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RESEARCH ARTICLE

Expression of Metallothionein as a Biomarker in Response to Various Stress Factors in Different Organisms

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Abstract

The present study was designed to evaluate expression of metallothionein (MTs) at protein level inplant and animal tissues after exposure tobiotic and abiotic factors. Test organisms, within different treatment groups were exposed to different kinds of biotic (Sex, age, hormones, microbial infection) and abiotic factors (e.g., oxidative stress; salt stress; cold conditions) as well as heavy metals stress. The main goal of this study is to investigate and compare the responses of metallothionein biomarker in the selected organisms to assess environmental stress. Two independent types of analyses were conducted utilizing spectrophotometric and SDS-PAGE methodologies.In *Brassica* MTs were detected in seedling tissues indicating their important roles in development while in mice, a high level of MT content in hepatic tissues was also observed. Spectrophotometric analysis proved the expression of metallothioneins and distinctive MTs concentrations were observed in tested organisms exposed to various stress factors. The analysis of the presence and intensity of bands revealed by SDS–PAGE electropherograms also confirmed the induction of stress proteins by different treatments in both organisms. Results of SDS-PAGE analysis for MTs expression at the protein level in *B. napus* and hepatic tissue of mice revealed differences in the protein expression pattern among different examined stress factors. Our data showed that metallothionein protein expression has the potential to be sensitive environmental biomarkers. Based on the results, a strong relationship can be inferred among metal exposure, oxidative stress, sex differences and MT protein levels. Therefore, this study represents an important step to understanding MT response processes as a whole. However, these results should be combined with results from genomic, transcriptomic, metabolomic, functional and physiological studies to unravel the complexity of metallothionein responses.

Introduction

Biomarkers are defined as "any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status". Biochemical, physiological, histological and morphological measurements in organisms that can be used to determine exposure to and/or toxic effects caused by chemicals are to be considered as biomarker. The use of sub-lethal stress indicators as biomarkers is an alternative approach that is potentially useful to assess the response of a single organism or a population of organisms to contaminants. Biomarkers are considered to be early warning signals because the changes at molecular level occur at a threshold that is less toxic than the levels that can be detected by monitoring change in a species, population, or community **(Won et al., 2008).** Therefore, biomarker studies can be used to assess toxicity of specific contaminants from an environment exposed to multiple anthropogenic and natural stressors **(Gastaldi et al., 2007).**

Many stress proteins are responsible for immediate stress protection or conduct cellular repair processes **(Gangwar et al., 2014)**, in a variety of organisms. Stress proteins including metallothionein (MT) have been reported to be induced in response to most situations of stress. Therefore, it has been considered to be an excellent environmental biomarker **(Linde et al., 2008)**

Metallothioneins (MTs) were discovered in 1957 by Margoshes and Vallee and identified as a family of cysteine-rich (20%~30%), heat stable, low-molecular-weight, non-enzymatic, sulfhydryl rich proteins of61 to 68 amino acid residues. The thiol groups (–SH) of cysteine residues enable MTs to bind particular metals **(Amiard et al., 2006)**. MTs are present in various eukaryotic organisms including fungi, invertebrates, mammals, and plants, as well as some prokaryotes. MTs can efficiently bind metals and some of them are transcriptionally regulated by metals; they are also thought to play important roles in metal tolerance, detoxification, and homeostasis **(Hassinen et al., 2011).** Many studies suggested that MTs might be involved in biological processes as diverse as apoptosis, growth, embryonic development, microspore development, senescence, fruit development, maturation and stress responses **(Akashi et al., 2004).** On the other hand, there were also intensive studies for the responses of MTs to stress conditions, such as reactive oxidant species and other abiotic factors **(Akashi et al., (2004); Wong et al., 2004).** Based on these studies, biotic and abiotic factors seem to have a significant influence on MT levels in some organisms.

Quantification of MTs is conclusive for the determination of the roles played by these metalloproteins in the organism and could be a useful biological indicator of the exposure of individuals **(Brulle et al., 2010).** Methods for quantification of MTs are based on a direct determination of the peptide moiety of MT or an indirect determination *via* the metal and sulphydryl (SH) content of MT **(Linde et al., 2008).** Various analytical techniques have been used for the evaluation of MT concentrations in tissue samples. However, these procedures often prove to be too sophisticated and/or expensive to be used in the routine analyses required in environmental monitoring studies **(Brulle et al., 2010).** On the other hand, a number of methods based on the determination of sulphydryl levels such as spectrophotometric method using Ellman's reagent, **(Brulle et al., 2010; Linde and Garcia-Vazquez 2006)** seem to be the most promising techniques for routine evaluation of MTs in living organisms. This method has been reported to be a sensitive, time saving, and low cost technique able to detect metallothionein content in the tissues of marine organisms and has been standardized by a number of laboratories **(Zorita et al., 2005).** Several modifications have been done to the original protocol introduced by **Viarengo et al., (Brulle et al., (2010); Linde and Garcia-Vazquez 2006; Galloway et al., 2004).** On the other hand, Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) belongs to the most commonly and routinely used methods for determination of MTs at the protein level **(Krizkova et al., 2009).**

Biotechnological applications of MTs are fundamental goal in MTs research; the recent advances regarding their functions and their use in phytoremediation are described in literature (**Ge et al., 2012; Ahn et al., 2012).** Therefore, the present study was designed to evaluate expression of MTs at protein level inplant and animal tissues after exposure tobiotic and abiotic factors. The main aim of this study is to investigate and compare the responses of metallothionein biomarker in the selected organisms to assess environmental stress throughout: 1. Assess the effectiveness of MTs to act as biomarkers in different organisms by assessing their correlation with various environmental stressors. 2. Identify any relationship between those stressors and metallotionein level in the selected species tissue. These aims were achieved by conducting two independent types of analyses utilizing spectrophotometric and SDS-PAGE methodologies.

Materials and methods

Experimental organisms

Based on previous studies in the same area of research, model species were chosen for the study including plants (Seeds of *Brassica napus*) and animal (Swiss Albino Mice, adult and young male and/or female).

Biomarker exposure

Test organisms within different treatment groups were exposed to different kinds of biotic (Sex, age, microbial infection) and abiotic (e.g., oxidative stress; salt stress; cold conditions) factors, as well as heavy metals stress.

Plant materials and growth conditions

Seeds of *B. napus* L. were germinated on plates in a growth chamber (22°C, 16 h photoperiod) for 7 days, and the seedlings were used for experiments. Growth conditions and mode of treatments were carried out according to **Ahn et al., (2012).**

Stress treatment

Seven-day-old *B. napus* seedlings were used in the assessment of the responses to all stress treatments. For the evaluation of responses to hormone treatment, seedlings were treated with 200 μM salicylic acid (SA), 50 μMgibrellicacid (GA), 50 μMIndole acetic acid (IAA). For heavy metal toxicity, seedlings were exposed to 300 μM each of NiCl2.6H2O, Cd $(NO_3)_2.4H_2O$. For abiotic stress assessment, seedlings were exposed to 10 mM hydrogen peroxide (H_2O_2), 300 mMNaCl. Cold tolerance was assessed through exposure to 4°C. For biotic stress, seedlings were exposed to bacterial infection of *Pseudomonasfluorescens* strain. Samples were collected 48 h after treatment, and stored at −40°C until analyzed.

Experimental animals and treatments

A total of 80 adult and young male or female Swiss albino mice (10 - 12 week-old, 25 g for adult male or female and 3-4 week-old, 15 g for young male or female) were randomly drawn from the stock colony of "King Fahd Center for Medical Research", King Abdul-Aziz University, Jeddah, Saudi Arabia. Animal groups were housed in a controlled atmosphere with a temperature range of 25±5C° and mean relative humidity of 50 ± 5%, in a light controlled room with an alternating 12 hrs light/dark cycle. Animals were allowed to become acclimatized to laboratory conditions for a week before experimentation with free access to water and food. All experiments inclusive of animal handling and sacrifice were conducted strictly in conformation with standard guidelines of the National rules on animal care.

Mice were randomly assigned into eight groups (each group included 8–10 mice): four experimental groups (adult male & female and young male & female) each of this groups received orally injection of $NiCl_2$ at a dose of 20 mg/kg for seven consecutive days (once a day) and four control groups received an oral injection of the same volume of distal water. At the end of the experiment, 24 h after the last injection, all animal groups were scarified by cervical dislocation and liver tissue were collected, and stored at –40 °C until use for protein extraction and determination.

We chose to administer the Ni Cl_2 treatments by oral route because it is the main mode of exposure to Ni in humans and animals. Doses were chosen on the basis of available literature data by **Hassan and Barakat (2008).**

Quantification of Metallothioneins

Total Metallothioneins accumulation were assayed through evaluation of total levels of MTs protein in tissues of treated organisms by spectrophotometric assay for metallothioneins using Ellman's reagent according to **Viarengo et al., (1997).** Metallothionein determination was based on the method described by **Cataldo et al., (2011).** In details, the tissue obtained from each experimental specimen was placed in 3 volumes of the homogenization buffer (0.5 M sucrose, 20 mMTris-HCl buffer, pH8.6, containing 0.01% β-mercaptoethanol). The tissues were homogenised using pestle and mortar. The homogenates were centrifuged at 10,000 x g for 30 min to obtain a supernatant containing metallothionein. Then, 1 ml of iced absolute ethanol and 80 μL of chloroform were added per 1 ml of resulting supernatant. The samples were centrifuged at 6000 x g for 10 min. Three volumes of cold ethanol were added to the resulting supernatant and stored at -20 °C for 1 h. For the purification and quantification of metallothionein, the supernatant was centrifuged at 6000 x g for 10 min. The resulting pellets washed with an ethanol:chloroform:homogenisation buffer (87:1:12 and then centrifuged again at 6000 x g for 10 min. The pellets were air dried, resuspended in 100 μL of 5 mMTris-HCl, 1 mM EDTA, at pH 7. The resuspended metallothionein fraction were added to 420 μl of 0.43 mM 5,5 –dithiobis (nitrobenzoic acid) in a 0.2 M phosphate buffer at pH 8; it was then left for 30 min at room temperature. The concentration of

reduced sulfhydryl evaluated by reading the absorbance at 412 nm in a Genesys 10UV spectrophotometer (Thermo Scientific).

A standard curve with GSH is used as a standard reference for a correct quantification of MTs in the samples. GSH contains one cystein per molecule; thus, it can be used as a standard for quantifying cysteins in protein analyses. Solutions with different concentrations of GSH were prepared, and their absorbance was measured at 412 nm. The amount of metallothionein in the samples was estimated using the GSH standard, assuming that 1 mol of MT contains 20 mol of cystein **(Linde et al., 2008).**

SDS-PAGE Analysis

Proteins extracted from experimental samples by the chemical method according to the procedure of **Tsugama et al., (2011).** Experimental tissues were boiled in the lysis solution for SDS- PAGE which prepared by mixing stock solutions of its components (0.5 M EDTA, pH 8.0, 1 M Tris-HCl, pH 6.8, 10% w/v SDS, 100% b-ME, 100% glycerol and bromophenol blue powder). The pH value of this solution was around 7.4. Cells were lysed by boiling tissues in the indicated solutions for 5-10 minutes (until the solution become cloudy by cell extracts). Extracted samples were stored at-40°C and then solutions can be directly loaded onto the gel. Proteins were subjected to SDS-PAGE according to **Laemmli (1970),** using 30 µg of protein per lane. Protein electrophoresis was carried out in 15% SDS-polyacrylamide gels. For the determination of the molecular mass, NEB's unstained Protein Marker, Broad Range (NEB #P7702) was used. The electrophoresis was run at 150 V at 78°C until the front dye reached the bottom of the gel. Gels were stained by Comassie brilliant blue G-250.Gels were scanned then analyzed via Gel pro analysis program image software to quantify maximum optical density of the bands of interest as OD/nm.

Statistical analysis

Each treatment was replicated 3 times for statistical validity. SPSS computer software was used for statistical analyses (SPSS release 21). Any differences between treatments were determined using one-way analysis of variance (ANOVA) and Duncan multiple comparison test. Differences are considered as statistically significant for a *p* value less than 0.05. The means and standard errors of the means (Mean±SE) are reported. Graphical plots were made by using Excel release 2007.

Results

Metallothionein concentration

In order to estimate the amount of MT, in different samples the standard curve with GSH was used as a standard reference for a correct quantification. MT content was then estimated by assuming the relationship of 1 mol MT = 20 mol GSH, as described by **Linde et al., (2008).** Results revealed that both biotic and abiotic factors, were correlated with the activity of GSH and hence the concentration of metallothioneins.

In *B. napus* seedlings, the metallothionein content varied significantly between samples in response to stress factors **(Fig.1).** Samples exposed to H_2O_2, NaCl, SA, Ni, Cd showed significant increase in MTs levels, compared to control. Low temperature and bacterial infection showed slight increase with insignificant values. On contrary, seedlings exposed to GA, IAA showed insignificant decrease in MTs levels. Within group of abiotic factors, the lowest concentrations were observed in seedling exposed to cold temperature while the highest concentration was pronounced in seedling exposed to H_2O_2 followed by NaCl. Phyto-hormone exposure caused lower values of MTs concentration in case of GA and IAA hormones, while the highest content was recorded in seedlings exposed to SA **(Table 1& Fig.1).** Heavy metals exposure to Ni and Cd showed a significant increase in MTs content. Finally, biotic factor represented by bacterial infection of *Pseudomonasfluorescens* caused insignificant increase of MTs concentration in *B. napus* tissue.

In hepatic tissues of mice, MTs content differed significantly among groups affected by biotic factors like age and sex as shown in **Fig.2 & Table 2**. For all sampling tissues, within control groups, adult male exhibited the lowest values of MTs content followed by young male, whereas an increase was observed in all female groups both adult and young individuals. However there were insignificant differences in metallothionein concentration between adult and young control male (untreated male). Within Ni exposed groups, male and female, adult or young, significant increase in the hepatic metallothionein content among groups were found after exposure to a heavy metal as abiotic factor. The results indicated a highly significant increase in hepatic MTs in adult and young male treated by Ni by 3.57 and 2.62 fold increase respectively in compared to the control groups; while in adult and young female treated by Ni, hepatic MT content increased by 2.0 and 2.36 fold respectively in compared to the control groups **Table 2**. Elevated values were more pronounced in the adult female followed by young female.

SDS-PAGE analysis

MTs were detected in the experimental organisms after exposure to different abiotic and biotic factors through evaluation of the maximum optical density of each band using densitometry measuring program (Gel pro analysis program). The gel is scanned and then analyzed via image software to quantify the bands of interest as OD/nm.

Results of SDS-PAGE analysis for MTs expression at the protein level in *B. napus* seedlings is presented in **Figs 3 &4**. Differences in the protein expression pattern were observed among different stress factors. The analysis of the presence and intensity of bands revealed by SDS–PAGE electrophoresis and specific staining indicates the induction of stress proteins induced by different types of stress factors.

The SDS-PAGE electropherogram showed the separation of various polypeptides ranging from 4KD-71 KD **(Fig.3).** There were 5 major regions in the gel where the polypeptides were much more intensely started. They were classified as 71 KD (only one fraction of polypeptide), 46- 58 KD polypeptide (four fractions were deserved), 25-30 KD polypeptides (only two polypeptides fractions were observed, 7-17 kD (three polypeptides fractions) and a 4 KD polypeptide **(Fig.3).** The comparison of protein profiles among control and various treatments showed significant differences in optical density of bands of interest at 7-17 KD polypeptides between different stress factors **(Fig.4).** It is obvious from **Figs 3&4** that an increase in density was occurred in samples exposed to oxidative stress (H_2O_2), salt stress (NaCl), hormones, heavy metals and bacterial infection, however, with variation in significance comparing to unexposed samples. In contrast, samples exposed to cold temperature exhibited no detectable decrease in intensity **(Fig.4).**Thus it was presumed that the region of 7-17 KD polypeptides might include the metallothionein at the lowermost polypeptide of 13.1KD

The typical denaturing SDS-PAGE gel of hepatic tissue samples of experimental groups are shown in **Fig.5**. The protein profile showed the separation of polypeptides ranging from 6KD -120 KD. Among various polypeptides there were 5 regions could be recognized in the gel: more intense region includes three polypeptides, (120 KD, 80 KD, 30 KD) followed by 12 KD, and 5 KD polypeptides. The lowermost polypeptide of 6KD molecular weight was the samples were heat treated prior to SDS-PAGE according to procedure mentioned before **(Fig. 5).** The OD of bands of interest were in the range of 0.5133 – 0.8078, considered adequate for this type of quantification. Statistical analysis revealed positive correlations between metallothionein concentration and optical density of the bands in different groups with both factors (biotic and abiotic) **(Figs5&6).** In general, it is clear that both biotic (sex and age) and abiotic (heavy metal exposure) factors were greatly affected the MTs levels. As sex bioindicator, female individuals exhibited higher levels of expression in comparison to male ones. With regard to age factor, adult mice expressed MTs in a higher levels compared to young mice. Within all experimental groups, Ni exposed individuals characterized by significant high levels of MTs expression compared to unexposed individuals.

Table 1: Metallothioneins content in *B. napus* seedlings treated with various stress factors estimated using the GSH standard curve

Sample	Absorbance at 412 nm	X-value	(x/20) MT content (µmole) $x10^{-5}$ Mean±SE
control	0.089	0.006	30 ± 1.15 ^{e}
Nacl	0.108	0.10	50 ± 1.15 ^{bc}
H_2O_2	0.128	0.014	70 ± 1.15 ^{a}
Cold treatment	0.093	0.007	35 ± 2.8d ^{e}
Bacterial infection	0.092	0.007	35 ± 1.7d ^{e}
GA	0.081	0.005	25 ± 1.15 ^{f}
IAA	0.080	0.005	25 ± 2.88 ^{f}
SA	0.099	0.008	40±2.3 ^{cd}
Ni	0.098	0.008	40±2.8 ^{cd}
Cd	0.098	0.008	45 ±2.8 ^{b}

Means with different superscripts (a, b, c, d e & f) between groups in the same column are significantly different at P<0.05.

Table 2: Metallothioneins content in hepatic tissue in different treatment estimated using the GSH standard curve.

Sample	Absorbance at 412 nm	X-value	(x/20) MT content (µmole) $\times 10^{-5}$ Mean±SE
Ad. M. c	0.093	0.007	35 ± 2.8d ^{f}
Yn. M. c	0.098	0.008	40 ± 1.44 ^{f}
Yn. F. c	0.112	0.011	55 ± 1.54 ^{e}
Ad. F. c	0.134	0.014	70 ± 1.8 ^{d}
Yn. M. N	0.172	0.021	105 ± 2.53 ^{c}
Ad. M. N	0.195	0.025	125 ± 2.81 ^{b}
Yn. F. N	0.195	0.026	130 ± 3.12 ^{ab}
Ad. F. N	0.207	0.028	140 ± 3.43 ^{a}

Means with different superscripts (a, b, c, d e & f) between groups in the same column are significantly different at P<0.05.

Ad.M.C:Adult male control**;** ***Yn.M.C:***Young male control; ***Yn.F.C:***Young female control**;** ***Ad.F.C:***Adult female control;***Yn.M.N*****:*** Young male treated by Ni**;** ***Ad.M.N*****:*** Adult male treated by Ni**;** ***Yn.F.N*****:*** Young female treated by Ni **and** ***Ad.F.N:***Adult female treated by Ni**.**

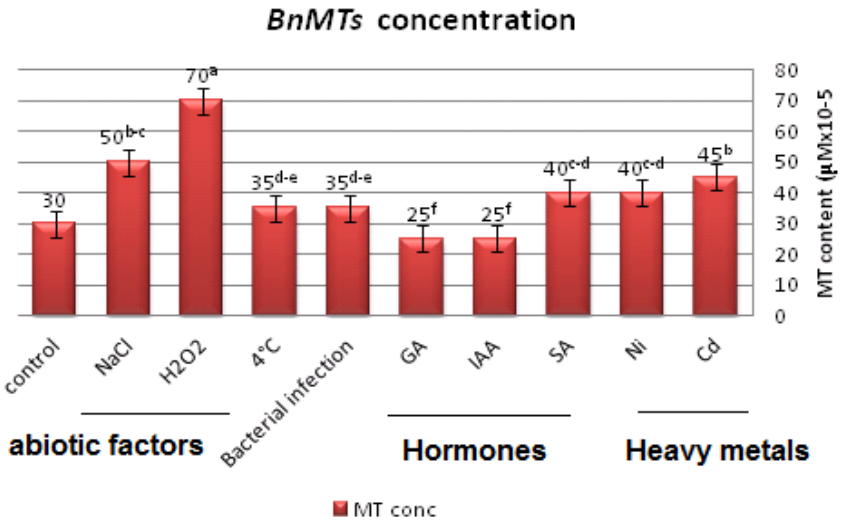

Fig.1:**MTs spectrophotometric determination in *B. napus* seedlings exposed to different stress factors.**

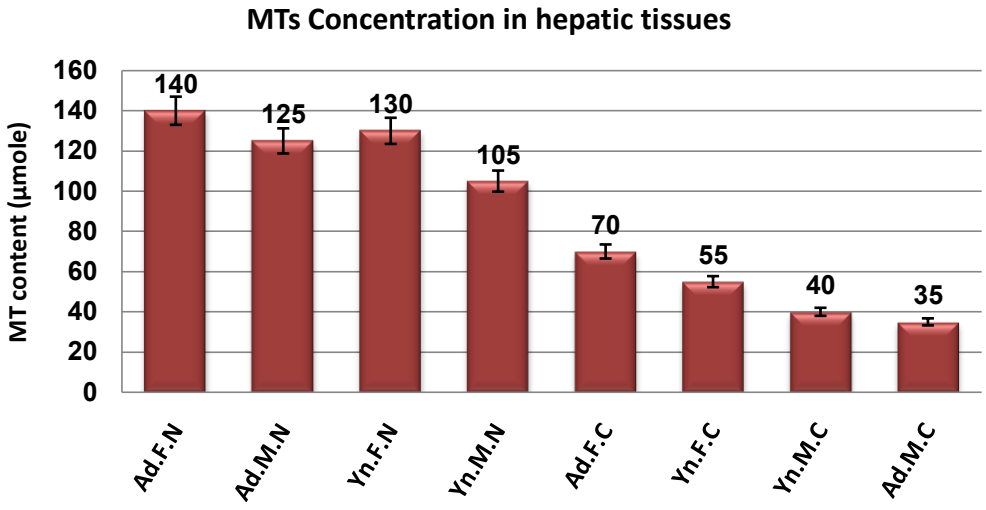

***Fig.2:*MTs spectrophotometric determination in *hepatic tissue* in different treated group.**
Ad.F.N*:**Adult female treated by Ni**;** ***Ad.M.N*:** Adult male treated by Ni**;** ***Yn.F.N*:** Young female treated by Ni**;** ***Yn.M.N*:** Young male treated by Ni**;** ***Ad.F.C:Adult female control;***Ad.M.C:***Adult male control**;** ***Yn.F.C:***Young female control**;** ***Yn.M.C:***Young male contro.

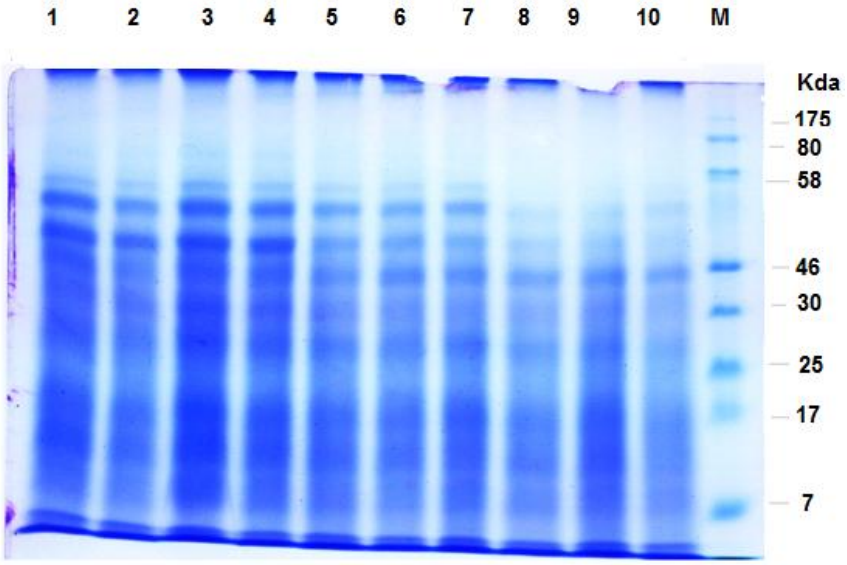

Fig.3: SDS-PAGE gel showing expression pattern of MTs in seedlings of *B. napus* in response to different abiotic and biotic factors. ***Lane 1:*** Control; ***Lane 2:***NaCl; ***Lane 3:*** H_2O_2; ***Lane 4:*** GA; ***Lane 5:*** IAA; ***Lane 6:*** SA; ***Lane 7:*** Ni; ***Lane 8:*** Cd; ***Lane 9:*** Bacterial infection; ***Lane 10***: 4°C and ***Lane M:*** Protein marker

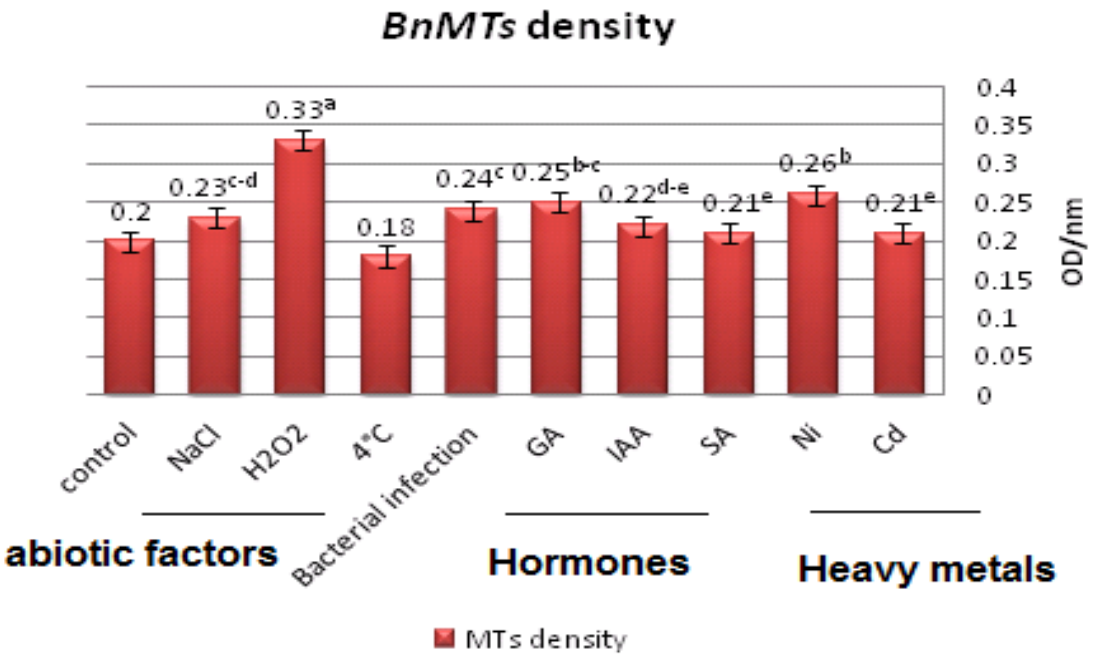

Fig.4: Optical density of MTs in *B. napus* seedlings, separated on SDS-PAGE gel.

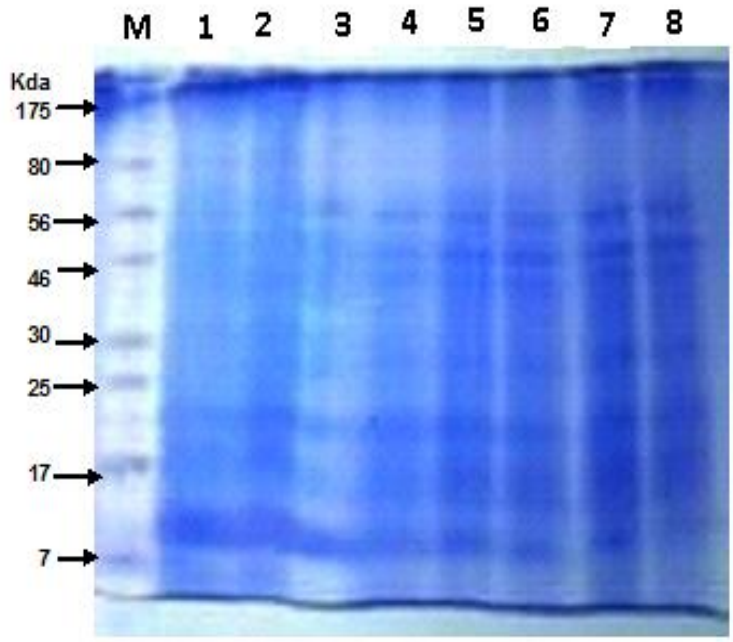

Fig.5: SDS-PAGE gel showing expression pattern of MTs in different groups of mice: ***Lane M:*** Protein marker; ***Lane 1:*** Adult female exposed to Ni; ***Lane 2:*** Adult male exposed to Ni; ***Lane 3:*** young female exposed to Ni; ***Lane 4:*** Young Male exposed to Ni; ***Lane 5:*** Adult female; ***Lane 6:*** young Female; ***Lane 7:*** Young male; ***Lane 8:*** young male

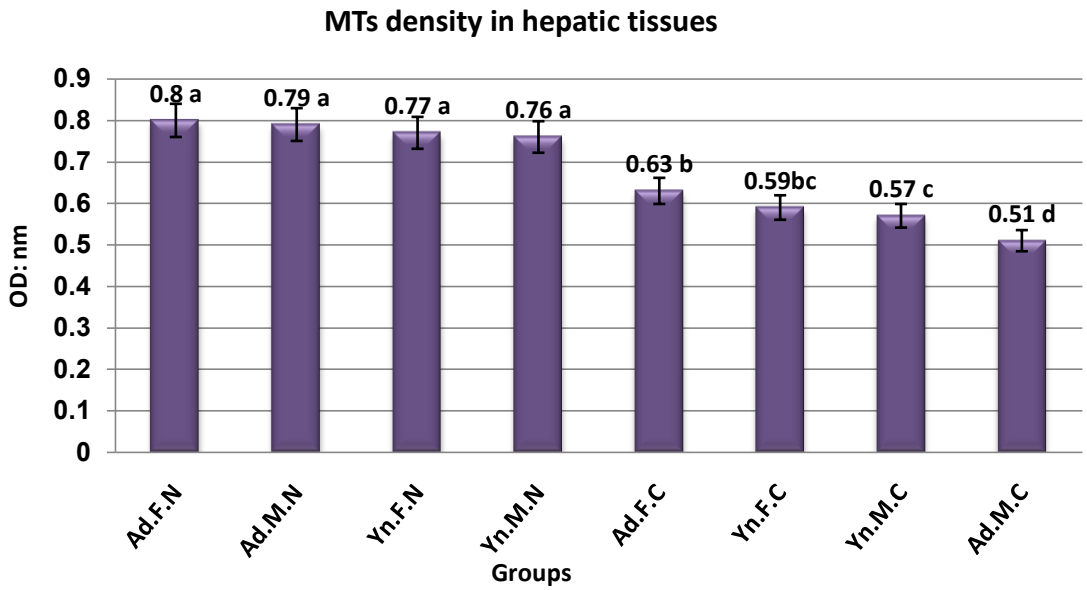

Fig.6: Optical density of MTs separated on SDS-PAGE gel in different groups of mice.
Ad.F.N*:**Adult female treated by Ni**;** ***Ad.M.N*:** Adult male treated by Ni**;** ***Yn.F.N*:** Young female treated by Ni**;** ***Yn.M.N*:** Young male treated by Ni**;** ***Ad.F.C*:**Adult female control;Yn.F.C*:**Young female control**;** ***Yn.M.C*:**Young male contro and ***Ad.M.C*:**Adult male control.

Discussion

This study was designed to assess the effects of different environmental stress factors in natural populations by monitoring MTs levels in tissues of different organisms at protein level. Furthermore, the utility of MT protein expression as a biomarker for metal exposure was tested. By combining these data, relationships between various factors and MTs expression could be established under natural exposure conditions. To prove that metallothionein is present in the tested organisms, two independent types of analyses were conducted. In each of these methods the presence of this metal-binding protein was verified in all the examined tissues. In *Brassica* MTs were detected in seedling tissues indicating their important roles in development. In *mice*, a high level of MT content in hepatic tissues was also observed.In previous studies, the synthesis of MTs has been reported in many kinds of human, animal, plant cells **(Gangwar et al., 2014; Hauser-Davis et al., 2012)** induced by the ions of metals, hormones, biotic factors, free radicals, physical stress and some abiotic agents.

In the present study, SDS-PAGE electrophoreograms of both plant and animal samples showed the presence of protein bands in the range of 14 kDa (Figs 3&5). MT have been described as presenting a 14 kDa molecular weight in liver **(Hauser-Davis et al., 2012)** and in the range of 6-13 kDa in plant species **(Gangwar et al., 2014).** Based on these findings, it can be presumed that the observed polypeptides within a region of 7-17KDa (Fig.3) include metallothionein.

When comparing the two different methods for MTs detection, the same trend seen in the spectrophotometric assay was observed in the SDS-PAGE gels in the exposed organisms.

Metallothionein expression in Brassica napus

Within *Brassica* samples, quantitative analysis of MTs activity in seedlings exposed to H_2O_2, NaCl, SA, Ni, Cd showed significant increase in MTs levels, compared to control. Low temperature and bacterial infection showed slight increase with insignificant values. On contrary, insignificant decrease in MTs levels was recorded in seedlings exposed to GA, IAA. Hence, oxidative stress, salinity and Cd exposed specimens exhibited the highest MT levels. Within 7-17KDa region SDS-PAGE electropherogram, revealed extremely visible bands and significant intensity increase in samples exposed to oxidative stress (H_2O_2), GA and bacterial infection. Moderate increase of band intensity was noticed in samples exposed to NaCl and IAA whereas SA and Cd- exposure exhibited insignificant increase in bands intensity. In contrast, samples exposed to cold temperature exhibited no detectable decrease in intensity. These findings are nearly consistent with data from quantitative spectrophotometric assay. Both types of analyses suggested that *BnMTs*, being more expressed in situation of oxidative stress exposure whereas less expression was detected in cold stress situation. Despite the variation in significance of MTs levels in Ni and Cd exposure, there is a consistence between both methods showing induction of MTs in response to heavy metal stress.

The present data at the protein level, suggested which kind of abiotic stress induced *Bn*MTs expression. It can be inferred from the results that MTs expression was induced not only under the condition of oxidative stress, but also by salt stress. Low temperature caused a non detectable increase in MT level.According to previous studies **(Wang et al., 2014; Gangwar et al., 2014),** it has been reported that the reactive oxygen species (ROS), such as H_2O_2 may lead to unspecific oxidation of proteins and membrane lipids or DNA injury and described MTs as a ROS scavenger in abiotic stress tolerance. Elevated expression of MT genes has been recorded in many plants under H_2O_2 treatment **(Ahn et al., 2012; Wang et al., 2014).** This is in accordance with the results of H_2O_2 stress experiment in this study. Based on these results, it is indicated that BnMTs-over expressing has acquired a more efficient antioxidant system to minimize the effect of oxidative stress **(Akashi et al., 2004).**

Low temperature is also one of the most common exogenous stress factors. Therefore, it is important to determine the expression patterns of the cold stress-inducible MTs genes for improving the stress tolerance of crops. Results in this study revealed insignificant variation in MTs expression as a response to cold treatment whether by increase or decrease. This is in accordance with previous study which suggested the exposure time as a critical factor affecting the expression pattern of MTs **(Ahn et al., 2012).** In short–term exposure, analysis of expression profiles of MTsexhibited gradual up-regulation of expression from 3-24h followed by slight down-regulation through 48h.

Sodium chloride (NaCl) represents the major soluble salt causing soil salinity which is considered a strong limitation of agricultural production worldwide, especially in arid and semi-arid regions. Plants actively respond to salinity stress by reprogramming their metabolism to induction of an enhanced stress tolerance. Many studies focused on salinity response in important crop plants and a comprehensive review on plant protein response to salinity including a database of salt-responsive proteins has been published **(Wang et al., 2014)**. Other studies confirmed that MTs play an important role in the tolerance of salt stress **(Ahn et al., 2012; Filiz and Dogan 2013)** and demonstrated that MT transcripts were significantly up-regulated under salt stresses. In this study, salt stress (NaCl) caused significant increase in MTs content.

Phytohormones (plant hormones) are organic substances that regulate plant growth and development. Their presence may stimulate reactions that are signal and/or causative agents for stress responses (**Gangwar et al., 2014)**. Plant hormones as signal molecules regulate cellular processes in targeted cells and even plant death. In addition, they are known to play important roles in response to multiple environmental stimuli including various biotic and abiotic stresses **(Ge et al., 2012; Ahn et al., 2012).** Results of this study revealed low to moderate induction of MTs in seedlings of *B. napus* by the application of SA. Exposure to IAA caused insignificant change in MTs levels either by decrease or increase However, variation in response to GA–exposure was detected depending on the method used. Spectrophotometric analysis exhibited insignificant decrease in MTs content whereas moderate induction of MTs is reported in SDS-PAGE analysis. Some studies have assumed that GA downregulates some stress related genes and others have reported up- regulation of them. These findings suggested that GA has an antagonistic function by alternative splicing variants can generate both an activator and a repressor (**Gangwar et al., 2014).**In agreement with other reports from animals and plants **(Hauser-Davis et al., 2012)**, the expression of MTs in *Brassica* seedlings was also up-regulated by the application of certain hormones and down regulated by others. Considering the importance of hormones in many plants there are some studies that support their role in plants under metal as well as other abiotic stress conditions **(Nejat et al., 2012; Gangwar et al., 2014)**.The application of exogenous hormones seems to initiate various biochemical pathways that enhance plant tolerance against various abiotic stresses. Therefore, elucidation of hormonal metabolisms in plants can contribute to developing physiological, biochemical, and biotechnological strategies against abiotic stresses in order to increase plant yield and crop productivity.

Based on biotic stress, several defense-response and pathogen-related proteins are induced by pathogen attack. MT genes are related to those plant defense and stress response proteins. In response to *Pseudomonas fluorescens* infection both quantitative and qualitative analyses of *BnMTs*proteins, exhibited low significant increase in MTs expression in a comparison to control seedlings. This is suggesting that bacterial infection had little effect at the protein level of *BnMTs* investigated. It is possibly that, *MTs* are not involved in *Pseudomonas* infection response and alternative mechanisms might include other stress proteins could be associated with pathogen attack in *Brassica napus* **(Nejat et al., 2012).**

In addition, induction of MTs represents a major means of metal homeostasis and therefore used as a potentially good biomarker of metal contamination in many organisms **(Ahn et al., 2012).** Previous research on MTs provides evidence of a correlation between MTgene expression and heavy metals such as Cd and Ni which are extremely toxic elements to biological systems **(Castiglione et al., 2007).** In the present work, quantitative analysis revealed higher MTs levels in response to heavy metals with moderate significant value in situation of Cd and low significant value in situation of Ni. Surprisingly, SDS-PAGE gel showed insignificant increase in expression of MTsin Cd- exposed seedlings whereas moderate significant increase was noticed in Ni-exposed

seedlings. However, both metals caused induction of MTs supporting their response to heavy metal stress as demonstrated for other plants **(Castiglione et al., 2007; Ahn et al., 2012).** This variation in metal stimulation for MTs could be explained on the basis of different assumptions: Firstly, a different threshold concentration of heavy metal might be required for causing effects on MTs expression **(Castiglione et al., 2007; Serenoa et al., 2007).** Secondly, the heavy metal could be detoxified and, therefore, the MT transcripts return to control levels. Finally, excessive cell damage which may lead to reduction of MTs.Further information regarding the structures and properties of MTs could clarify their role in heavy metal detoxification.

Metallothionein protein expression in Mice

Experience and fundamental similarities in cell structure and biochemistry between animals and humans provide general valid bases for the prediction of likely effects of chemicals on human population. Metallothionein exists in tissue for various animal species and are known to detoxify heavy metals and are thought to play essential, but as yet unknown, roles in cellular processes **(Satoh et al., 2003)** MTs in mice are usually analyzed in liver, and some studies also determine MT levels in muscle and kidney **(Khalifa et al., 2003).** It was reported that liver is the first target organ for cadmium or other heavy metal accumulation, whereas kidneys are the final target organs. Liver measurements remain the most suitable tissues, since they detect early exposure to contaminants, as it is the main detoxification organ of the body and plays a main role in the metabolism of metalloproteins **(Henriques and Cozzolino 2001).** In this study, hepatic tissues were confirmed as a useful tool for monitoring the biological effects of metal exposureby detecting MTs concentrations. The concentrations observed using Ellman's reaction are also in accordance with the one dimension SDS-PAGE gels, in which protein bands from nickel treated groups, adult and young male or female, were more intense than those of the control groups. These findings indicate that hepatic MT protein synthesis follows the same trend as in spectrophotometer measurement. In this regard, **Henrique and Cozzolinot (2001)** reported that total liver metallothionein is almost five times greater than kidney and testes MT as the liver tissue plays a main role in the metabolism of the minerals.

Peixoto et al., (2007) reported that a direct relationship exists between MT and metals for both hepatic and renal tissues, which indicates that the increase in metal levels occurs in parallel to the increase in MT content. The result in this study proveda highly significant increase in hepatic MTs in adult and young male treated with Ni by 3.57 and 2.62 fold increase respectively in compared to the control groups; while in adult and young female treated by Ni, hepatic MT content increased by 2.0 and 2.36 fold respectively compared to the control groups; hence MT concentration in hepatic tissue increase ranging from 2 to 3.57 fold after treatment with Ni. These are in agreement with **Šveikauskaitė et al., (2014)** who found that **N**i activated MTs synthesis in mice liver and MTs content increased by 55% compared with control. Also **Man and Woo (2008)**, reported that primary hepatocyte culture from silver sea bream sparussarba directly exposed to sub lethal level of cadmium in vitro showed up regulation of metallothionein mRNA expression. In this concern, **Andreani et al., (2011)** showed that metallothionein mRNA concentrations were significantly higher in hepatopancrease of molluscusscaphorcainaequivalvis exposed to heavy metal.

In kidney and liver most of heavy metals (76%- 87%) in cytosoles was bound to metallothionein as they contain heavy metal binding protein which participates in their accumulation and distribution so liver and kidney show a tendency to accumulate high level of cadmium and other heavy metals **(Vasatkova et al., 2009)**. Possible mechanisms by which MT may protect against metal toxicity include: (1) reduction of metal uptake into cells; (2) sequestration of metal within the cells; and (3) enhanced metal export out of cells. MT is an inducible intracellular protein. It is possible that metallothionein, as a transporter of zinc (II) ions, is intensively redistributed to tissue-specific transcriptional factors; hence it belong to key molecules participating in protection of an organism against xenobiotics **(Vasatkova et al., 2009; Andreani et al., 2011).**

As mentioned above; several studies have described metal toxicity and the role of metallothioneins in the detoxification and regulation of metal homeostasis. However, little data exist on this topic during the specific post-natal developmental phase in young mammals **(Peixotoa et al., 2007)**. This developmental phase is particularly important since young animals are more sensitive to toxicants than adults. Present study revealed that there were insignificant differences in metallothionein concentration between adult and young control male (untreated male), however a significant increase in MT concentration in treated groups with Ni were observed in both adult and young female or male mice. In the same manner, **Hunt and Clarke (1993)** stated that estimates of thionein protein content of tissues from mutant and normal mice demonstrated that the levels are significantly elevated in both young and adult mutant liver, in parallel therefore with the changes in tissue metal levels which confirmed our results.

During fetal development, tissue MT concentrations change dramatically. Metallothioneins are detected in rat fetal liver by day 18 of gestation, reaching maximum hepatic concentrations at birth **(Peixotoa et al., 1990).** MT concentrations in liver of newborn rats are 20-fold higher than in adult rats. This high level of hepatic MT is

maintained during the first 2 weeks postpartum. Thereafter, the hepatic concentration of MT decreases, with adult expression levels exhibited by 35 days of age **(Peixotoa et al., 1990; Hunt and Clarke 1993).** One explanation for a consistently high level of MT during development is that MT is localized in the nucleus during development, and thus it is not available to the intracellular degradation machinery. In general, it is clear that both biotic (sex and age) and abiotic (heavy metal exposure) factors were greatly affected the MTs levels. As sex bioindicator, female individuals exhibited higher levels of expression in comparison to male ones. An early study proved that the progesterone-induced increases in MT synthesis provide one example of MT expression being under hormonal control observed in rat-liver cells *in vitro* by **Shimada et al., (1997).**

Conclusion

In conclusion, metallothionein regulation is fairly complex, considering certain factors such as the period of sexual maturation, ambient temperature fluctuation, exposure time and threshold concentration, it promises to be a potent indicator for environmental monitoring. Our data show that metallothionein protein expression has the potential to be sensitive environmental biomarkers in both tested organisms. A strong relationship was found among metal exposure, oxidative stress, sex differences and MT protein levels. Although results of this study demonstrated that biotic and abiotic factors seem to have a significant influence on MT levels in some organisms inhabiting natural environments, it does not allow universal generalisation regarding its utility as a biomarker applicable to all species and/or in all types of environments. Taken together, the results from this study represent an important step to understanding MT response processes as a whole. However, these results should be combined with results from genomic, transcriptomic, metabolomic, functional and physiological studies to unravel the complexity of metallothionein responses

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