

Regulation of the Expression of Nitrate Reductase Genes in Leaves of Medical Plant, *Foeniculum vulgare* by Different Nitrate Sources

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Abstract: The effect of different sources of nitrate salts (ammonium nitrate, sodium nitrate and potassium nitrate) on nitrate reductase (NR) activity and nitrate reductase expression in the leaves of *Foeniculum vulgare* seedlings grown in pots containing perlite. Two week old seedlings were treated with 30, 60 and 120 mM aqueous solution of ammonium nitrate, sodium nitrate or potassium nitrate. After 21 days of nitrate supply, the leaves were harvest and kept in liquid nitrogen for analysis of nitrate/nitrite ratio, nitrate reductase activity and mRNA expression. Result showed that ammonium nitrate, sodium nitrate and potassium nitrate, as inducers, had significant effects on both nitrate reductase activity and nitrate reductase expression in some rates. In high ammonium nitrate, sodium nitrate and potassium nitrate supplies, nitrate reductase activity and nitrate reductase expressions were suppressed apparently due to nitrogen metabolite response inhibition and toxicity.

Key words: Nitrate reductase • Ammonium nitrate • Nitrate reductase gene expression • *Foeniculum vulgare*

INTRODUCTION

Nitrogen is a very important compound in higher plants. It is the most consequential inorganic nutrient for plant growth, such as root branching, leaf growth and flowering time [1]. Range of nitrogen concentration in the soil solution can be extremely different [2]. In many natural and agricultural ecosystems, NH_4^+ is the predominant N source [3] and is usually present to some extent in the majority of ecosystems.

Nitrate is the major source of inorganic nitrogen in most plants [4] and is converted to ammonium [5] via the Glutamate synthesis cycle in two successive steps catalyzed by nitrate reductase (EC 1.6.6.2, Nitrate Reductase-NR) and nitrite reductase (EC 1.7.7.1, Nitrite Reductase-NiR) in the cytosol and chloroplasts of leaves. The acquired ammonia can go into amino acids, proteins,

secondary metabolites and other important nitrogenous compounds. Since the reduction of nitrate to nitrite catalyzed by NR is the major rate-limiting step; it is supposed to be the key enzyme in the process of nitrate assimilation by plants [6]. The Studies in higher plants have shown that nitrate is the primary factor regulating nitrate reductase activity (NRA) [7] and NR expression [8]. NR is an inducible enzyme and there is a close relationship between NRA and NO_3 content in plants [9].

Foeniculum vulgare is an important medicinal plant widely used in pharmaceutical, perfumery, cosmetic, health and food industry. It has properties like anti-cancer, anti-psychotic, anti-oxidant, anti-platelet, anti-clotting treatment for infant colic, liver protection, immune regulators, pesticide and mosquito repellent etc. [10]. In this study, the effect of different sources of nitrogen on the NR gene expression and enzyme activity

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of *F. vulgare* seedlings are studied to explore the possibility of its future manipulation in secondary metabolite production for medicinal and pharmaceutical use.

MATERIALS AND METHODS

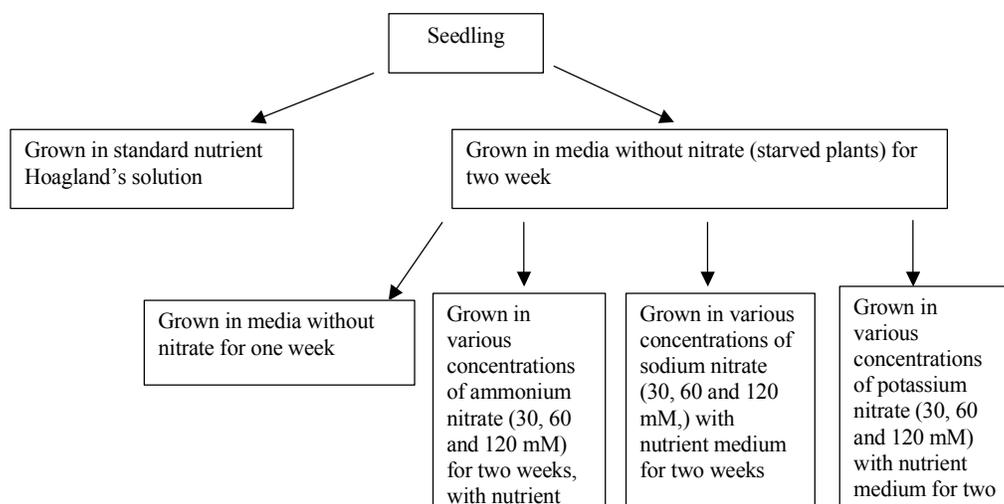
Plant Material and Nitrate Treatments: The seeds of *F. vulgare* were surface sterilized for 20 minutes with 5% (w/v) sodium hypochlorite and germinated on wet filter paper in Petri dishes at 20°C. The 3-day-old seedlings were transplanted in pots (20×15 mm) containing 1 kg/pot perlite (particle size: 3-7 mm) and placed 2-3 cm deep. Each pot contained 20 seedlings. Seedlings were irrigated with 1/4 Hoagland's solution for two weeks (14 days) in a greenhouse at 25/18°C (day/night), 16-h photoperiod (560 μmol/m²/s) and 60% relative humidity.

Nitrate/nitrite Estimation: The leaves frozen in liquid nitrogen were powdered with a mortar and pestle, solubilized in phosphate buffered saline with a pH 7.4 and the extract was centrifuged at 8,000 rpm for 20 min. The supernatant was stored at -80°C. Nitrate was determined by Griess reagent using sodium nitrate as standard [11]. Briefly, 100 μl of vanadium chloride (III) (8 mg/mL) was added to 100 μl of the extract, After 50 min, 60 μl of Griess reagents [1:1 (v/v) of 0.1% naphthylethylenediaminedihydrochloride (NED) in H₂O⁺ 2% sulphanilamide in 5% H₃PO₄] was added and incubated at 37°C for 15 min and the absorbance was recorded at 540 nm by spectrophotometer. For nitrite assay, vanadium chloride (III) was eliminated from the reaction mixture.

Nitrate Reductase Activity: The nitrate reductase activity assays were done as described by the reduction of nitrate to nitrite [12] explain briefly. One unit of enzyme activity is defined as the production of 1 μM nitrite per min.

RNA Extraction and Reverse Transcription: Total RNA was extracted from plant leaves using RNX-Plus (CinaGen, Iran) as per the manufacturer's protocol. DNase treatment was done using Fermentas DNase Kit according to the manufacturer's directions and the purity of RNA was examined after extraction of RNA on the agarose gel and visualize by ethidium bromide staining and estimating the purity of the extract by OD260/OD280 absorption ratio (>1.8). 2 μg of DNase-treated RNA was used for the first strand. cDNA synthesis using 100 pmol oligo-dT (18 mer), 15 pmol dNTPs, 25 U RNase Inhibitor and 200 U M-Mulv reverse transcriptase in a 25 μl final volume.

Primers, Probes and Real-Time PCR Conditions: Real-time PCR for the quantification of nitrate reductase mRNA was directed using SYBR Premix Ex Taq II reagent (Takara, Japan). cDNAs were amplified by SYBR Premix Ex Taq II in a 20 μl reaction mixture containing 0.4 μl of each gene-specific primer (10 μM), 2 μl cDNA, 10 μl 2x buffer and 7.2 μl ddH₂O. The specific primer pairs for nitrate reductase were designed as follows: 5'-G A C A T C A T T C T C G C C T A T -3' (forward) and 5'-C A C T T C A C C A T T C T A C C A -3' (reverse). The glyceraldehyde-3- phosphate dehydrogenase (GADPH) gene was used as the reference gene with the primers 5'-G A G G A G T T C G G C A T C G T G A A G G G A -3' (forward) and 5'-T G G G G C A A C A C A A G G G A C A C A G -3' (reverse). The amplification reactions were



carried out in a line GeneK thermal cycler (Bioer, China) under the following conditions: 2 min at 94°C, 40 cycles of 94°C for 10 sec, 57°C for 15 sec and 72°C for 30 sec. After 40 cycles, the specificity of the amplifications was tested by heating from 50°C to 95°C, resulting in melting curves. Data were analyzed as delineated before [13].

Statistical Analyses: The experimental designs were randomized complete block with three replicates. Analysis of variance (ANOVA) test was done using the statistical software SPSS 11.5. P rates less than 0.05 were considered statistically significant, Duncan's multiple range test at $P < 0.05$ was used to determine significant differences among treatments.

RESULTS

In this study, results showed that ammonium nitrate in 30m M more increased the accumulation of nitrate

compared with sodium nitrate and potassium nitrate (Fig 1). Ammonium nitrate with increasing the concentration 60 and 120 mM led to decrease in accumulation of nitrate. Sodium nitrate in 30 and 120 mM, caused decrease in nitrate accumulation. Potassium nitrate had the similar process to sodium nitrate. Also in 60 mM Sodium nitrate caused more accumulation of nitrate in plants.

Ammonium nitrate in 30 mM showed the maximum nitrite accumulation in plant compared to other concentrations. Sodium and potassium nitrate in 60 mM had both maximum nitrite accumulations in leaves (Fig 2). In plant under treatment with ammonium nitrate, with increase in concentration, reduce in nitrite accumulation in the leaves was observed. In all treated concentrations 120mM had minimum nitrite production in the leaves. Thus, ammonium nitrate, Sodium and potassium nitrate supplying increased nitrite accumulation that it is dependent manner in a dose.

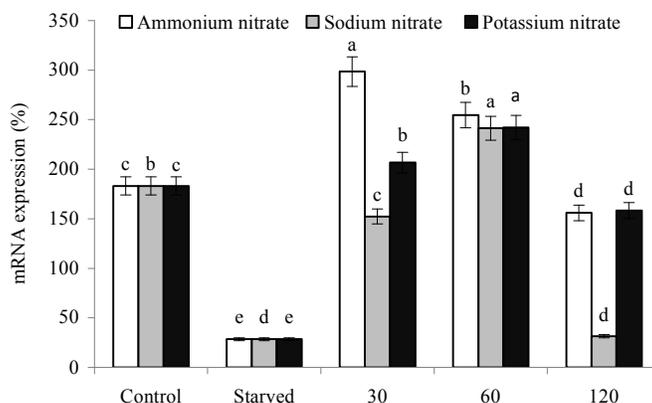


Fig. 1: Effect of various concentrations of ammonium nitrate, sodium nitrate and potassium nitrate on the nitrate accumulation in the leaves. Means with the same letter are not significantly different as determined by SPSS software version 11.5 at $p < 0.05$.

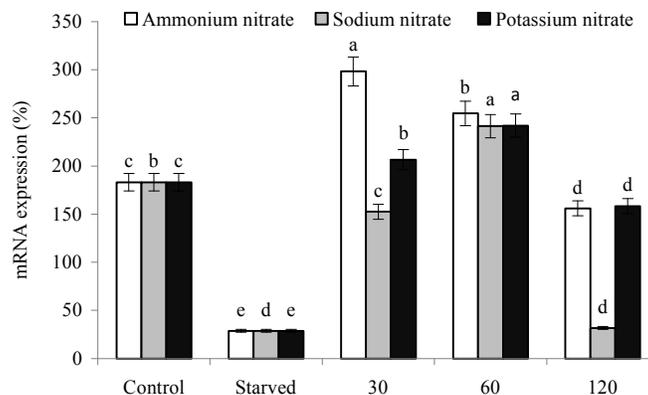


Fig. 2: Effect of various concentrations of ammonium nitrate, sodium nitrate and potassium nitrate on the nitrite accumulation in the leaves. Means with the same letter are not significantly different as determined by SPSS software version 11.5 at $p < 0.05$.

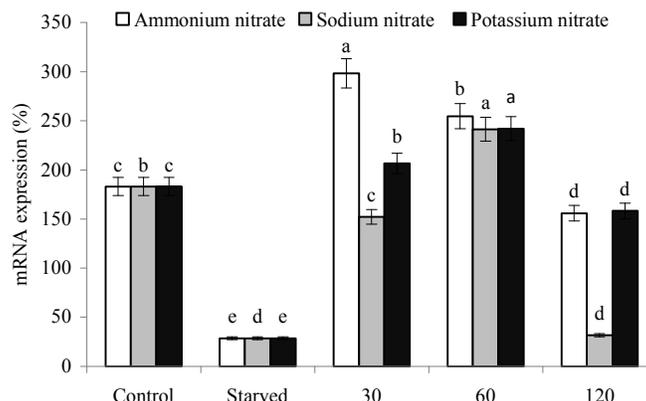


Fig. 3: Effect of various concentrations of ammonium nitrate, sodium nitrate and potassium nitrate on the nitrate reductase activity in the leaves. Means with the same letter are not significantly different as determined by SPSS software version 11.5 at $p < 0.05$.

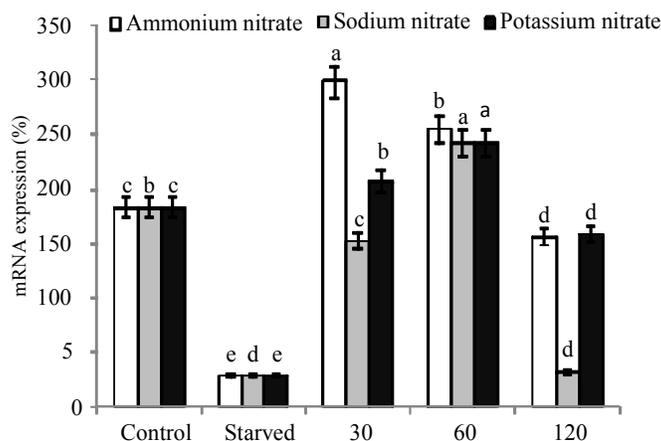


Fig. 4: Effect of various concentrations of ammonium nitrate, sodium nitrate and potassium nitrate on the mRNA expression in the plants. Means with the same letter within an assay are not significantly different as determined by SPSS software version 11.5 at $p < 0.05$.

The result showed that ammonium nitrate in high concentration (60 and 120 mM) lead to decrease in nitrite reductase activity (Fig 3). Sodium and potassium nitrate in concentration of 60 mM caused increase in nitrite reductase activity (Fig 3). Ammonium nitrate in concentration of 30 mM showed more mRNA expression in the leaves than other treatment (Fig 4). mRNA expression in the plants leaves under treated ammonium nitrate decreased with increase in 120mM concentrations.

DISCUSSION

The purpose of the study was to explain the changes in activities of the key enzymes responsible for nitrate reduction in *Foeniculum vulgare* leaves. Nitrate available in soil is absorbed by root cells mediated by nitrate transporter. Nitrate is reduced in the roots or stored in

root leaf, cell cytoplasm and the excess of which is stored in leaves cells vacuoles. However, the excess nitrate is transported to the leaves where it is reduced in the formulation in the leaves increased with nitrogen supply. The efficiency of net nitrate uptake is under negative response control by nitrate accumulation [14]. Therefore, when nitrogen supply is higher than the plant demand, the decrease in nitrate accumulation might be due to the decrease of nitrate uptake as a result of the negative feedback regulation by accumulated nitrate [15]. The rate of nitrate uptake relies on the activity of nitrate transport systems in the plasma membrane of root cells. External factors, such as nitrate concentration as well as internal factors such as nitrogen metabolites (ammonium and glutamine) all regulate the rate of nitrate uptake [16]. Exposure of roots to nitrate causes the induction of nitrate transport 2 transcripts, which leads to nitrate

uptake by positive feed forward, whereas metabolites resulting from nitrate reduction, most likely ammonia and glutamine, down regulate NRT2 [17, 18].

CONCLUSION

In conclusion, our results indicate that nitrogen supply stimulates nitrate uptake, nitrate reduction, nitrite reduction and nitrate reductase activity at low concentrations of ammonium nitrate, sodium nitrate and potassium nitrate by positive effects of nitrate on the nitrate assimilation pathway. But at high nitrogen supplies, nitrate metabolites including ammonium and glutamine suppress nitrate assimilation pathway by inhibiting the related rate-limiting enzymes in the biosynthetic pathway.

REFERENCES

1. Zhang, H. and B.G. Forde, 1998. An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. *Science*, 279(5349): 407-409.
2. Jackson, R.B. and M.M. Caldwell, 1993. The scale of nutrient heterogeneity around individual plants and its quantification with geostatistics. *Ecology*, 74: 612-614.
3. Bijlsma, R.J., H. Lambers and S.A.L.M. Kooijman, 2000. A dynamic whole plant model of integrated metabolism of nitrogen and carbon. 1. Comparative ecological implications of ammonium-nitrate interactions. *Plant Soil*, 220: 49-69.
4. Li, G.J., F.T. Peng, L. Zhang, X.Z. Shi and Z.Y. Wang, 2010. Cloning and characterization of a SnRK1-encoding gene from *Malus hupehensis* Rehd. and heterologous expression in tomato. *Molecular Biology Reports*, 37: 947-954.
5. Stitt, M., C. Muller, P. Matt, Y. Gibon, P. Carillo, R. Morcuende, W.R. Scheible and A. Krapp, 2002. Steps towards an integrated view of nitrogen metabolism. *Journal of Experimental Botany*, 53: 959-970.
6. Campbell, W.H., 2001. Structure and function of eukaryotic NAD (P) H: nitrate reductase. *Cellular and Molecular Life Sciences*, 58: 194-204.
7. Kaiser, W.M., H. Weiner and S.C. Huber, 1999. Nitrate reductase in higher plants: a case study for transduction of environmental stimuli into control of catalytic activity. *Physiologia Plantarum*, 105: 385-390.
8. Daniel-Vedele, F. and M. Caboche, 1996. Molecular analysis of nitrate assimilation in higher plants. *C R Acad Sci Paris, Life Sci.*, 319: 961-968.
9. Skrdleta, V., A. Gaudinova and M. Nemcova, 1979. Relationships between nitrate level, nitrate reductase activity and anaerobic nitrite production in *Pisum sativum* leaf tissue. *Plant Biology*, 21: 307-310.
10. Jeet Kour, G. and D. Singh Arora, 2010. "Bioactive potential of *Anethum graveolens*, *Foeniculum vulgare* and *Trachyspermum ammi* belonging to the family Umbelliferae - current status." *Journal of Medicinal Plants Research*, 4(2): 87-94.
11. Miranda, M.K., M.G. Espey and D.A. Wink, 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*, 5: 62-71.
12. Brunetti, N. and R.H. Hageman, 1979. Comparison of in vivo and in vitro assays of nitrate reductase in wheat (*Triticum aestivum* L.) seedlings. *Plant Physiology*, 58: 583-587.
13. Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *NAR*, 29: 2002-2007.
14. Chen, B.M., Z.H. Wang, S.X. Li, G.X. Wang, H.X. Song and X.N. Wang, 2004. Effects of nitrate supply on plant growth, nitrate accumulation, metabolic nitrate concentration and nitrate reductase activity in three leafy vegetables. *Plant Science*, 167: 635-643.
15. Sivasankar, S., S. Rothstein and A. Oaks, 1997. Regulation of the accumulation and reduction of nitrate by nitrogen and carbon metabolites in maize seedlings. *Plant Physiology*, 114: 583-589.
16. Orsel, M., S. Filleur, V. Fraiser and F. Daniel-Vedele, 2002. Nitrate transport in plants: which gene and which control? *Journal of Experimental Botany*, 53: 825-33.
17. Walch-Liu, P. and B.G. Forde, 2008. Nitrate signaling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate- induced changes in root architecture. *Plant Journal*, 54: 820-828.
18. Remans T., P. Nacry, M. Pervent, T. Girin and P. Tillard, 2006. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in Arabidopsis. *Plant Physiol*, 140: 909-921.