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Expression of GSH Gene Related to Heavy Metals Tolerance and Accumulation in *Brassica sp*. Plant Genotypes

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A B S T R A C T

The present study included two experiments related to heavy metals tolerance and accumulation in *Brassica sp* plant genotypes *Brassica juncea* and *Brassica nigra*. The first one concerned with the study of two genotypes for Seedling characters. The second experiment was carried out under different molecular markersto characterize the relationship between treatment and over expression of GSH gene. The effect of treatment on the level of GSH hence, the highest expression as 5.3 fold of GSH was obtained in treatment with Cd, and 3.5 fold of GSH was obtained in treatment with Ni in *B. juncea*. While 1.4 fold was obtained in treatments Pb. The same results were obtained with treatment in *B. nigra*.

Introduction

The *Brassica* genus is very essential agricultural crops in all the world and are also recognized to be heavy metal accumulators. There had been a big number of research regarding the tolerance, uptake and protection mechanism in numerous of those species, considerably *Brassica juncea* and *B. napus*, against to the stress caused by heavy metals (Miguel *et al.*, 2015).

Phytoremediation uses green plant to clean up poisonous amount of inorganic and organic pollutants from the environment. The rapid industrialization and urbanization of many evolved and recently growing international locations (as an example China, India, Brazil etc.) have extended heavy metal and natural pollution inside the environment (Memon and Schroder, 2009). Metal infected soils pose an excessive danger to environment, agriculture and therefore thru the meals chain to human and animal health (Schwitzguébel *et al.*, 2011). plants that hyper acquire heavy metals such as Zn(2+) and Cd(2+) are currently of notable medical hobby as they can be used for cleaning up contaminated soils, a process known as phytoremediation (Salt *et al.*, (1995); Raskin *et al.*, (1997). Environmental

pollution from business effluents and other natural pollutants has turn out to be a primary environmental and human situation international (Li and Yi (2012); Dong *et al.*, (2013).

Metals are essential components of all ecosystems and arise evidently inside the earth's crust (Pinto *et al.*, 2003). They appear in a huge variety of oxidative states and coordination numbers, influencing their chemical characteristics and for this reason their bioavailability and toxicity (Pinto *et al.*, 2003; Verbruggen *et al.*, 2009). Positive metals along with iron (Fe), copper (Cu) and zinc (Zn) are taken into consideration vital nutrients to flora and are wished for photosynthesis and as cofactors for many enzymes (e.g. Kovacik *et al.*, 2010; Shanmugam *et al.*, 2011). Plant life soak up vital elements from their environment, however they may be also in a position to accumulate factors, which haven't any recognized organic feature, which include heavy metals like cadmium (Cd), chromium (Cr) or lead (Pb) Peralta-Videa *et al.*, 2009). Those nonessential metals are capable of input plant cells through metallic transporters and vendors for the uptake of crucial metals (Clemens, 2001; Shanker *et al.*, 2005).

Phytoextraction is based on the use of pollutant-accumulating plants for trace element removal from soils by concentrating them in the harvestable parts (Salt *et al.*, 1998). An ideal plant for trace element phytoextraction should possess the following characteristics: (a) tolerance to the trace element concentrations accumulated, (b) fast growth and highly effective trace element accumulating biomass, (c) accumulation of trace elements in the above ground parts, (d) easy to harvest (Vangronsveld *et al.*, 2009). Plant species generate a range of defense mechanisms to resist Cd induced toxicity and to recover the subsequent damages (Meharg, 1993; Mohamed *et al.*, 2012). Among heavy metals cadmium (Cd), a soil pollutant with a strong toxicity (Waalkes, 2000), inhibits photosynthesis (Qian *et al.*, 2009) and prevents the growth of roots and stem. Cd inactivates some enzymes by a strong affinity with the thiol group (Mendoza-Cozatl *et al.*, 2005) and it forms the active oxygens such as hydrogen peroxide (H_2O_2), superoxide anion (O_ 2_), and hydroxyl radical (_OH) (Romero-Puertas *et al.*, 2004) together with lipid peroxide, causing damage to biopolymer and cell membrane by inducing the oxidative stress (Heyno *et al.*, 2008).

Materials and Methods

In the present work two species of the Indian mustard were used. The first species (*Brassica juncea*) was obtained from the Agriculture Research Center (A.R.C.), Giza, Egypt, while the second species (*Brassica nigra*) was kindly obtained from (IPK), Germany.

Treatments and experimental design

The experiment was arranged in factorial experiment in Randomized Complete Block Design (RCBD) with three replications. The experiment included two different Indian mustard which were gotten the first species (*Brassica juncea*), while the second species (*Brassica nigra*).

Four different concentrations of lead (Pb) i.e. 0, 100, 200 and 400 ppm, cadmium (Cd) i.e. 0, 10, 20 and 40 ppm and nickel (Ni) i.e. 0, 20, 50 and 150 ppm were used. Fifty seeds of uniform size in each cultivar were allowed to germinate to primary roots of 2 mm length in a Petri dish containing a filter paper of 9 cm diameter; the Petri dishes were placed in a growth chamber for 7 days at ± 28°C, germinated seeds were selected and transferred to pots (diameter 10 cm)

containing quartz sand. Then, these pots transferred to the green house with day and night temperature of 25°C and grown in plastic pots. Seeds were germinated, in the presence of lead (Pb), cadmium (Cd) and nickel (Ni). Each dish contained fifty seeds and a total of three dishes were used for each treatment. After One week from the initiation of germination, selected seedlings (stressed and non-stressed) were carefully transferred to suitable pots containing quartz sand to continue their growth in the greenhouse. They were irrigated with Hoagland's solution, containing macro and micro nutrient. After 21 days of sowing, plantlets were harvested and were homogenized in liquid nitrogen; the plant material was stored in liquid nitrogen until used.

Electrophoresis and isozyme techniques

Peroxidase (Prx.) E.C.1.111.1.7 was determined by using polyacrylamide gel electrophoresis according to the method of Davis (1964).

SDS Protein Electrophoresis

SDS – Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970).

Total RNA isolation

Total RNA was isolated from leaves of both species of the treated and untreated Indian mustard. Plants using the SV Total RNA isolation kit (Promega) according to the technical manual-TM 048.

RT-PCR amplification

Total RNA was subjected to reverse transcriptase polymerase chain reaction RT-PCR by using specific primers for the GSH gene (www.nbci.com). RT-PCR Ready To Go kit (Bio Basic Inc.) was used to isolate the cDNA fragment according to the manufacture system as following:

1- The following reagents were added to the tubes which contained the kitreagents pellet. These reagents contained 1 µg. of total RNA and 10 µM of each specific primer.

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

5'- CCC CTC GAG TTA GCG CTC GAT TTG TAT - 3' a total volume of 50ul.Reaction was completed using nuclease free water.

The PCR conditions were as follows; the tubes were incubated at 42ºC for 15 minutes to activate the reverse transcriptase enzyme, and 94°C for 5 minutes to inactivate the enzyme, then followed by 35 cycles of 94°C for 1 min, 67°C for 1min, and 72°C for 1min. Finally 72º C for 10 minutes.

The PCR products were electrophoresed in 1% agarose gel. A 100 bp DNA marker was used with the samples and photographed.

Results and Discussion

The present study for both Indian mustard *Brassica nigra* and *Brassica juncea*. study the response of germinate under heavy metals; Lead (Pb), Cadmium (Cd) and Nickel (Ni) stress to confirm the seedling growth performance to examine a range of genetic variability for heavy metals tolerance among both Indian mustard *Brassica nigra* and *Brassica juncea*.

Seedling characters

Lead tolerance evaluation

The results shown in Table (1) revealed highly significant differences for root/shoot

ratio (R/S), germination index (GI), root length (RL), shoot length (SL) and germination % (FGP) traits between the genotypes and Lead application as well as their interaction.

With respect to root/shoot ratio, significant differences were detected among the two studied Indian mustard species (Table 2).

The species *Brassica juncea* gave highly significant mean (33.481), while, second species *Brassica nigra* provide decreased mean (29.456). These differences among two studied Indian mustard species might be attributed to their genetic background.

The analyses of variance indicate that root/shoot ratio was significantly influenced by Pb application levels. As shown in Table (3), the treatment without Pb application produced the highly mean of root/shoot ratio (75.449), while the application Lead with 200 ppm gave the lowest value for root/shoot ratio (6.145). The change in root growth due to the consequences of the exposure of the radical to toxicity and accumulation of metals in the roots followed by slow mobility to the plant shoots (Godzik, 1993 and Fargasova, 1994). According to (Chaignon and Hinsinger 2003), higher concentrations of heavy metals can inhibit root growth before shoot growth and can accumulate in the roots without any efficient increase in its content of the aerial parts. Heavy metals are found to be more toxic for root growth because they accumulate on roots and retard cell division and cell elongation.

Cadmium tolerance evaluation

The results shown in Table (4) revealed highly significant differences for final germination percentage (FGP), germination index (GI), root length (RL), shoot length (SL) and root/shoot ratio (R/S) traits between the genotypes and Cd application as well as their interaction.

Regarding to root/shoot ratio, significant differences were detected among the two studied Indian mustard species (Table 5).

The species *Brassica juncea* gave highly significant mean (35.240), while, second species *Brassica nigra* provide decreased mean (25.546). These differences among two studied Indian mustard species might be attributed to their genetic background.

The analysis of variance indicates that final germination percentage was significantly influenced by Cadmium application levels. As shown in Table (6), the untreated seedling (control) gave the highly mean value of final germination percentage (91.88%), while the application Cadmium with 400 ppm gave the lowest mean for final germination percentage (68.36%).

Nickel tolerance evaluation

In Table (7), the analysis of variance revealed highly significant differences among the genotypes tested for final germination percentage (FGP), germination index (GI), root length (RL), shoot length (SL) and root/shoot ratio (R/S) traits by the genotypes and nickel application as well as their interaction.

Estimates of final germination percentage, significant differences were detected among the studied cultivars (Table 8), Indian mustard species *Brassica juncea* gave highly significant mean (76.833%), while *Brassica nigra* species provide decreased mean (75.00%).

As shown in Table 9, the untreated seedlings (control) produced the highly mean of final germination percentage (91.889%), while the application nickel treated with 150 ppm gave the lowest mean for final germination percentage (29.611%).

Biochemical and Molecular Genetic Analysis

Isozyme Analysis

The biochemical methods of investigation, especially isozyme studies, have provided valuable tools for breeders. Isozyme can serve as unique molecular genetic markers for biochemical characterization of genotypes (Tanksley and Orton, 1983). In the present study an attempt was made to assay the variation of number and activity of one isozyme patterns; peroxidase, in the leaves crude extract of three weeks old seedlings. Two varieties; three heavy metals pb, Cd, Ni and three different concentration were chosen for isozymes studies of all the materials. Variations in number and activity of bands are shown in Figures 1, and 2 for the peroxidase.

Figure one of peroxidase isozyme patterns of *Brassica juncea* as well as their treated with different concentrations of lead, cadmium and nickel. In total, Figure 1 shows a total number of 68 bands detected in the present materials. The untreated genotype showed seven bands as a total. The treated concentration with 40 ppm Cd showed the lowest number of peroxidase isozyme bands (Prx 2, Prx 3, Prx 4 and Prx 6). While the treated concentration with 200 ppm Pb, 20 ppm Ni and 150 ppm Ni showed the highest number of bands (Prx 1, Prx 2, Prx 3, Prx 4, Prx 5, Prx 6, Prx 7 and Prx 8). The band (Prx 1) was present in the treated with heavy metals; 200 ppm Pb, 20 ppm Ni and 150 ppm Ni and was absent in the control so, it could be considered as potential markers associated with heavy metals tolerance under treatment. While the band (Prx 5) was present in the control and was absent in 40 ppm Cd, the two bands Prx 7 and Prx 8 were present in the control and were absent in 40 ppm Cd and 50 ppm Ni.

Figure 2 of peroxidase isozyme patterns of *Brassica nigra* as well as their treated with different concentrations of lead, cadmium and nickel. In total, Figure 2 shows a total number of 70 bands detected in the present materials. The untreated genotype showed nine bands as a total. The treated concentration with 50 ppm Ni showed the lowest number of peroxidase isozyme bands (Prx 4, Prx 5, Prx 6, Prx 7 and Prx 9). While the untreated (control) showed the highest number of bands (Prx 1, Prx 2, Prx 3, Prx 4, Prx 5, Prx 6, Prx 8, Prx 9 and Prx 10). The band (Prx 7) was present in the treated with heavy metals; 200 ppm Pb, 400 ppm Pb, 10 ppm Cd, 20 ppm Cd, 40 ppm Cd, 20 ppm Ni, 50 ppm Ni and 150 ppm Ni and was absent in the control and 100 ppm Pb so, it could be considered as potential markers associated with heavy metals tolerance under treatment. While the bands (Prx 1 and Prx 2) were present in the control and were absent in their treated with some heavy metals; 100 ppm Pb, 200 ppm Pb, 400 ppm Pb, 10 ppm Cd, 20 ppm Cd, 40 ppm Cd, 20 ppm Ni and 50 ppm Ni.

The activity of isoenzyme in the two cultivars genotypes treated with heavy metals (Pb, Cd and Ni) and control determined electrophoretically the intensity of activity of the peroxidase. Results summarized in Figure 2 where the appearance band of Prx 7 under treatment with Pb, Cd and Ni and in *Brassica nigra* could be considered as a potential marker for Pb, Cd and Ni tolerance.

An increase in peroxidase activity probably represents an induced protective reaction delaying senescence (Birecka *et al.*, 1977). Since, as we know the importance of peroxidase isoenzyme to catalyze the reaction that protects the plants, against damage by free radicals. Populations showing low peroxidase activity indicated that it may not adapt them at wider range because plants may lose the permeability of membrane and proceed toward the end of life due to the harmful action of free radicals. Lipid of membranes where a peroxidation of

unsaturated fatty acids takes place is main cellular components susceptible to damage by free radicals (Monk *et al.,* 1989).

SDS Protein Electrophoresis

Electrophoretic analysis was carried out using SDS – PAGE for water soluble protein fraction and was stained to detect the whole protein banding pattern SDS – PAGE was achieved to screen the water soluble leaf protein extracted from the two species of the Indian mustard *Brassica juncea* and *Brassica nigra* under the treatments of heavy metals (Pb, Cd, and Ni) and control. The protein banding patterns showed a maximum number of 20 bands which were characterized with molecular weights (MW) ranging from 116 to 12.5 KDa, and shown in Figure 3 and 4 and Tables 10 and 11.

Concerning protein banding pattern treated of *Brassica juncea* for the heavy metals (Pb, Cd, and Ni) are shown in Table 10 and Figure 3. The control without any treatment has 15 bands ranging from 116 to 14 KDa. The treatment with 100 ppm Pb metal showed 15 bands ranging from 116 to 14 KDa, and also the treatment with 200 ppm Pb showed 15 bands ranging from 116 to 14 KDa. For the treatment with 400 ppm Pb showed 13 bands ranging from 116 to 14 KDa. On other hand the treatment with 10 ppm Cd exhibited 16 bands which ranged from 116 to 12.5 KDa, and also the treatment with 20 ppm Cd showed 12 bands ranging from 116 to 14 KDa. For the treatment with 40 ppm Cd showed 12 bands ranging from 116 to 14 KDa. On other hand the treatment with 20 ppm Ni exhibited 12 bands which ranged from 116 to 14 KDa, and also the treatment with 50 and 150 ppm Ni showed 12 bands ranging from 116 to 14 KDa, The band with MW 59, 39.1 and 16.2 KDa was found in the control and the treatment of 100 and 200 ppm Pb only.

Concerning protein banding pattern treated of *Brassica nigra* for the three heavy metals (Pb, Cd, and Ni) are shown in Table 11and Figure 4. The control without any treatment has 14 bands ranging from 116 to 12.5 KDa. The treatment with 100 ppm Pb showed 16 bands ranging from 116 to 12.5 KDa, also the treatment with 200 ppm Pb showed 16 bands ranging from 116 to 12.5 KDa. Treatment with 400 ppm Pb showed 15 bands ranging from 116 to 12.5 KDa. On other hand the treatment with 10 ppm Cd exhibited 13 bands which ranged from 116 to 14 KDa, also the treatment with 20 ppm Cd showed 12 bands ranging from 116 to 14 KDa, and also the treatment with 40 ppm Cd showed 13 bands ranging from 116 to 12.5 KDa. While, the treatment with 20 ppm Ni exhibited 14 bands which ranged from 116 to 12.5 KDa, also the treatment with 50 and 150 ppm Ni showed 13 bands ranging from 116 to 12.5 KDa. The band with MW 29.8 KDa which was present in Pb treatments except the control can be considered as potential markers associated with lead tolerance under treatment. While, the band with MW 17.5 KDa which was present in all treatments except the control can be considered as potential markers associated with heavy metals tolerance under treatment.

In general, protein bands which induced in SDS-PAGE banding patterns of the two species of the Indian mustard after treatment with three heavy metals (Pb, Cd and Ni). Meanwhile, these bands were absent from the control. This clearly suggests that these protein bands might represent an important enzyme and or structural protein that could be involved either directly or indirectly in the process of heavy metals tolerance of Indian mustard plants. The excess of toxic heavy metal ions induces several cellular stress responses and damage to different cellular components such as membranes, proteins and DNA (Waisberg *et al.,* 2003 and Jimi *et al.,*

2004). SDS-PAGE for total soluble proteins and isozyme analysis revealed moderate and moderate to high degree of polymorphism, respectively (Patra and Chawla, 2010), studied Eighteen traditional and improved basmati rice (*Oryza sativa* L.) varieties for morphological descriptors, total soluble proteins as biochemical markers for determining distinctive features.

Gene expression and Gene screening analysis

RT-PCR and PCR

cDNA of Glutathione GSH gene which might be induced after treatment with heavy metals was amplified from the two Indian mustard genotypes (*Brassica juncea* and *Brassica nigra*) after treatment with heavy metals; Cd, Ni and Pb. Many strategies have been conducted to isolate genes from plants. All techniques for gene isolation exploit one or more of the characteristics that define genes. Some techniques may permit the isolation of genes from any plant, while others are only applicable to one or few. Isolation of plant genes can be done via construction of cDNA library and protein purification. Based on the mentioned clues our was to take advantage of the heavy metal tolerant genotype of Indian mustard plants and to use it for identifying one of the heavy metal tolerance genes using RT-PCR .Pure preparations of the isolated total RNA were found to have 260/280 ratio between 1.8 and 2.0 RNA integrity was examined on 1.2% agarose-formaldehyde denaturing gels before proceeding in RT-PCR. The total RNA (2µg.) showed sharp and clear 28S and 18S rRNA bands. This indicates that the total (poly A-RNA) isolated for both *Brassica juncea* and *Brassica nigra*have no serious signs of degradation.

The expression activity was detected for glutathione GSH gene from the two species (*B. juncea* and *B. nigra*) using RT-PCR. The RT-PCR product of GSH gene of *Brassica juncea* and *Brassica nigra* treatment with heavy metals separated at about 240 bp on the 1.5% agrose gel. Signals intensities of the separated fragment were different and the band intensity will be indicating the variation in GSH gene expression as a response to different treatments with heavy metals.

Table.1 Analysis of variance for final germination percentage, germination index, root length, shoot length and root/shoot ratio affected by Pb.

S.O.V	df	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Replication	2	3.215 ^{ns}	0.0001 ^{ns}	0.002 ^{ns}	0.284 ^{ns}	2.044 ^{ns}
Genotypes	2	205.704 **	0.017 **	2.604 **	7.893 **	175.329 **
Treatments	3	614.414 **	0.070 **	391.523 **	195.902 **	37990.940 **
G x T	3	24.404 **	0.003 **	2.738 **	3.195 **	200.603 **
Error	24	4.534	0.001	0.081	0.133	17.410

ns: Not significant; *** , **** : Significant at 5% and 1% , respectively.

Table.2 Mean performance of Lead for final germination percentage, germination index, root length, shoot length and root/shoot ratio of the twelve rice cultivars.

Genotypes	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Brassica juncea	91.000 [a]	0.968 [a]	2.933 [a]	7.250 [a]	33.481 [a]
Brassica nigra	90.333 [ab]	0. 0.943 [ab]	2.450 [ab]	6.350 [b]	29.456 [ab]

Means followed by the same latter in each column or significantly different by the least significant at p 0.05 according to Duncan's test**.**

Table.3 Mean performance for final germination percentage, germination index, root length, shoot length and root/shoot ratio affected by Pb application levels.

Treatment	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Control	91.889 [a]	1.000 [a]	7.297 [a]	9.667 [a]	75.449 [a]
100 ppm	86.833 [b]	0.948 [b]	2.158 [b]	7.450 [b]	29.499 [b]
200 ppm	84.611 [c]	0.922 [c]	0.350 [c]	5.758 [c]	6.145 [c]
400 ppm	82.194 [d]	0.897 [d]	0.292 [c]	4.231 [d]	6.937 [c]

Means followed by the same latter in each column or significantly different by the least significant at p 0.05 according to Duncan's test.

Table.4 Analysis of variance for final germination percentage, germination index, root length, shoot length and root/shoot ratio affected by Cd.

S.O.V	df	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Replication	2	0.444 [ns]	0.006 [ns]	0.057 [ns]	0.001 [ns]	9.337 [ns]
Genotypes	1	438.310 [**]	0.062 [**]	1.872 [**]	5.555 [**]	183.429 [**]
Treatments	3	3782.840 [**]	0.410 [**]	386.433 [**]	275.628 [**]	36730.476 [**]
G x T	3	284.759 [**]	0.038 [**]	2.575 [**]	2.094 [**]	266.640 [**]
Error	24	4.48	0.006	0.072	0.125	22.369

ns: Not significant; *** , **** : Significant at 5% and 1% , respectively.

Table.5 Mean performance of Cadmium for final germination percentage, germination index, root length, shoot length and root/shoot ratio of the twelve rice cultivars.

Genotypes	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Brassica juncea	84.083[ab]	0.972 [a]	3.033 [a]	5.942 [b]	35.240 [a]
Brassica nigra	84.500 [b]	0.851 [b]	2.442 [b]	6.850 [a]	25.546 [b]

Means followed by the same latter in each column or significantly different by the least significant at p 0.05 according to Duncan's test.

Table.6 Mean performance for final germination percentage, germination index, root length, shoot length and root/shoot ratio affected by Cd application levels.

Treatment	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Control	91.889 [a]	1.000 [a]	7.297 [a]	9.667 [a]	75.449 [a]
10 ppm	87.528 [b]	0.929 [b]	2.053 [b]	4.878 [c]	43.863 [b]
20 ppm	84.194 [c]	0.919 [b]	0.533 [c]	5.278 [b]	10.506 [c]
40 ppm	68.361 [d]	0.749 [c]	0.236 [d]	3.172 [d]	7.654 [d]

Means followed by the same latter in each column or significantly different by the least significant at p 0.05 according to Duncan's test.

Table.7 Analysis of variance for final germination percentage, germination index, root length, shoot length and root/shoot ratio affected by Ni

S.O.V	df	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Replication	2	1.799 [ns]	0.001 [ns]	0.051 [ns]	0.051 [ns]	32.565 [ns]
Genotypes	1	1127.035 [**]	0.195 [**]	2.504 [**]	3.944 [**]	1614.818 [**]
Treatments	3	29967.30 [**]	3.467 [**]	364.392 [**]	719.956 [**]	39857.13 [**]
G x T	3	759.453 [**]	0.081 [**]	2.470 [**]	1.902 [**]	1312.858 [**]
Error	24	3.054	0.0001	0.060	0.054	27.708

ns: Not significant; *** , **** : Significant at 5% and 1% , respectively.

Table.8 Mean performance of Nickel for final germination percentage, germination index, root length, shoot length and root/shoot ratio of the twelve rice cultivars.

Genotypes	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Brassica juncea	76.833 [a]	0.887 [a]	2.958 [a]	3.836 [ab]	41.789 [a]
Brassica nigra	75.000 [b]	0.823 [b]	2.400 [b]	3.963 [a]	22.167[b]

Means followed by the same latter in each column or significantly different by the least significant at p 0.05 according to Duncan's test.

Table.9 Mean performance for final germination percentage, germination index, root length, shoot length and root/shoot ratio affected by Ni application levels.

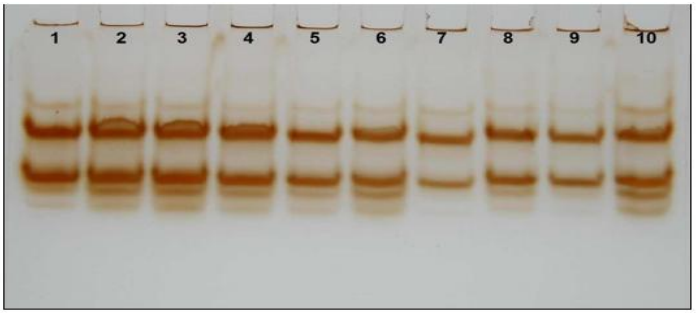
Treatment	Germination percentage (FGP)	Germination index (GI)	Root length (cm)	Shoot length (cm)	Root/Shoot ratio
Control	91.889 [a]	1.000 [a]	7.297 [a]	9.667 [a]	75.449 [a] *
20 ppm	88.833 [b]	0.969 [b]	1.361 [b]	3.014 [b]	48.473 [b]
50 ppm	63.556 [c]	0.703 [c]	1.158 [c]	0.374 [c]	18.282 [c]
150 ppm	29.611 [d]	0.330 [d]	0.433 [d]	0.010 [d]	0.00 [d]

Means followed by the same latter in each column or significantly different by the least significant at p 0.05 according to Duncan's test.

Table.10 Effect of heavy metals (Cd, Ni and Pb) and control on the level of GSH gene transcription in *B. juncea* using RT-PCR.

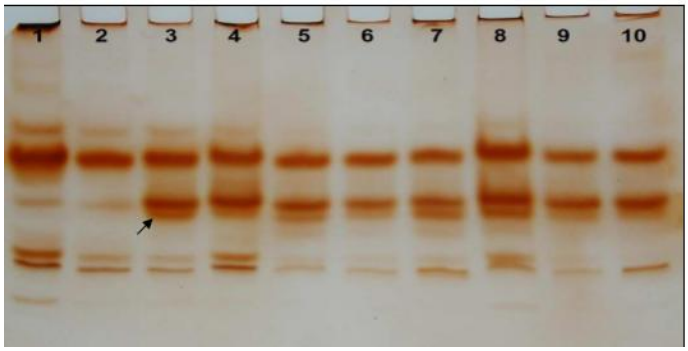
MT 1 Kb	Control	Pb	Ni	Cd
240 bp	1 fold	1.4 fold	3.5 fold	5.3 fold

Fig.1 Peroxidase isozyme pattern *Brassica nigra* genotype under treatment with heavy metals.



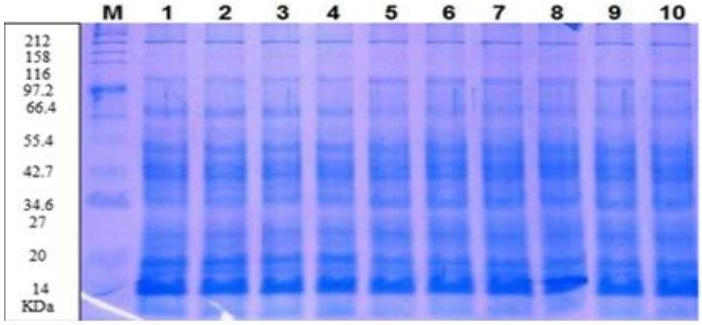
Lane 1: control
Lane 2: 100 ppm Pb
Lane 3: 200 ppm Pb
Lane 4: 400 ppm Pb
Lane 5: 10 ppm Cd
Lane 6: 20 ppm Cd
Lane 7: 40 ppm Cd
Lane 8: 20 ppm Ni
Lane 9: 50 ppm Ni
Lane 10: 150 ppm Ni

Fig.2 Peroxidase isozyme pattern *Brassica nigra* genotype under treatment with heavy metals



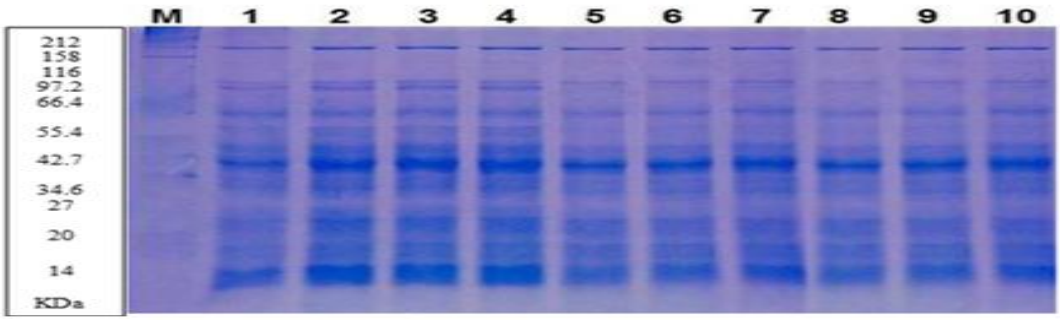
Lane 1: control
Lane 2: 100 ppm Pb
Lane 3: 200 ppm Pb
Lane 4: 400 ppm Pb
Lane 5: 10 ppm Cd
Lane 6: 20 ppm Cd
Lane 7: 40 ppm Cd
Lane 8: 20 ppm Ni
Lane 9: 50 ppm Ni
Lane 10: 150 ppm Ni

Fig.3 SDS-PAGE protein banding pattern for *Brassica juncea* genotype under treatment with heavy metals.



Lane **1**: control - Lane **2**: 100 ppm Pb – Lane **3**: 200 ppm Pb – Lane **4**: 400 ppm Pb – Lane **5**:10 ppm Cd - Lane **6**: 20 ppm Cd – Lane **7**: 40 ppm Cd – Lane **8**: 20 ppm Ni – Lane **9**: 50 ppm Ni – Lane **10**: 150 ppm Ni

Fig.4 SDS-PAGE protein banding pattern for *Brassica nigra* genotype under treatment with heavy metals



Lane **1**: control - Lane **2**: 100 ppm Pb – Lane **3**: 200 ppm Pb – Lane **4**: 400 ppm Pb – Lane **5**:10 ppm Cd - Lane **6**: 20 ppm Cd – Lane **7**: 40 ppm Cd – Lane **8**: 20 ppm Ni – Lane **9**: 50 ppm Ni – Lane **10**: 150 ppm Ni

Fig.5 Agrose gel for RT-PCR amplified fragment on RNA isolated from leaves Indian mustard under treatment with heavy metals

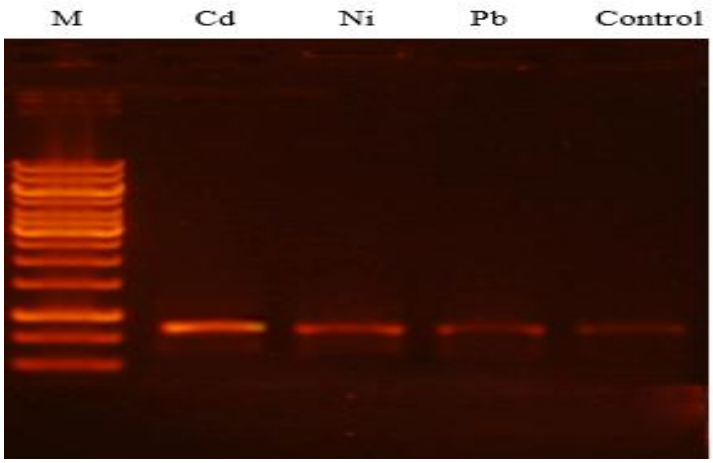
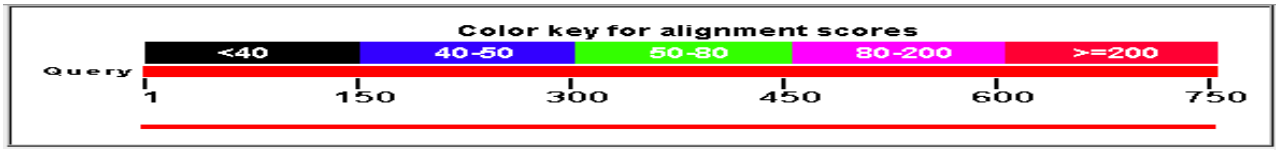


Fig.6 Database sequences aligned to the query sequence



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus niger CBS 513.88 MFS transporter, mRNA	1397	1397	100%	0.0	100%	XM_001402501.3

```
Query  1    ATGAACGGCAATCCATCCTCTTCATCAGCTACAGAGACAAACGCCGCCGATCTAGAAAAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGAACGGCAATCCATCCTCTTCATCAGCTACAGAGACAAACGCCGCCGATCTAGAAAAA  60
Query  61   GAGCCGAACCAAGATGCACCACCTAGGCTGGAGGTATTTTCAGTCTTCACAGTCAACCAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GAGCCGAACCAAGATGCACCACCTAGGCTGGAGGTATTTTCAGTCTTCACAGTCAACCAG  120
Query  121  AAGAGAGCCATGGTGGCGACCGGCTCAATGGCGAGCTTTTTCTCGCCTTTGTCTTCAAGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  AAGAGAGCCATGGTGGCGACCGGCTCAATGGCGAGCTTTTTCTCGCCTTTGTCTTCAAGC  180
Query  181  ATCTACTTCCCTGCCATGGACACGATCGCAACAGCGCTGAATGTCTCCACATCCAAGATT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  ATCTACTTCCCTGCCATGGACACGATCGCAACAGCGCTGAATGTCTCCACATCCAAGATT  240
Query  241  GATTTGACTGTCACGACCTACCTCATAATGCAAGGCATAGCTCCCATGTTGATCGCCGGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GATTTGACTGTCACGACCTACCTCATAATGCAAGGCATAGCTCCCATGTTGATCGCCGGA  300
Query  301  TTCTCAGACACAGCCGGCAGACGTCCCGCCTACATAATCTGCTTCACCGTCTATATCGCC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  301  TTCTCAGACACAGCCGGCAGACGTCCCGCCTACATAATCTGCTTCACCGTCTATATCGCC  360

Query  361  GCCAACCTGGGCCTCGGCATCCAGAACGACTACGGTTCGCTACTCGGTCTGCGATGCCTC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GCCAACCTGGGCCTCGGCATCCAGAACGACTACGGTTCGCTACTCGGTCTGCGATGCCTC  420
Query  421  CAAAGTGCAGGAAGCAGCGGCACCGTGGCCCTGGCCAACGGCCTCGTTGGAGACCTAGTA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CAAAGTGCAGGAAGCAGCGGCACCGTGGCCCTGGCCAACGGCCTCGTTGGAGACCTAGTA  480
Query  481  ACATCAGCGGAACGCGGTACATACATCGCCTTCGCTTCGCTTGGGAGCATGCTCGGCCCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  ACATCAGCGGAACGCGGTACATACATCGCCTTCGCTTCGCTTGGGAGCATGCTCGGCCCC  540
Query  541  TCTCTGTCGCCCATCATCGGCGGACTCCTCAGCCAATATCTCAACTGGCACTGGATATTT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  TCTCTGTCGCCCATCATCGGCGGACTCCTCAGCCAATATCTCAACTGGCACTGGATATTT  600
Query  601  TGGTTCCTTCTCATCTTCTCGGGTGCCTTCTTCCTCCCTCTATTACTCTTCCTCCCGGAG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  TGGTTCCTTCTCATCTTCTCGGGTGCCTTCTTCCTCCCTCTATTACTCTTCCTCCCGGAG  660
Query  661  ACCTGCCGCAAAGTCGTAGCCGATGGCTCTGTCCCACCCCCCACACTAAACAAAAATATA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  ACCTGCCGCAAAGTCGTAGCCGATGGCTCTGTCCCACCCCCCACACTAAACAAAAATATA  720
Query  721  AGCGACACACTCCGGCACCGCAACCGAAAAGCCAAA  756
             ||||||||||||||||||||||||||||||||||
Sbjct  721  AGCGACACACTCCGGCACCGCAACCGAAAAGCCAAA  756
```

The untreated control plants (C) of *B. juncea*and *B. nigra* did not show any expression of GSH gene, meanwhile the gene up-regulated in varied levels as a response to stress, is shown in figure 5 and table 12.

Table (12) show the effect of treatment on the level of GSH hence, the highest expression as 5.3 fold of GSH was obtained in treatment with Cd, and 3.5 fold of GSH was obtained in treatment with Ni in *B. juncea*. While 1.4 fold was obtained in treatments Pb. The same results were obtained with treatment in *B. nigra*. These results are in agreement with those obtained by Vido *et al.,* (2001) who reported that (GSH) is strongly increased in response to cadmium treatment, Schützendübel and Polle, (2002), GSH is apparently a critical step in cadmium sensitivity since plants with improved capacities for GSH synthesis displayed higher Cd tolerance. These results also disagreement with those obtained by Liang *et al.,* (1999), In the presence of Cd, the GS enzyme is rate limiting for the biosynthesis of glutathione and phytochelatins.

Sequence analysis of GSH gene

A multiple sequence alignment of GSH nucleotide sequence (current study) was carried out with GSH sequences published in the GenBank. Sequence comparison showed that GSH of the current study had sequence homology of about 100% with other GHS isolates, mRNA sequence homology of about 100% with *Aspergillus Niger* isolates. These results are in agreement with those obtained.

An overview of the Gene bank sequences is shown in figure 6.

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