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NICKEL AFFECTS PROLINE ACCUMULATION, PROTEIN CONTENT AND GENE EXPRESSION OF ANTIOXIDANT ENZYMES OF MAIZE (*ZEA MAYS* L.) GENOTYPES

Enas Fahd Naji* and Dhia S. Hassawi

Departement of Biology, College of Sciences, University of Anbar, Al-Anbar, Iraq.
*e-mail: SC.enas-fahad@uoanbar.edu.iq

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ABSTRACT : This study was conducted to investigate the effects of nickel concentrations on gene expression and certain physiological parameters of maize (*Zea mays* L.) genotypes. Three Iraqi varieties (5018, Baghdad-3 and Fajr-1) were exposed to four concentrations of nickel (0, 200, 300 and 400 ppm). The results showed high proline and protein contents of Fajr-1 variety, which recorded the values of 3.58 µmole/g and 8.699%, respectively. There was a gradual increase in proline content under the influence of the elevated concentrations of nickel (18.75%, 25.83% and 49.16%). In addition to significant increase in the total protein content in response to nickel concentrations as 31.78%, 54.05% and 59.61%. These findings were consistent with gene expression results, which revealed that nickel affected the gene expression levels of three major antioxidant enzymes (*SOD*, *GPX* and *MDAR*). In general, nickel concentrations of 300 and 400ppm increased gene expression, where Fajr-1 variety recorded the highest level of *SOD* gene (5.314) and *MDAR* gene (354.588), when exposed to 300 ppm of nickel; while Baghdad-3 variety with 400 ppm scored the highest expression level for *GPX* gene (16.679).

Key words : Antioxidant enzymes, gene expression, maize, nickel, proline.

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INTRODUCTION

Abiotic stresses have an impact on growth, development and productivity and significantly limit worldwide agricultural yields, mainly by stimulating the generation of reactive oxygen species (ROS) in cells (Abu-Romman, 2016a). That is, ROS exceeds the situation of antioxidants if not metabolized, causing damage to DNA, proteins, lipids and other macromolecules and eventually arresting cellular metabolism. ROS is considered aerobic life's inevitable chemical entity (Halliwell, 2006). Excessive heavy metal exposure is known to increase the generation of reactive oxygen species (ROSs) in plants and if the equilibrium between ROS generation and removal were disrupted, oxidative stress would occur (Mittler, 2002).

Oxidative stress is an aspect of general stress that occurs when an organism changes its homeostasis by experiencing multiple external or internal influences. In answer, by triggering the corresponding defensive mechanisms, an organism either aims to preserve the previous status or goes to a new stable state (Mittler, 2002). Hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) are the major ROSs. After the reduction of molecular O_2, superoxide ($O_2^{\bullet-}$) is the first species to be produced and is considered to have strong reactivity and oxidizing capabilities. It exists in many cell compartments, but the photosynthetic electron transport chain located in the chloroplast and mitochondrial electron transport (respiration) are the key producers of superoxide. Moreover, cell-wall-bound peroxidases and NADPH oxidase found in the plasma membrane and can also generate $O_2^{\bullet-}$ radicals (Arora *et al*, 2002).

Metal tolerant plants, which are capable of subsisting under such challenging conditions and exclude plantsthat are capable of storing the metal in the root system in order to prevent moving it into the food chain are widely used in metal stabilization strategies (Kidd *et al*, 2009). Nickel (Ni) is an important plant micronutrient as it is the enzyme activity urease center needed in higher plants

for nitrogen metabolism. Excess Ni, however is considered to be toxic and several studies have been carried out on the toxicity of Ni in different plant species. Development inhibition, photosynthesis, mineral nutrition, sugar transport and water relationships are the most common signs of nickel toxicity in plants (Seregin and Kozhevnikova, 2006).

Plants use antioxidant defense machinery including enzymatic and non-enzymatic defense systems to reverse the inhibitory effects of reactive oxygen species (Gill and Tuteja, 2010). The enzymatic one, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), as a major scavenger of superoxide (O_2), hydrogen peroxide (H_2O_2) and oxygen (O_2). Antioxidant enzymatic system normally operates in plant, however their activity is increased during stress to resist the damage caused by reactive oxygen species produced during abiotic stress (Abu-Romman, 2016b).

The aim of this research was to study the impact of different nickel levels on :

1. The accumulation of proline and total protein content in the vegetative parts of the maize plant.

2. The level of gene expression of antioxidant enzymes (*SOD*, *GPX* and *MDAR*).

MATERIALS AND METHODS

Seeds of three maize varieties (5018, Baghdad-3 and Fajr-1) were obtained from the center of seeds certification at Abu Ghraib city, Iraq. The seeds were surface sterilized with 10% sodium hypochlorite for 2 minutes and washed extensively with distilled water for three times. About ten seeds were germinated in each of plastic pots filled with16 Kg planting media, which were prepared from sandy loam soil and peat moss in 5:1 ratio. After 10 days of planting, the germinated seedlings were thinned to five, as the most active and homogeneous plants were kept. The growing plants were irrigated with normal water, up to the field capacity and then watering daily according to the needs of the plants.After 30 days of planting, nickel was added to the soil in the form of nickel nitrate hexahydrates $Ni(NO_3)_2.6H_2O$ with concentrations of 0, 200 and 300, 400 mg/ kg. Nickel was applied after dissolving specified weight of nickel nitrate in the appropriate volume of distilled water in three batches with a period of 10 days interval between additions.

Proline content

Extraction and determination of free proline were achieved from fresh leaves according to Bates *et al* (1973). A sample of 0.5 g leaf material was homogenized in 10 ml of 3% 5-sulfosalysilic acid solution. The homogenate was filtered via Whatman No. 1 filter paper. Proline was evaluated by reacting 2 ml of the extract with 2 ml of glacial acetic acid and 2 ml of ninhydrin solution. The mixture was incubated in a boiling water bath for 1 h. After cooling, 4 ml of toluene was added and agitated. The absorbance of the toluene phase was determined at 520 nm in a Jenway (PD-6315). The proline content was determined using the following equation:

(ìgproline/ ml × ml toluene) / 115.5 ìg / ìmoles]/ [(g sample)/5] = ìmoles proline/g of fresh weight material.

Protein estimation

First, the total nitrogen was estimated in the dry vegetative parts for maize plants according to Kjeldahl method. From nitrogen values, protein content was calculated by the following equation:

Protein % = Nitrogen% × 6.24 (AOAC, 1975).

Statistical analysis

The experiment was conducted according to the Complete Randomize Design (CRD) with three replicates for each treatment. The data were subjected to statistical analysis using the statistical software GenStat, (12^{th}) Edition. Analysis of variance (ANOVA) was used to analyze the experimental results of treatments. The least significant difference (LSD) was calculated at $P \leq 0.05$.

Expression analysis of antioxidant genes

Total RNA was extracted using the QIAzol ® reagent kit from maize fresh leaves, which were collected after the last addition of nickel, and the concentration was quantified using Qubit 4 flourometer. The transcription levels of all genes were analyzed using quantitative real-time PCR (RT-PCR, Bioer, Japan) and the melting curve evaluation indicated that all primers produced only a single amplicon and no primer dimers or unexpected products were detected. Actin's gene (ZmActin1) was used to check the equivalent loading of cDNA as an internal guide. Primers were constructed using Primer 3program (Table 1).

A total of 20ìl of PCR reaction mixture was prepared for the reaction mix by combining 10ìlofGoTaq® Probe qPCR Master Mix with dUTP with 0.4ìl of GoScript™ RT Mix for 1-Step RT-qPCR. 2ìl of each gene-specific primer, 5ìl of 10 pg RNA template and Nuclease-free water were added to make up the final volume of 20ìl. Amplifications were performed for 1 cycle of 15 minutes at 37°C for reverse transcription, 1 minute at 95°C for activation of GoTaq® DNA polymerase. For denaturation 40 cycles of 10 seconds at 95°C, 40 cycle at 60°C for 30 seconds for the Anneal/Collect data, 40

cycles at 72°C for 30 seconds to Extend, Melting Stage 1 cycle at 95°C for 5 minutes, 1 cycles at 60°C for 5 minutes to annealing and final denaturation (Read) step were performed by 1 cycle 10 minute at 95°C. The relative quantification of the relative expression was carried out using the comparative threshold cycle method as described by Livak and Schmittgen (2001).

Table 1 : List of primers that were used in the present study.

Gene	Accession Number	Sequence (5'→3')	Amplicon size (bp)
Cu/Zn-SOD	EU963633	Forward-TGCATATCGACAGGACCACA	158
		Reverse-TGGGCCAGTCAAAGGAATCT	
GPX	NM_001153000	Forward-CTTCAAGGCTGACTACCCCA	182
		Reverse-GAAGTGGTTGGAGCATAGCG	
MDAR	NM_001196274	Forward-CTGTAAAGGCGATCAAGGGC	189
		Reverse-TCCTTGATCCAGTACGAGCC	
***Zm*Actin 1**	NM_001155179	Forward-AAGTACCCGATTGAGCATGGCA	257
		Reverse-CATCACAATACCAGTTGTTCGCCC	

RESULTS AND DISCUSSION

Proline content

The results of the differential responses of maize varieties to nickel are presented in Table 2, indicating that Fajr-1 variety had higher content of proline in leaves, which accounted to 3.58 ìmole/g, with significant differences compared to Baghdad-3 and 5018 varieties which accounted 2.69 ìmole/g and 2.62 ìmole/g, respectively. Exposing maize plants to different levels of nickel resulted in a gradual increase in the content of proline in their leaves by increasing the concentration of nickel in the soil. This increase was estimated in percentages of 18.75%, 25.83% and 49.16% under the influence of concentrations 200, 300 and 400 ppm, respectively. The first and second concentrations were insignificant, and the third concentration was significant for the control treatment of 2.40 ìmole/g.

The results of the interaction revealed the superiority of treatment 400 ppm with variety 5018 for proline content, as it has the highest content, reaching 4.09 ìmole/g, with a significant difference comparing to control treatment (1.76 ìmole/g). the same variety showed low proline content (2.06 ìmole/g) when treated with 200 ppm of nickel. These results are in agreement with Yildirim *et al* (2019), who confirmed that proline and sucrose content increased after heavy metal treatments.

Proline accumulation is known to play a role in the detoxification of heavy metals;it could be involved in metal chelating in the cytoplasm (Costa and Morel, 1994) by increasing biosynthesis of proteins and increasing the activity of the antioxidant enzymes (catalase and peroxidase). Proline seems indirectly to reduce the toxic impact of heavy metals and increases stress tolerance of plants through such mechanisms as osmoregulation, protection of enzymes against denaturation, and stabilization of protein synthesis (Zengin and Munzuroglu, 2005).

Total protein content

The results of the maize varieties mentioned in Table 3 showed that Fajr-1 variety scored significantly the highest protein content of 8.699% compared to other two varieties, followed by 5018 variety with a value of 7.168%, while the Baghdad-3 variety scored the lowest protein content of a value of 5.324%. These findings indicated that exposing maize plants to high concentrations of nickel stimulated the plant to accumulate proteins in the vegetative system. The maize plants recorded a significant increase in their protein content at all added concentrations; this increase was 31.78%, 45.05% and 59.61% in response to concentrations 200, 300 and 400 ppm, respectively.

The results of interaction between maize varieties and the concentrations of the nickel element confirmed that treatment 400 ppm nickel with variety Fajr-1 had the highest value in the total protein content, which increased significantly by 99.89% over the control treatment of 5.535%. On the other hand, the minimum value of the protein content appeared when treating Baghdad-3 variety with 400 ppm of nickel, which was significantly lower than the control treatment.

The increase in the amount of proline, which plays a role in reducing osmotic stress, acts as a source of C and N, stabilizes protein synthesis and functions as an antioxidant and pH regulator, is caused by many forms of stress. In maize, treatment with Cd resulted not only in a decrease in protease activity, but also in amino acid and proline accumulation. (Nagoor, 1999). Cuypers (2005) explained that plants build new proteins under stress, such

as phytochelates and the metallicthionine also referred to the ability of heavy metals to induce plants to develop antioxidant enzymes that play an important role in reducing the toxicity of those minerals and/or preserving the stability of those minerals.

Expression analysis of antioxidant genes

Heavy metals impact plants in two different ways. First, they change reaction rates and affect the kinetic properties of enzymes that contribute to changes in the metabolism of plants. Second, excessive heavy metals result in stress from oxidants. Plants evolve various resistance mechanisms to escape or tolerate metal stress during the time of metal treatment, including changes in lipid composition, isoenzyme patterns and enzyme activity, sugar or amino acid content and the level of soluble proteins and gene expressions. Such changes include qualitative and/or quantitative metabolic changes that also provide a competitive advantage and influence the survival of plants (Schützendübel and Polle, 2002). Ni has capability to produce OH via a Fenton/Haber–Weiss reaction (Kehrer, 2000). Hao *et al* (2006) reported that excessive Ni results in significant rises in hydroxyl radicals, superoxide anions, nitric oxide and hydrogen peroxide concentrations. Gajewska and Sklodowska (2005) mentioned that Ni is not a redox-active metal, these reactive oxygen species cannot be directly produced (ROS). It indirectly interferes, however with a number of antioxidant enzymes.

Singh *et al* (2019) mentioned that chloroplasts, mitochondria, peroxisomes, apoplasts and plasma membranes are the major cellular ROS generation sites. While ROS is produced as part of normal cellular metabolism in the plant, over accumulation due to stress seriously destroys required cellular ingredients such as carbohydrates, proteins, lipids, DNA, etc. due to their highly reactive nature (Raja *et al*, 2017). Plants primarily deal with oxidative stress via an endogenous defensive mechanism consisting of different enzymatic (superoxide dismutase, SOD; glutathione peroxidase, GPX; monodehydroascorbate reductase (MDHAR) antioxidants (Kaur *et al*, 2019).

Hasanuzzaman *et al* (2012) reported the antioxidant defense mechanism and ROS accumulation maintain a steady-state equilibrium in plant cells. Mittler (2017) added that maintaining an optimal cell ROS level allows adequate redox biology responses and the control of numerous processes that are important for plants, such as growth and development. The balance between ROS creation and ROS scavenging maintains this intermediate stage (Hasanuzzaman *et al*, 2019).

Table 2 : Changes in proline content (µmole/g) of maize varieties under different nickel levels

Variety name	Ni concentrations (ppm)				Average
	Control	200	300	400	
5018	1.76	**2.06**	2.55	**4.09**	2.62^{b}
Baghdad-3	2.09	2.51	3.21	2.97	2.69^{b}
Fajr-1	3.36	3.98	3.28	3.68	3.58^{a}
$LSD_{P\leq0.05}$	1.459				**0.730**
Average	2.40^{b}	2.85^{ab}	3.02^{ab}	3.58^{a}	**Grand mean**
$LSD_{P\leq0.05}$	0.843				**2.96**

Table 3 : Changes in total protein content (%) of maize varieties under different nickel levels.

Variety name	Ni concentrations (ppm)				Average
	Control	200	300	400	
5018	4.150	6.408	7.488	10.627	7.168^{b}
Baghdad-3	6.115	5.972	5.678	**3.532**	5.324^{c}
Fajr -1	5.535	8.443	9.753	**11.064**	8.699^{a}
$LSD_{P\leq0.05}$	0.5535				**0.2767**
Average	5.267^{d}	6.941^{c}	7.640^{b}	8.407^{a}	**Grand mean**
$LSD_{P\leq0.05}$	0.3196				**7.064**

As represented in Table 4, variety 5018 appeared down-regulation of *SOD* gene under graduated concentrations of nickel (2.531-fold, 19.607-fold and 21.276-fold). When treated Baghdad-3 variety with 200 and 300 ppm of nickel, the plants show up-regulation of *SOD* gene as 0.221-fold and 0.443-fold respectively, while 400 ppm concentration recorded a decrease in the expression level of *SOD* gene as 15.625-fold. Fajr-1 variety scored the highest level of *SOD* gene expression under 300 and 400 ppm concentrations of nickel as 4.314-fold and 3.789-fold, respectively.

SOD (SOD; EC 1.15.1.1.1) gene is broadly spread among organisms that consume O_2 and is responsible for dismuting $O_2^{\bullet-}$ into H_2O_2 and thus affecting the concentration of $O_2^{\bullet-}$ and H_2O_2 reducing the probability of OH formation. In higher plants, *SOD* isoenzymes are compartmentalized and three isoenzymes have been found in plants identified by their metal cofactor: Mn, Fe, and Cu/Zn (Gill *et al*, 2005). In the mitochondria and peroxisomes, Mn-SODs are found, Fe-SOD has been shown to be correlated with chloroplasts, whereas in the cytosol, chloroplasts and peroxisomes Cu/Zn-*SOD*s are located (Gratão *et al*, 2005). The enzyme SOD in plants is directly linked to stress, which creates the first line of protection, turning $O_2^{\bullet-}$ into H_2O_2 (Gupta *et al*, 2018).

Maize varieties that treated with 200 ppm of nickel scored down-regulation with *GPX* gene of 1.592-fold, 1.197-fold and 1.818-fold for 5018, Baghdad-3 and Fajr-1 varieties, respectively. In contrast, 300 and 400 ppm

nickel recorded an increase in expression level of *GPX* gene with the three varieties as 0.547-fold and 4.133-fold for 5018; 7.876-fold and 15.679-fold for Baghdad-3; 4.426-fold and 4.617-fold for Fajr-1, respectively (Table 5).

GPX (EC 1.11.1.9) is a non-heme member of the POX family. An antioxidant enzyme with an extremely reactive thiol group that using GSH and TRXs to scavenges H_2O_2, Lipid reduction and Organic hydroperoxides (Bela *et al*, 2015).

According the results that demonstrated in Table 6, variety 5018 undergoes down-regulation for *MDAR* gene under elevated levels of nickel as 23.255-fold, 5.102-fold and 4.878-fold, respectively. Regarding variety Baghdad-3, it was shown down-regulation for *MDAR* gene by 2.364-fold in response to 200 ppm nickel, but the level of gene expression begins to rise slightly above the normal level in response to the concentrations 300 and 400 ppm as 0.375-fold and 3.856-fold. Under the influence of graded concentrations of nickel (200, 300 and 400 ppm), there was an obvious increase in gene expression in Fajr-1 variety as 20.406-fold, 353.588-fold and 149.122-fold, respectively.

Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) containing a thiol group responsible for MDHA converting to AsA by a NADPH-dependent flavin adenine dinucleotide enzyme-MDHAR which found as two isoforms in various cellular locations (Hasanuzzaman *et al*, 2019). When pigeon pea plants were treated with 0.5-1.5mM Ni, malondialdehyde (MDA, a lipid peroxidation product) content increased in the roots and shoots (Rao and Sresty, 2000). In corn, similar results have also been recorded by Baccouch *et al* (2001). Ni stress-induced increase in performance of SOD, GPX and MDHAR which was further up regulated by Si supplementation that helped to minimize Ni toxicity (Ahanger *et al*, 2020).

Table 4 : Relative expression of *SOD* gene in maize under different levels of nickel.

Variety name	Heavy metal concentration (ppm)		
	200	300	400
5018	0.395	0.051	0.047
Baghdad-3	1.221	1.443	0.064
Fajr-1	0.157	5.314	4.789

Table 5 : Relative expression of *GPX* gene in maize under different levels of nickel.

Variety name	Heavy metal concentration (ppm)		
	200	300	400
5018	0.628	1.547	5.133
Baghdad-3	0.835	8.876	16.679
Fajr-1	0.550	5.426	5.617

Table 6 : Relative expression of *MDAR* gene in maize under different levels of nickel.

Variety name	Heavy metal concentration (ppm)		
	200	300	400
5018	0.043	0.196	0.205
Baghdad-3	0.423	1.375	4.856
Fajr-1	21.406	354.588	150.122

CONCLUSION

The present results indicated a significant increase in the shoot content of total proteins corresponding with the increase of nickel concentrations; this could be related to the increase in the level of gene expression of the antioxidant enzymes (*SOD*, *GPX* and *MDAR*) under the influence of elevated concentrations of nickel. This increase was also accompanied by the accumulation of proline in the plant. Fajr-1- variety recorded the highest level of proline, total proteins and gene expression of *SOD* and *MDAR* enzymes; this reflects the ability of this variety to withstand high concentrations of nickel. Baghdad-3 variety scored the highest level of *GPX* gene expression under nickel stress.

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