

## ***IN VITRO* EFFECT OF HUMIC FERTILIZER ON ACTIVITY OF NITRATE REDUCTASE, UNDER DROUGHT STRESS MEDIATED THROUGH POLYETHYLENE GLYCOL IN WHEAT**

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### **ABSTRACT**

**Activity of nitrate reductase changes rapidly in response to stresses. Humic substances can indirectly and directly affect the physiological processes of plant growth. They have selective effects on enzyme activities. An *in vitro* experiment was conducted for finding the effects of humic fertilizer on nitrate reductase activity in wheat plantlets. Four genotypes were cultured in Petri dishes in different environmental conditions (normal; normal + potassium humate; drought; drought + potassium humate). Polyetylen glycol 6000 was applied as drought inducing agent. Statistical analysis showed that potassium humate increased nitrate reductase activity in both primary roots and the first young leaves in stressed and non stressed environments. The highest enzyme activity in both primary roots and the first young leaves was observed in Gobustan, compared to other genotypes.**

**Key words:** *Triticum aestivum* L., humate, humic, nitrate reductase, drought, PEG.

### **INTRODUCTION**

**N**itrogen, in one form or another, accounts for about 80% of total mineral nutrients absorbed by plants (Marschner, 1995) and it is the second constituent by quantity of plant tissue after carbon constituting 1.5% of plant's dry material. Nitrogen is one of the elements playing a major role in plant metabolism, as it becomes directly involved in germination of the seeds, structure of tissues (walls and membrane), protein molecules, nucleic acids and pigmentation. Plant absorbs nitrate or ammonium solution available in the soil using its root system. Assimilation of nitrate involves a reduction phase, which takes place prior to real assimilation. Thus, in theory, ammonium must be remarkably more economical than nitrate in energetic terms, as it is readily digestible. However, nitrate produces the highest yield of growth and production, whereas ammonium often reveals the signs of toxicity. Of all the various factors differentiating the nutrition of nitrate and

ammoniac, the most noticeable ones are the way these two ions are absorbed and the changes of pH of intracellular space during their replication (Hopkins and Norman, 2004).

Once the absorption of nitrate into the cell is complete, nitrate reductase enzyme should reduce it to nitrite. This enzyme is found in cytosol of epidermic, root crust and mesophile cells (Fedороva, 1994). Partitioning of nitrate reductase enzyme between root and aerial organ varies depending on the species and age of the plant, as well as on environmental factors. In most forage plants, the reduction of nitrate takes place in the leaves, whereas in woody species this often takes place in the roots. The contributions of root and aerial organ in the reduction of nitrate taking place in a complete plant, depends upon the relative size of the organ, age of tissue and in general on the ability of root to mobilize nitrate into the aerial organ (Carelli and Fahl, 2005). When nitrate is used as the source of nitrogen nutrition, the activity of nitrate reductase enzyme increases. With

ammonium, authors believe that the above mentioned enzyme plays a regulatory role. It is known that ammonium in family *Lemnaceae* and in species such as *Chlorella* and *Neurospora* counteracts the activity of nitrate reductase. However, in most higher plants, it has either had no effect on, or increases the activity of the nitrate reductase (Poonacchita and Darnell, 2004).

Nitrate reductase (NR), is a regulatory enzyme restricting the rate of nitrate consumption, and there have been numerous studies investigating its catalytic functions. In higher plants, an oligomeric complex depends upon NAD (P) H containing FAD, hem (cytochrome b566) and a molybdenum-pterin prosthetic. It is known that there are three molecular forms of nitrate reductase within the roots of wheat (Kenjebavea and Rakova, 1995). Nitrate reductase is recognized as a limiting factor for growth and protein production within the plants. This enzyme is inevitably affected by environmental conditions. Physiological investigations suggest that there is a complex regulatory system controlling gene expression of nitrate reductase against environmental factors (Botella et al., 1993). Activity of nitrate reductase changes rapidly in response to lower temperature, salinity or osmotic stress. Unfortunately, it is unknown how different stresses regulate the activity of this enzyme (Bungard et al., 1999).

Nitrate and ammonium ions are most abundant nitrogen sources for higher plants and their availability in the soil usually constitutes a limiting factor for plant growth (Causin and Barneix, 1993).

Potassium humate causes increase in tolerance of plant to drought stress (Shahriari et al., 2008). Humic substances (HS) are natural organic compounds. Organism development, hormone-like activity, nutrient carriers, catalysts of biochemical reactions and antioxidant activity have been proposed as their principal ways of action (Kulikova et al., 2005). HS are known to possess bioactivating properties in relation to plants. Humic preparations are increasingly applied as stimulators in plant breeding (Shahryari et al., 2009). Research has confirmed that humic

substances can indirectly and directly affect the physiological processes of plant growth. They have effects on photosynthesis, formation of ATP, amino acids, carbohydrates and proteins, nucleic acids synthesis, and selective effects on enzyme activities (Gadimov et al., 2009).

This study was focused on: 1) Effect of potassium humate on activity of nitrate reductase in the primary roots and young wheat plant under *in vitro* condition; 2) Investigating the effect of drought on synthetic activity of nitrate reductase enzyme in various wheat genotypes; 3) The role that nitrate reductase can play in developing drought tolerance in the studied genotypes.

## MATERIAL AND METHODS

Four bread wheat (*Triticum aestivum* L.) genotypes, namely Gascogne, Sabalan, 4057 and Gobustan were planted in a factorial experiment based on completely randomised block design. One factor was conditions: normal; normal + potassium humate; drought; drought + potassium humate. Other factor was wheat genotypes.

Liquid humic fertilizer (potassium humate) derived from peat was applied as 2 ml/l. There were 3.3% w/v humic acids, 0.9% w/v fulvic acids and totally 4.2% w/v humic extract in it.

Polyethylene glycol 6000 (PEG) was used to apply drought under *in vitro* condition and a pressure as high as -7 bar was created.

Treatments by PEG solution were done for drought conditions after germination. Temperature of laboratory was about 23±1 °C during the experiment.

Measurements of nitrate reductase activity (NRA) and its related characters were as described by Bybordi (2010). So, NRA was measured in the young leaves (third or fourth from top) according to Klepper et al (1971). The leaf tissue (0.2 g fresh weight) was placed in reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.5), 0.02 M KNO<sub>3</sub>, 50% isopropanol, 0.05 chloramphenicol at 30°C for 1 h in the dark. The indicative Grease reagent containing 0.001 g/l

naphtyl-ethylene diamine, 0.01 g sulfanilic acid, and 0.9 g tartaric acid was added to each sample. The concentration of nitrite ( $\text{NO}^{3-}$ ) formed during the reaction was measured spectrophotometrically at 540 nm. Nitrate in tissue samples was determined by nitration of salicylic acid (Cataldo et al., 1975). Approximately 0.2 g of dried tissue powder was placed in 125 ml container and 25 ml hot water was added. The samples were shaken for 30 min on a Wristaction shaker and filtered through Whatman No 42 filter paper. Nitrate in the filtered solution was determined by adding a 0.2 ml sample aliquot containing 0.8 ml of 5% (w/v) salicylic acid  $\text{H}_2\text{SO}_4$  mixture and 19 ml 2 N NaOH. Samples were allowed to cool at room temperature for 1 h, and developing colour was measured at 410 nm by spectrophotometer (JENWAY, China). The concentration of total nitrogen (N) in the youngest fully expanded leaves was determined by Kjeldahl method. The same above mentioned method was used for measurements in primary roots.

A statistical analysis was made using analysis of variance by MSTATC software and the means were compared by Duncan's Multiple Range Test at 5% probability level.

## RESULTS AND DISCUSSION

When in plant experiences any stress, is present the amount of nitrate reductase enzyme increases. Tolerance against drought is a complex trait, and its development involves the interaction of various morphological (early maturity, reduced leaf area, leaf rolling, wax rate, functional root system, awn presence, sustainable yield and reduced tillering), physiological (reduced transpiration, increased water consumption efficiency, closed stomata and osmotic regulation), and biochemical (accumulation of proline, polyamine, Trehalose, etc., increased activity of nitrate reductase enzyme and increasing stored carbohydrates) traits. Genetic mechanisms controlling these traits are not well completely understood.

Kondrat'ev and Lebedinskaya (1995) believed that the activity of nitrate reductase

enzyme is not genetically controlled very strictly and environmental factors are by far more important. Botella et al. (1993) and Cramer et al. (1995) reported that the nitrate reduction mainly takes place in the leaves of cereal crops.

Results from analysis of variance on values of nitrogen, nitrate and activity of nitrate reductase enzyme in the presence of various levels of polyethylene glycol and humate are presented in table 1 for four wheat genotypes. Results indicated that there was a significant difference between the studied genotypes in terms of all evaluated traits. This represents a high genetic variation between genotypes, which can be exploited for selection based on traits of interest. Studies showed that there was a significant difference between the conditions of this research for all three evaluated traits in both primary roots and young leaves. In the primary roots, effect of conditions  $\times$  genotypes was only significant for enzyme activity at probability level of 5%.

Mean comparisons for measured traits in different conditions of this experiment for primary roots (Table 2) revealed that potassium humate increased N from 0.95 to 1.375%;  $\text{NO}^{3-}$  from 1.043 to 1.301  $\text{mg kg}^{-1}$  and NRA from 1.858 to 3.000  $\mu\text{mol h}^{-1}\text{g}^{-1}$  in non stressed condition. Also, potassium humate increased N,  $\text{NO}^{3-}$  and NRA in stressed condition, from 0.81 to 1.383%; 1.050 to 1.223  $\text{mg kg}^{-1}$  and 4.508 to 5.692  $\mu\text{mol h}^{-1}\text{g}^{-1}$ , respectively. Potassium humate increased nitrate reductase enzyme activity of primary roots in both stressed and non stressed conditions. It increased nitrogen content by 0.425%, nitrate amount by 0.258  $\text{mg kg}^{-1}$  and NRA by 1.142  $\mu\text{mol h}^{-1}\text{g}^{-1}$  in non stressed condition, and increased these parameters by 0.573%, 0.173  $\text{mg kg}^{-1}$  and 1.184  $\mu\text{mol h}^{-1}\text{g}^{-1}$ , respectively in the stressed condition.

In the young leaves, potassium humate also increased all of three measured characters in both non stressed and stressed conditions (Table 2), increasing: N from 2.95 to 3.56%;  $\text{NO}^{3-}$  from 1.626 to 1.852  $\text{mg kg}^{-1}$  and NRA from 6.592 to 8.092  $\mu\text{mol h}^{-1}\text{g}^{-1}$  in non stressed condition, and also, potassium

humate increased N,  $\text{NO}_3^-$  and NRA; respectively, from 2.142 to 2.810%; 1.489 to 1.713  $\text{mg kg}^{-1}$  and 10.320 to 11.640  $\mu\text{mol h}^{-1}\text{g}^{-1}$  in stressed condition. The increases was for nitrogen content by 0.61%, for nitrate amount by 0.226  $\text{mg kg}^{-1}$  and for NRA by 1.500  $\mu\text{mol h}^{-1}\text{g}^{-1}$  in non stressed condition, and by 0.668%, 0.224  $\text{mg kg}^{-1}$  and 1.320  $\mu\text{mol h}^{-1}\text{g}^{-1}$  respectively, in the stressed condition.

Mean values (Table 3) indicated that Gobustan (1.208%) and Gascogne (1.017%) had the highest and lowest value of nitrogen in their primary roots, respectively. Gobustan (1.210  $\text{mg kg}^{-1}$ ) and 4057 (1.118  $\text{mg kg}^{-1}$ ) had the highest, whereas Gascogne (1.096  $\text{mg}$

$\text{kg}^{-1}$ ) had the lowest amount of nitrate in their primary roots. Gobustan (4.158  $\mu\text{mol h}^{-1}\text{g}^{-1}$ ) and Sabalan (3.667  $\mu\text{mol h}^{-1}\text{g}^{-1}$ ) produced the highest and lowest mean levels of enzyme activity in the primary roots, respectively.

Gobustan (3.033%) and Gascogne (2.717%) had the highest and lowest amount of nitrogen in their young leaves, respectively. 4057 had the highest mean amount of nitrate in its young leaves (1.727  $\text{mg kg}^{-1}$ ) followed by Gobustan (1.699  $\text{mg kg}^{-1}$ ), whereas Gascogne (1.596  $\text{mg kg}^{-1}$ ) had the lowest amount. Gobustan (9.592  $\mu\text{mol h}^{-1}\text{g}^{-1}$ ) and Gascogne (8.692  $\mu\text{mol h}^{-1}\text{g}^{-1}$ ) produced the highest and lowest values in terms of enzyme activity, respectively.

Table 1. ANOVA for measured characters at different levels of PEG and humate in primary roots and first young leaves

S. O. V.	Df	MS					
		Primary roots			The first young leaves		
		N	Nitrate	Nitrate reductase activity	N	Nitrate	Nitrate reductase activity
R	2	0.411**	0.065**	0.158**	0.684**	0.16**	75.164**
Conditions (C)	3	1.04**	0.198**	33.941**	4.057**	0.278**	60.937**
Genotypes (G)	3	0.095*	0.035**	1.301**	0.226**	0.039*	1.907*
G × C	9	0.009	0.001	0.055*	0.012	0.001	0.039
E	30	0.025	0.003	0.019	0.033	0.004	0.627
CV (%)		14.01	5.04	3.67	6.33	3.62	8.64

\* and \*\*= significant at 1% and 5% probability level, respectively.

MS= mean of squares, R= replication, E= error, CV= coefficient of variations

Table 2. Mean comparisons for nitrogen content, nitrate amount and nitrate reductase activity at different conditions of this investigation in primary roots and first young leaves of wheat

Conditions	Primary roots			The first young leaves		
	N (%)	Nitrate ( $\text{mg kg}^{-1}$ )	Nitrate reductase activity ( $\mu\text{mol h}^{-1}\text{g}^{-1}$ )	N (%)	Nitrate ( $\text{mg kg}^{-1}$ )	Nitrate reductase activity ( $\mu\text{mol h}^{-1}\text{g}^{-1}$ )
Normal	0.950 b	1.043 c	1.858 d	2.95 b	1.626 c	6.592 d
Normal + humate	1.375 a	1.301 a	3.000 c	3.56 a	1.852 a	8.092 c
Stress	0.810 b	1.050 c	4.508 b	2.142 c	1.489 d	10.320 b
Stess + humate	1.383 a	1.223 b	5.692 a	2.81 b	1.713 b	11.640 a

Differences between averages of each column which have common letter(s) are not significant at probability level of 5%.

Table 3. Mean comparisons of wheat genotypes for nitrogen content (N), nitrate amount and nitrate reductase activity in primary roots and first young leaves

Wheat genotypes	Primary roots			The first young leaves		
	N (%)	Nitrate (mg kg <sup>-1</sup> )	Nitrate reductase activity (μmol h <sup>-1</sup> g <sup>-1</sup> )	N (%)	Nitrate (mg kg <sup>-1</sup> )	Nitrate reductase activity (μmol h <sup>-1</sup> g <sup>-1</sup> )
Gascogne	1.017 b	1.096 b	3.375 d	2.717 b	1.596 b	8.692 b
Sabalan	1.100 ab	1.122 b	3.667 c	2.800 b	1.658 ab	8.992 ab
4057	1.192 a	1.188 a	3.858 b	2.908 ab	1.699 a	9.367 ab
Gobustan	1.208 a	1.210 a	4.158 a	3.033 a	1.727 a	9.592 a

Differences between averages of each column which have common characters are not significant at probability level of 5%.

## CONCLUSION

In conclusion, our results indicate that application of potassium humate as a natural fertilizer increases nitrate reductase activity in wheat plantlets, especially in drought stressed environment. Genotypes also had different responses, the highest enzyme activity, both in primary roots and in the first young leaves being found in Gobustan, relative to the other genotypes.

## REFERENCES

- Botella, M.A., Cruz, C., Martins-Loucao, M.A., Cerda, A.L., 1993. Nitrate reductase activity in wheat seedlings as affected by NO<sub>3</sub>/NH<sub>4</sub><sup>+</sup> ratio and salinity. *J. Plant Physiol.*, 142: 531-536.
- Bungard, R.A., Wingler, A., Morton, J.D., Anderws, M., Press, M.C., Scholes, J.D., 1999. Ammonium can stimulate nitrate and nitrite reductase in the absence of nitrate in *Clematis vitalba*. *Plant Cell Environ.*, 22: 859-866. DOI: 10.1046/j.1365-3040.1999.00456.x
- Bybordi, A., 2010. Effects of salinity and N on the growth, photosynthesis and N status of canola (*Brassica napus L.*). *Not. Sci. Biol.*, 2 (2): 92-97.
- Carelli, M.L.C., Fahl, J.I., 2005. Partitioning of nitrate reductase activity and its relation to carbon assimilation under different irradiance regimes. *Environ. Exp. Bot.*, 120: 65-72.
- Cataldo, D.A., Haroon, M., Schrader, L.E., Youngs, V.L., 1975. Rapid colorimetric determination of nitrate in plant tissues by nitration of salicylic acid. *Common Soil Sci. Plant Ana.*, 6: 71-80. DOI:10.1080/00103627509366547
- Causin, H.F., Barneix, A.J., 1993. Regulation of NH<sub>4</sub><sup>+</sup> uptake in wheat plants: Effect of root ammonium concentration and amino acids. *Plant and Soil*, 151: 211-218.
- <http://link.springer.com/article/10.1007%2F978-94-007-1628-6?LI=true>
- Cramer, M.D., Lewis, O.A.M., 1993. Influence of nitrate and ammonium nutrition on growth of wheat and maize plant. *Annals of Botany*, 72: 359-365. <http://dx.doi.org/10.1590/S0103-84782008000300008>
- Fedorova, E., Greenwood, J.S., Oaks, A., 1994. In-situ localization of nitrate reductase in maize roots. *Planta*, 194: 279-286. <http://link.springer.com/article/10.1007%2F978-94-007-1618-9?LI=true>
- Gadimov, A.G., Shahryari, R., Garayeva, A.G., 2009. A perspective on humic substances as natural technological products with miraculous biological effect on crops. *Transaction of the Institute of Microbiology of Azerbaijan National Academy of Sciences*, V, 7: 121-128.
- Hopkins, W.G., Norman, P.A., 2004. *Introduction to plant physiology*. Third edition. Chapter 8. The University of Western Ontario, John Wiley. <http://notulaebiologicae.ro/nsb/article/viewFile/3532/4331>
- Kenjebavea, S., Rakova, N., 1995. Multiple forms of nitrate reductase and their role in nitrate assimilation in roots of wheat at low temperature or high salinity. *Physiol. Plant*, 93: 249-252.
- Klepper, L.A., Flesher D.E., Hageman, R.H., 1971. Generation of reduced nicotinamide-adenine dinucleotide for nitrate reduction in green leaves. *Plant Physiol.*, 48(5): 580-590. DOI: <http://dx.doi.org/10.1104/pp.48.5.580>
- Kondrat'ev, M.N., Lebedinskaya, S.O., 1995. Nitrate reductase and protease activities in wheat during reproductive growth. *Russ. J. Plant Physiol.*, 42(3): 399-407.
- Kulikova, N.A., Stepanova, E.V. and Koroleva, O.V., 2005. Mitigating Activity of humic substances: direct influence on biota. In: I.V. Perminova et al. (ed.). Use of humic substances to remediate polluted environments: from theory to practice. Springer Netherlands, 52: 285-309. DOI:10.1007/1-4020-3252-8\_14

- Marschner, H., 1995. *Mineral nutrition of higher plants*. 2<sup>nd</sup> Ed. Academic Press. London.
- Poonacchita, U., Darnell, R., 2004. *Effect of ammonium and nitrate of chelate reductase and nitrate reductase in Vaccinium species*. *Annals of Botany*, 93: 399-405.
- Shahriari, R., Gurbanov, E., Gadimov, A., Hassanpanah, D., 2008. *In vitro effect of potassium humate on terminal drought tolerant bread wheat*. 14<sup>th</sup> Meeting of the International Humic Substances Society From molecular understanding to innovative applications of humic substances. Russia, 706-710. <http://www.humus.ru/ihss-14/eng/index.htm#sec1>
- Shahriari, R., Gadimov, A., Gurbanov, E., Valizade, M., 2009. *Application of potassium humate in wheat for organic agriculture in Iran*. *Asian Journal of food and Agro Industry*, V: 2. <http://www.ajofai.info/index.asp>