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Article

Gene expression of a nitrogen tolerance gene ZmNR1 under the influence of different levels of nitrogen in maize

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Abstract: A field experiment was carried out in the College of Agricultural Engineering Sciences - University of Baghdad, during the fall season of 2021 to find out which cultivated cultivars of maize are efficient under nitrogen fertilization. The experiment was applied according to an RCBD (split-plot design with three replications). The cultivars of the experiment (Baghdad, 5018, Sarah) supply three levels of nitrogen fertilizer, which are N1 (100 kg.N/ha), N2 (200 kg.N/ha) and N3 (300 kg.N/ha). The statistical analysis results showed the superiority of the Sarah genotype, which gave the highest value of SOD and CAT enzymes, reaching 11.59 units mg-1 and 10.76 units mg^{-1}. Protein sequentially, while cultivar5018 outperformed as it gave the highest value of POD enzyme, which was 5.43 units mg-1.protein, and there were no significant differences between genotypes in NR enzyme. The increase in nitrogen fertilizer caused an increase in the values of oxidation and reduction enzymes, as the nitrogen level N3 gave the highest value for SOD, POD, CAT and nitrate reduction enzymes NR, reaching 11.59 mg^{-1} and 6.94 mg^{-1} units. Protein and 16.40 mg-1 units. Protein and 6.30 mg^{-1} units. Protein sequentially. The results of the molecular analysis using the Real-Time PCR technique showed the expression of the ZmNR1 gene. The analysis showed that the cultivated genotypes contained the gene in varying proportions as the gene expression increased in the compositions to which the nitrogen fertilizer was added. The value ranged from (0.16) to (49.46) times (a copy of the gene), where the highest expression of the gene was (49.46) for the Sarah cultivar when The nitrogen level N2 also gave the same gene expression ZmNR1 (15.01) folds. The cultivars of maize varied among them in their tolerance to excess or deficiency of nitrogen and in their ability to express the ZmNR1 gene, one of the most important nitrogen-carrying genes for maize crops.

Keywords: maize, gene expression, nitrate reductase, antioxidant enzymes

Introduction

Nitrogen (N) is a major limiting factor in most agricultural systems because the availability of nitrogen in abundance affects crop productivity, and a large amount of fertilizer is added to increase the yield. However, the nitrogen use efficiency (NUE) for grain is as low as 33%, which means that More than 60% of the manure added is lost to the environment. Nitrogen is absorbed as nitrate (NO_3^-) and (NH_4^+) by radicals through nitrate transporters (NRT1 and NRT2); the absorbed nitrate is first reduced to nitrite by nitrate reductase (NR) and finally to ammonium by nitrite reductase (NiR) in the chloroplasts. Then, the ammonium is incorporated into the organic form by the glutamine complex (GS) and glutamate

synthase/glutamine-2-oxoglutarate aminotransferase (GOGAT), also known as GS/GOGAT[2]. To develop nitrogen use, Effective genotypes should be used to understand plants' responses to nitrogen deficiency or excess and adaptive changes to reduce the adverse effects of stress conditions. Changes in the expression of key genes play an essential role in nitrogen metabolism. In this study, knowing the genes associated with nitrogen tolerance and metabolism in corn is vital. Increasing the level of gene expression for nitrogen tolerance increases the effectiveness of metabolic pathways that directly depend on light energy, as the reduction of nitrate to nitrite through the enzyme nitrate reduction to nitrite or to ammonium, which is carried out by the compound NADPH as well as the Ferrodoxin protein, which is required to raise the level of nitrogen tolerance. Gene expression to absorb large amounts of nitrogen, which is converted into amino acids and sucrose synthesis, and increasing the gene expression activity of the NR gene requires ATP energy and light[3]. The aim of this search:

They are measuring the gene expression of the ZmNR1 gene under each effort level and determining the amount of fold gene expression to find out which genotypes are more tolerant of a decrease or increase in nitrogen fertilizer.

I am studying the activity of oxidation and nitrate reductase enzymes among the genotypes of maize under the influence of excess or deficiency of nitrogen.

Materials and Methods

A field experiment was carried out during the autumn season of 2021 in the experimental field - College of Agricultural Engineering Sciences - University of Baghdad, to use some molecular parameters to identify some genes related to plant nitrogen tolerance, such as ZmNR1, and to measure the gene expression of the ZmNR1 gene under each nitrogen stress level and to determine the amount of several fold gene expression to identify which cultivars are more tolerant of decreased or increased nitrogen fertilization in maize. The land was plowed with a flip-flop plow on 15/07/2021, and soil samples were taken to analyze and measure ready nitrogen in the soil, total nitrogen, soil pH and soil EC. Then the process of tillage and leveling of the field area was carried out and divided into identical experimental units (3×9 m); three cultivars of maize were used(Baghdad,5018, Sarah) according to the RCBD (split-plot design), and the seeds were planted on the distance between of furrows and another 0.75 m and between one hole and another 0.25 m at the rate of 2-3 seeds/hole on 7/26/2021. The field was irrigated every 4-5 days. The plants were thinned to one plant after 15 days from emergence; urea (N46%) was supplied in three levels at the rate of 100 kg N/ha, 200 kg N/ha and 300 kg N/ha in two periods of time, the time 16/8/2021 and the second 2021/ 9/4, and the wedding was carried out as needed for this process. The activity of enzymes such as nitrate reductase was measured according to superoxide dismutase[5], while peroxidase[6] and catalase[7] samples were collected for molecular analysis. Each sample was extensively pulverized in liquid nitrogen using a mortar and pestle to produce a fine powder. 100 mg (±1) was transferred to a 2 ml microcentrifuge tube to extract RNA using the RNeasy Mini kit (No. 74104k-Qiagen, USA) and according to the manufacturer's instructions. The RNA cleanup method was then carried out using 100 l of RNA and adhering to the RN simple mini kit's manufacturer's recommendations. The high-capacity RNA-to-cDNA kit was used to convert clean RNA samples to cDNA (No. 4387406- Applied biosystem, Thermo Fisher Scientific, USA). 2 µg from RNA was used in this reaction of a total of 20 µl reaction. The mixture of this reaction was 10 µl RT buffer, 1 µl RT enzyme, up to 9 µl from RNA samples, and then the mixture to 20 µl using Nuclease-free H2O. Quickly

centrifuge the mixture and RNA sample to remove any air bubbles. The mixture was then incubated in the thermal cycler at 37°C for one hour, and then the action was stopped by raising the temperature to 90 °C for 5 min. Samples were stored at -20° C for use in RT-PCR. Primers' sequences were designed in Blast software as follows: ZmNR1 Forward:

5`-ATGGGGTACGACCTCGACAA-3`,dhn1Reverse:5`-TCGACACGTTACTAGGGTTCA-3` and for actin Forward: 5`-CAGACATAGACCCAAACCCGAT-3`andReverse: 5`-ACAGTTGCCCATTGTCAAAGAA-3`[8].The following compounds were combined to perform real-time PCR: SYBR Green master mix (10 l, twice), forward and reverse primers (0.5 l each), and reference dye (0.3 l), 1 µl experimental cDNA and 7.7 µl Nuclease-free PCR-grade water (according to Brilliant III Ultra-Fast SyBR Green-No. 600882- Agilent Technologies-USA). Thermal cycling was performed: 1 cycle at 95°C for 3 minutes, 5 sec. at 95°C, and 15 sec. at 60°C (40 cycles). The LSD test at 0.05 was used to see whether there were any significant differences between means after the data had been statistically analyzed using ANOVA.

Results

The efficacy of oxidation and reduction enzymes

Nitrate reductase enzyme NR (unit mg^{-1}. Protein) :

Table 1 showed significant differences between the cultivars of the nitrate reductase, where the cultivar (Sara) gave the highest rate of (4.144) unit mg^{-1}.protein compared to cultivar (5018), which gave the lowest rate of (3.144) unit mg^{-1}.protein. Protein, increasing the level of nitrogen fertilization increases the efficiency and effectiveness of NR in converting nitrate into nitrite since the enzyme consists of a metal part, nitrogen[10]It was also shown from the results of the statistical analysis in Table 1 that there was a significant effect of the interaction between of cultivars and the levels of nitrogen fertilization, where the cultivar (Sara) at the nitrogen level N3 gave the highest rate of (6.50unit mg^{-1}.proteincompared to the cultivar (Baghdad).

Cultivars	Nitrogen level			Mean Culti-vars
	N1	N2	N3	
Baghdad	1.17	4.47	6.06	3.90
5018	1.63	3.08	6.34	3.69
Sarah	2.23	4.65	6.50	4.46
L.S.D5%	0.616			0.357
N	1.67	4.07	6.30	
L.S.D5%	0.482			

Table 1. Effect of nitrogen fertilization levels on nitrate reductase in cultivars of maize.

Super oxide dismutase (unit mg^{-1}):

The results of Table 2 showed that there were significant differences between the cultivar in the SOD enzyme, where the cultivar (Sara) gave the highest rate of (11.59) mg^{-1} units compared to the cultivar (Baghdad), which gave the lowest rate of (8.38) unit mg^{-1}. That the increased concentration of SOD in the cultivars is due to an increasing in genetic parameters of mitochondria, that serve of protect of plant from aging[12] The results of Table 2 also showed that there were

significant differences between the levels of nitrogen fertilization in the character of SOD enzyme, as the level of fertilization N3 gave the highest rate of (15.03 unit mg^{-1}compared to the level of nitrogen fertilizer N1 which gave the lowest rate of (4.23) unit mg^{-1}, that nitrogen works to increase the N-terminal region of the SOD enzyme, as well as the formation of the amino acids Aspartic acid, histidine, which are the basis in the synthesis of SOD[13]The results of Table 2 also showed that there were significant differences in the interaction between the genotypes and the levels of nitrogen fertilization, as the cultivar(Sara) at the level of nitrogen fertilization N3 gave the highest rate of (17.79)unit mg^{-1}compared to the cultivar (Baghdad) at the level of nitrogen fertilization N1, which gave the lowest rate of (2.98)unit mg^{-1}.

Cultivars	Nitrogen level			Mean Culti-vars
	N1	N2	N3	
Baghdad	2.98	9.07	13.45	8.50
5018	3.48	7.81	13.86	8.38
Sarah	6.23	10.74	17.79	11.59
L.S.D5%	3.180			1.440
N	4.23	9.21	15.03	
L.S.D5%	3.012			

Table 2. Effect of Nitrogen Fertilization levels on superoxide Dismutase of Maize genotypes.

Peroxidase (unit mg^{-1}. protein):

Table 3 showed significant differences between the genotypes in the concentration of peroxidase enzyme, where the cultivar(5018) gave the highest rate of (5.43)unit mg^{-1}. Compared to the cultivar (Sara), protein gave the lowest rate of (3.89) unit mg^{-1}. Protein. The difference in the concentration of the enzyme peroxidase is caused by the presence of the TPX2 gene that encodes this enzyme and increases the concentration of Peroxidase that protects the plant cell from ROS[15]. The results of Table 3 also showed a significant effect of nitrogen fertilization on the concentration of POD enzyme, as the highest rate was at the nitrogen level N3 (6.94) unit mg^{-1}. Protein compared to the nitrogen level N1, which gave the lowest rate of (2.75) unit mg^{-1}. Protein. Increasing the nitrogen fertilization level increases the POD enzyme's activity to the permissible limit since nitrogen is included in the construction of this enzyme[16]. It was also shown from the results of Table 3 that there was a significant effect of the interaction between the genotypes and the levels of nitrogen fertilization, as the cultivar (5018) at the level of nitrogen fertilization N3 gave the highest rate of (8.56) unit mg^{-1}. Protein compared to the cultivar (Baghdad) at The nitrogen level N1, which gave the lowest rate was (1.62) unit mg^{-1}. Protein.

Cultivars	Nitrogen level			Mean Cul-tivars
	N1	N2	N3	
Baghdad	1.62	3.45	7.06	4.05
5018	3.68	4.04	8.56	5.43
Sarah	2.95	3.52	5.21	3.89
L.S.D5%	6.90			0.488
N	2.75	3.67	6.94	
L.S.D5%	1.515			

Table 3. Effect of nitrogen Fertilization levels on Peroxidase Enzyme Genotypes in maize.

Catalase enzyme (unit mg^{-1}. protein):

Table 4 showed significant differences between the genotypes in the enzyme catalase, where the cultivar (Sara) gave the highest rate of (10.76) unit mg^{-1}. Protein compared to the cultivar (Baghdad), which gave the lowest rate of (7.59) unit mg^{-1}. Protein. The difference in CAT concentration between the genotypes is due to the genetic nature of the cultivars in producing CAT enzyme[18]. The results of Table (4) also showed a significant effect of nitrogen fertilization in increasing the concentration of CAT enzyme, as the level of N3 fertilization outperformed the rest of the fertilization levels by giving it the highest rate of (16.40) unit mg^{-1}. Protein compared to the level of N1 fertilization, which gave an average of (4.43) units of mg^{-1}. Protein.

Cultivars	Nitrogen level			Mean Culti-vars
	N1	N2	N3	
Baghdad	3.54	7.30	11.93	7.59
5018	4.14	6.88	17.90	9.64
Sarah	5.61	7.28	19.38	10.76
L.S.D5%	1.275			0.820
N	4.43	7.15	16.40	
L.S.D5%	0.775			

Table 4. Effect of Nitrogen Fertilization Levels on Catalase Super Enzyme for Genotypes in Yellow Maize.

Gene expression of NR gene in maize cultivars under different nitrogen levels:

All enzymatic processes are affected by the activity of the enzyme (NR), which requires a high absorption of nitrogen by the plant, which helps in the development of plant roots, leaf expansion and regulation of expression, which is involved in nitrogen and carbon metabolism[21]As the nitrogen used in glutamine is used to represent many amino acids, nucleic acid, proteins as well as secondary metabolites[22] Although the plant did not absorb high levels of nitrogen in a gaseous form. This indicates that the plant absorbs nitrogen as nitrate and ammonia from the inorganic nitrogen element through the roots[23] And that the biological nitrogen formula and the high concentrations in the soil lead to plants participating in several processes to absorb nutrients in several ways[23]. Thus regulating the cooperation between plant functions, the enzyme NR (NR, EC 1.6.6.1) is the first enzyme involved in the representation of nitrogen, where it is reduced by NO3- to -NO2 in the plant cell cytosol through the catalyst of NR, which participates with the enzyme glutamine synthetase (GS, EC). 6.3.1.2) as well as glutamate synthetase (GOGAT, EC 1.4.1.14), which then forms a glutamine and glutamate layer, the essential amino acids required to build proteins[24].The plant's tolerance of nitrogen deficiency is tantamount to managing the nitrogen stress signal and stimulating the signal, which causes the activation of various physiological and metabolic processes. There are hundreds of genes and their metabolic pathways identified as essential genes for tolerance to nitrogen deficiency, as the ZmNR1 gene is essential for converting NO3-to-NO2 by the enzyme nitrate reductase. Also, some of the gene products needed by the plant are necessary under all conditions, which are called (housekeeping genes, endogenous, control or reference genes), including $Actin^{-1}$, DNA polymerase and RNA polymerase, and these genes that the plant requires their products under certain conditions such as (conditions of nitrogen deficiency) The plant does not always need it, such as the conditions of the plant's sufficiency of nitrogen, and that these genes can be expressed in high or low or may not be expressed at all,

and that the use of Housekeeping is used to calibrate mRNA between different samples. The gene expression value of the target gene called ZmNR1 was calculated as a result of its expression under nitrogen addition factors as shown in Table (5). It is noted from the table data that this gene has increased its expression in some genotypes to which nitrogen is added, but at different degrees from (0.16) to (49.96) times (a copy of the gene) more than its expression in plants without nitrogen added every 100 kg.N/ha.

Cultivars	Nitrogen level	ΔCT	ΔΔCT	NR	ACT	Folding
Baghdad	control	-7.39	0	27.82	35.21	**1**
	N2	-7.97	-0.575	27.725	35.695	**15.0125**
	N3	-5.69	1.7	30.21	35.91	**0.4075**
5018	control	-7.515	0	29.085	35.1	**1**
	N2	-4.87	2.645	31.04	35.91	**0.1665**
	N3	-7.54	-0.03	28.56	36.105	**1.2465**
Sarah	control	-3.135	0	32.875	36.01	**1**
	N2	-1.85	1.29	34.6	36.445	**49.9695**
	N3	-4.19	-1.055	30.825	35.015	**29.024**

Table 5. Gene expression of the ZmNR1 gene for maize genotypes under three levels of nitrogen fertilization.

Discussion

The difference between cultivars in increasing nitrate reductase is due to the genotypes containing high levels of NADPH, NAD critical in NR biosynthesis[9]. The results of Table 1 showed that there was a significant effect of nitrogen fertilization on the characteristic of nitrate reduction enzyme, where the level of nitrogen fertilizer N3 gave the highest value of (6.30) unit mg^{-1}.protein.compared to the level of nitrogen fertilizer N1, which gave the lowest value of (1.67)unit mg^{-1}.protein.

At the nitrogen level N1, the lowest rate was (1.17) units mg^{-1}.protein. The increase in nitrogen fertilization caused the effectiveness of NR in some cultivars for having high NAD[11].

The increase in nitrogen fertilization and its response varies according to the cultivar and its effectiveness, as it increases the FSOD enzyme, which regulates the concentration of O_2, which in turn works to remove the effect of superoxide ethion before it interacts with H_2O_2, which is OH and increases plant aging[14]

The increase in the concentration of POD enzyme in the cultivar by increasing the level of nitrogen fertilization is because nitrogen increases the formation of the POD enzyme, which in turn leads to the oxidation of acid oxidation of IAA and the elongation of plant cells[17].

Moreover, increasing the concentration of catalase enzyme in the presence of nitrogen, which is included in the compound NADPH-binary, leads to an increase in the activity of photosynthesis in leaves. It was clear from the results of the same Table that the cultivar (Sarah) at the N3 level was superior by giving the highest rate of (19.38) unit mg $^{-1}$. Protein compared to the cultivar (Baghdad) at the N1 level gave the lowest rate of (3.54) unit mg $^{-1}$. Protein. The increase in the activity of the enzyme CAT catalase in the cultivars is due to the increase in the gene expression of CAT1 and CAT2 genes by increasing the nitrogen concentration in the plant[20].

Furthermore, increasing the expression of the ZmNR1 gene increases nitrogen tolerance. It represents ammonium to amino acid and chloroplast glutamine synthetase (GS2), which binds with ferredoxin-dependent isoenzyme (Fd-GOGAT, as well as represents ammonium (NH4+) and returned it to the leaves[26] of. It was also found that this gene plays a significant role in the aspartate aminotransferase (AspAT; EC 2.6.1.1) in making green tissues by preparing Aspartate (ASP) in the biosynthesis of amino acids[27] Using the total polymerase chain reaction (Real-Time PCR) Quantitative levels of mRNA by using actin-1 as gene reference (gene expression constant under all agents to which the plant is exposed). The results of gene expression diagnosis showed in Table (5) the differences between the genotypes used for the levels of added nitrogen mRNA in the expression of the ZmNR1 gene when the nitrogen addition was increased every 100 kg. The clone of ZmNR1 doubled in maize plant leaf samples to show the adequacy and insufficiency of the added nitrogen; the expression increased in all the genotypes under study when adding 100 kg. 49.96-fold for this gene in N2 of Sarah cultivar, while the lowest expression level was (0.1665)-fold for cultivar 5018 at N2.The increase in the ability of gene expression in both the cultivar (Sarah)under the (N2 level) as well as (Baghdad N2) led to an increase in the levels of NR to the permissible limit, which has a role in converting NO3- to -NO2 and thus giving them a good result. These results agreed with the findings[28,29,30].

Conclusion

That maize was exposed to nitrogen deficiency, or excess nitrogen, led to the activity of the enzyme NR, which amounted to 5.13 units mg^{-1}.protein at the level of N3. The ZmNR1 gene has an important role in nitrogen tolerance to increase its expression in some cultivars, especially when exposing plants to an excess or deficiency of nitrogen. Exposing plants to nitrogen deficiency in the early stages of plant life leads to an increase in the gene expression of the ZmNR1 gene.

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