes	64.2	11.2 ± 0.7*
Zn-wild-type control	59.2	9.9 ± 0.6

* Significant ($P < 0.05$)

Protein analysis: Figure -1 and Table- 2 show the total water-soluble protein SDS–PAGE electrophoretic patterns for the four hyperaccumulator genotypes lines and the wild type control under heavy metal stress. In general, maximum of 14 bands have been visualized, with molecular weights (MW) ranged from 116 to 5.8 kDa. The wild-type control showed ten bands ranged from 116 kDa to 10.0 kDa. Ni – hyperaccumulator line showed eight bands ranged from 97.2 kDa to 8.2kDa, Mo hyper accumulator line showed eight bands ranged from 97.2 to 5.8kDa, Cd hyper accumulator line showed seven bands ranged from 97.2 to 5.8kDa, and Zn hyper accumulator line showed six bands which ranged from 97.2 to 8.2kDa. From these patterns, it can be seen that four protein bands of 97.2, 27, 8.2 and 5.8kDa were present in the four hyper accumulator genotypes lines, while they were absence in the wild-type controls. Thus, these proteins can be considered as a primary markers associated with heavy metals tolerance under treatment. More specifically, the 66.4kDa protein band was found in the wild-type control and the Ni –hyperaccumulator lines only, the 116, 62, 34.6, 21.6, 14.3 and 10kDa bands appeared in the control and were absent in all four hyper accumulator genotypes. The bands with molecular weights of 55.6, 44.1 and 12.6kDa appeared in the Ni – hyper accumulator genotypes only, and can be considered as primary markers associated with Ni tolerance, the bands with molecular weights of 48.2, 30.3and 7.2kDa appeared in the Mo –hyper accumulator genotypes only, and can be considered as a primary markers associated with Mo tolerance. Band with MW 42.3KDa was found in the treatment with Cd and the control only. The band with MW 24.6KDa was appeared in the treatments with Zn and the control only. The 20kDa protein band was found in the wild-type control and other genotypes except the Ni –hyper accumulator genotypes. These results are in agreement with those reported by Liu *et al.,* (2005), who used barley (*Horden vulgare* L) seedling reduced total soluble protein content in root tips as bio-indicator for Cd pollution. Such protein altered expression included the appearance of new bands or the disappearance of other bands, and could be used as markers associated with heavy metals stress tolerance in some plants. The bands with molecular weights ranging between 8.2 and 5.8kDa (detected in the present study in all four mutant lines) may suggest that these protein bands are metallothioneins (MTs). Since MTs are small proteins having molecular weights ranging between 4 and 8kDa. MTs genes have been shown to be differentially expressed during plant development and in response to different stress including heavy metals exposure **(**Zhang *et al.,* 2003; Mir *et al.,* 2004).

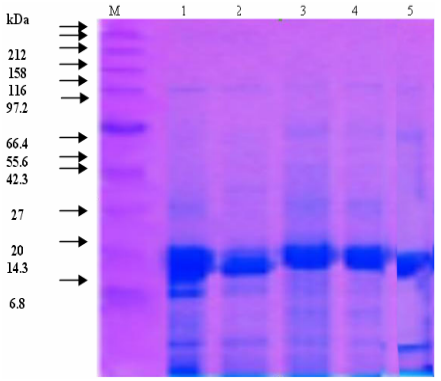

Figure-1: SDS-PAGE soluble protein banding patterns for the present four genotypes and the wild-type control under heavy metal stress. M: molecular weight marker, Lane 1: Zn - Lane 2: Cd, Lane 3: Mo, Lane 4: Ni and Lane 5: control.

Table-2: Presence (1) versus absence (0) of SDS-PAGE soluble protein bands of four hyperaccumulator genotypes and wild-type control under heavy metals stress.

MW Marker (kDa)	Zn-hyper accumulator	Cd-hyper accumulator	Mo-hyper accumulator	Ni-hyper accumulator	WT
212	0	0	0	0	0
156	0	0	0	0	0
116	0	0	0	0	1
97.2	1	1	1	1	0
66.4	0	0	0	1	1
62	0	0	0	0	1
60	1	1	1	1	0
55.6	0	0	0	1	0
46.2	0	0	1	0	0
44.1	0	0	0	1	0
42.3	0	1	0	0	1
34.6	0	0	0	0	1
30.7	0	0	1	0	0
27	1	1	1	1	0
24.6	1	0	0	0	1
21.6	0	0	0	0	1
20	1	1	0	0	1
14.3	0	0	0	0	1
12.6	0	0	0	1	0
10	0	0	0	0	1
8.2	1	1	1	1	0
7.2	0	0	1	0	0
5.8	1	1	1	1	0

* WT = wild-type

Isozyme patterns: In another attempt, the role of oxygen radical scavengers in heavy metal tolerance in plants, peroxidase and super oxide dismutase isozymes were assayed in the present genotypes.

Peroxidase isozymes: Figure-2 showed peroxidase isozyme patterns of the four hyperaccumulator genotypes and the wild-type control under heavy metal stress. In total, seven bands were detected; four migrating towards the anode (Prx-A1, Prx-A2, Prx-A3 and Prx-A4) and three bands migrating towards the cathode (Prx-C1, Prx-C2, and Prx-C3). The wild type control plants showed the lowest number of peroxidase isozyme bands (Prx-A1, Prx-A4 and Prx-C2). Mo and Ni hyper accumulator showed the highest number of peroxidase iso-zymes (five bands). Mo–hyper-accumulator plants showed Prx-A1, Prx-A3, Prx-C1, Prx-C2 and Prx-C3 bands. The nickel hyper-accumulator plants showed Prx-A1, Prx-A2, Prx-A4, Prx-C1 and Prx-C2 bands. On the other hand, the cadmium hyper accumulator plants showed four bands (Prx-A1, Prx-A3, Prx-A4 and Prx-C2); the zinc hyper accumulator plants showed four bands (Prx-A1, Prx-A2, Prx-A4 and Prx-C3). On the basis of peroxidase isozyme patterns, it can be said that cadmium, molybdate, zinc and nickel hyper accumulator *Brassica juncea* plants are genetically different from the wild-type control.

The result of heavy metals tolerance was found to be associated with (Lagriffoul *et al.,* 1998; M-Kalantari and Oloumi 2005) response to heavy metal toxicity, which is an increase in the production of enzymatic and non-enzymatic antioxidants lipophylic anti-oxidans include earotenoids and tocopherols, while water soluble antio-xidants include ascorbate and gluta-thione as well as many antioxidative enzymes like superoxide dismutase, peroxidase and catalases. These antioxidants have a major role in reducing the oxidative stress and quenching free radicals.

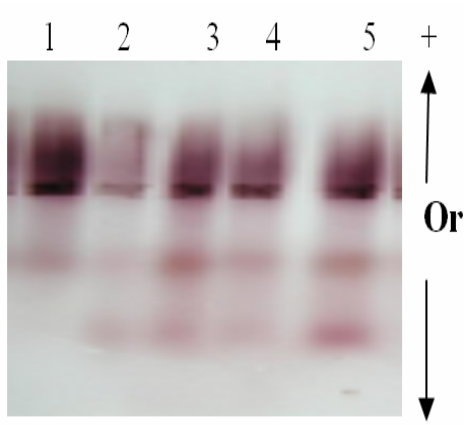

Figure-2: Isozyme Zymogram of peroxidase isozyme pattern for the four hyperaccumulator genotypes and control. Lane 1: control, Lane 2: Cd, Lane 3: Mo, Lane 4: Ni and Lane 5: Zn.

Superoxide dismutase isozymes: Figure -3 shows the isozyme patterns of super oxide dismutase (SOD) of the four hyperaccumulator genotypes and the wild-type control. The bands No.1, No.2 and No.10 appeared in all four genotypes and the control. Bands No.3, No.4, No.7 and No.8 were present in all treatments and not in the wild-type, higher activities for some was noticed. Bands No.5 and No.6 were found in the wild-type control and all treatments except in Zn-hyperaccumulator plants. The band No.9 appeared in all treatments except in the Cd- hyper accumulator plants and the wild-type control.

Similar results were obtained by (Dat *et al.*, 2000; Vranova *et al*., 2002) where they showed that the products of active oxygen radicals is stimulated by biotic and abiotic stresses, but due to the highly cytotoxic and reactive nature of these radicals but their accumulation must be under tight control. Plants possess very efficient defense systems that allow their detoxification and protect plant cells from oxidative damage. Among these defense systems, the enzyme super oxide dismutases (SOD) catalyze the dismutation of O_2 to H_2O_2. Although the results obtained in the present study, indicated the important role of oxygen radicals scavengers and the induction of phyto- chelatins gene and antioxidative enzymes like superoxide dismutase, and peroxidase, it seems that these defense mechanisms are general to several biotic and a biotic stresses.

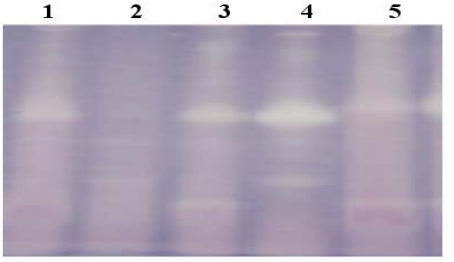

Figure- 3: Isozyme Zymogram of Super oxide dismutase isozyme pattern for the four hyperaccumulator genotypes and control. Lane 1: control, Lane 2: Cd, Lane 3: Mo, Lane 4: Ni and Lane 5: Zn.

Gene expression (RT-PCR) of GSH gene (s): In a third attempt, the expression of GSH gene(s) in the present four genotypes lines and the wild-type of B. *juncea*, using RT-PCR were carried out. The RT-PCR products of GSH gene of these genotypes under heavy metal stress are shown in Figure- 4. The (240Bp) fragment most probably candidate for the GSH gene according to Yang *et al.,* (2000) and Liang *et al.,* (1999) was clearly visualized.

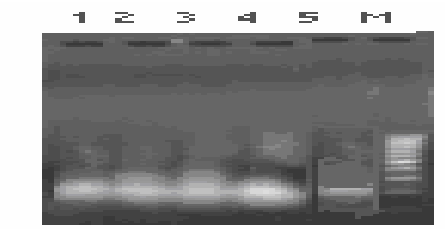

Figure -4: Agarose gel for RT-PCR amplified fragment of GSH- gene (s) for the present *Brassica juncea* genotype under heavy metal stress. Lane 1: Zn – hyperaccumulator; Lane 2: Ni – hyperaccumulator; Lane 3: Cd – hyperaccumulator; Lane 4: Mo– hyperaccumulator; Lane 5: wild-type; and M: molecular marker.

Table-3: Effect of (Cd, Mo, Ni and Zn) hyperaccumulator genotypes and the wild-type control under heavy metal stress on the level of GSH gene transcription in *B. juncea* and *B. nigra* using RT-PCR

	hyperaccumulator mutants			
Mr control 1 kb	Cd	Mo	Ni	Zn
~240 ------	5.3	3.5	2.1	2.5

The expression of the gene was estimated as fold compared to the low level (analyzed using Phortix v.6 gel documentation software).

The relative optical density scanning of agarose gel digital photos, using the algorithm dialogs of the computer program Phortix v.6, was used to compare the expression of GSH gene(s) in response to heavy metal stress, since it is known to be up regulated by exposure to heavy metals. The expression of the 240bp fragments of Cd-hyper accumulator line was found to be 5.3folds, Mo-hyper accumulator line was 3.5folds, zinc-hyper accumulator line was 2.5 folds and Ni-hyper accumulator line was 2.1 folds more than the wild-type expression.

These result are congruent with that of Yang *et al.,* (2000) and Liang *et al.,* (1999) who found that the *E.coli* GSH gene encoding the 240 bp glutathione (GS) in the cytosol of Indian mustard (*Brassica juncea*) plant treated with Cd. Increase in GSH expression has also been reported by Zenk, (1996) and Xiang and Oliver (1998). Exposure to sublethal Cd concentrations resulted in the recovery of cellular glutathione concentration and was accompanied by 4 and 30fold increased γ-EC synthetase and glutathione synthetase mRNA transcript levels (Xiang and Oliver, 1998; Schützendübel *et al.,* 2001). GSH gene products are directly involved in the detoxification of metals by conjugating them, forming a non-toxic complex through GSH.GSH is the precursor of the heavy metal binding peptides (phytochelitens-PCs), which are involved in heavy metal tolerance. Glutathione is the major non-protein thiol in plants and has many functions in plant metabolism. It is involved in the detoxification of heavy metals and xenobiotics and plays a role in gene activation and in the protection from oxidative stress (Noctor *et al*., 1998). The experiment conducted by Zhu *et al*., (1999) showed the over expression of GS in Indian mustard plants with increased of GSH and PCs when the plants were treated with cadmium (Cd). Thus; over expression of enzymes GS and γ-ECS are positively correlated to improvements in heavy metal stress response (Lee, 2003). Previous studies with *Pisum sativum* and *Zea mays* have shown that after Cd exposure the activities of the enzymes γ-glutamyl cysteine synthetase (γ-ECS) and GSHS are both increased several – fold.

In conclusion, it seems that the *B. juncea* hyperaccumulator genotypes, obtained in the present study, have

evolved a number of mechanisms to cope with heavy metal stress such as the expression of several metallothioneins and the over expression of GSH gene(s). However, the results of oxygen radical scavengers were not conclusive, and need further justifications.

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