1. Background

Polyamines (PAs), such as putrescine (Put), spermidine (Spd), and spermine (Spm), are generally present in all organisms and have essential roles in certain physiological processes (1, 2). PAs are present in all parts of the plant cells and are mostly known for involvement in the regulation of genome activity, cell division and expansion, and plant growth. PAs have important antioxidant functions, growth regulatory and essential interactions with components of the cell wall (3-5). Because of PAs participation in cell division processes and regulation of genome transcription, they are widely studied in cancer of animal cells (6).

2. Objectives

This study aimed to evaluate effect of ornithine enantiomers on expression of certain polyamine (PAs) biosynthetic genes in tobacco cells.

3. Materials and Methods

Suspension-cultured tobacco cells were treated with different concentrations of L- and D- Orn for 24 h. Cell viability was assayed by Evans Blue and hydrogen peroxide content. The changes of gene expression were analyzed by semi-quantitative RT-PCR.

4. Results

Exogenous D-Orn resulted in enhancement of expression of genes involved in Orn, arginine and S-adenosyl methionine metabolism. Additionally, exogenous D-Orn treatment resulted in sustained viability of cultured tobacco cells and normal levels of hydrogen peroxide were maintained. Supplied L-Orn increased the hydrogen peroxide level and lowered viability of cells. Treatment with L-Orn had a negative effect on the transcript levels for most analyzed PA-related genes. It was also illustrated that transcription of putrescine methyl transferase, key enzyme for nicotine production, was highly upregulated by L-Orn.

5. Conclusions

Based on the results, D-Orn was shown to have a stereo-selective function in regulation of the PAs-related genes.

Keywords: Arginine decarboxylase, D-amino acids, Nicotiana tabacum, Ornithine decarboxylase.
regulated in many processes of living cells (8-10). ODC is shown to be present in all living organisms and necessary for cell division, transcription process and growth under normal condition. ADC is considered for taking role under stress conditions for the biosynthesis of PAs (1).

L-Orn is a non-protein amino acid and has an essential role in PAs biosynthesis (11). It also has main roles in arginine (Arg) and urea production (12). In addition, Orn has important function in the production of proline (Pro), and nicotinic alkaloids (7).

The content of PAs in plants are associated with several physiological processes, such as N:C ratio and stress responses. PAs catabolism/anabolism, transport and conjugation define the PAs hemostasis (13).

It was previously shown that the “uncommon enantiomer”, D-Orn, can cause upregulation of free and conjugated PAs, as opposed to its enantiomer (14). In addition, D-Orn potently induces production of Spd and Spm. While D-Orn has no effect on nicotine levels, L-Orn enhances nicotine production in vivo (14). Results of that study were the first observations suggesting a Amino Acid (D-AA) may play a critical role in plant metabolism and development. Generally, plants produce D-AAs due to microbial infection and racemization of L-AAs (15, 16). Mostly, D-AAs are thought to be toxic or futile compounds for plant metabolism, growth and development (17). D-Serine and D-Alanine are of those D-AAs, which strongly inhibit plants growth at 3 mM concentration (15).

It has long been accepted that only L-AAs can effectively enter biosynthetic pathways and be incorporated with secondary metabolites and proteins structures of cells. Although the functions of L-AAs on plant metabolism are well known, there are few reports on effects of D-AAs.

2. Objectives
The effects of L- and D-Orn on PAs biosynthetic related genes were evaluated. Following our previous report, here we planned to determine the role(s) of Orn enantiomers in suspension-culture of Nicotiana tabacum.

3. Materials and Methods

3.1. Suspension Culture and Treatments
Tobacco (Nicotiana tabacum) cells were cultivated in a modified MS medium (18). The 5-day-old cells were treated with 0, 1, 5, 10 mM of L- and D-Orn (Sigma-Aldrich) for 24 h according to (19, 20). Cells were harvested and frozen for further analysis. Evans Blue was used to assay the cells viability (18).

3.2. Hydrogen Peroxide (H₂O₂) Content Determination
H₂O₂ concentrations were assayed according to Velikova et al. (21). Briefly, 0.2 g fresh cells were crushed with trichloroacetic acid (0.1% w/v), followed by centrifugation at 12,000 ×g for 10 min. The supernatant (0.5 mL) was mixed with 1 mL of 1 M potassium iodide and 0.5 mL of 10 mM potassium phosphate buffer. Absorbance measurement was carried out at 390 nm.

3.3. RNA Extraction and Semi Quantitative Reverse Transcriptase-PCR Analysis
Gene expression changes were measured by semi-quantitative RT-PCR. Total RNA was isolated from cells through RNX™ Plus kit (Cinna Gen Inc). The integrity and quantity of RNA was estimated using gel electrophoresis and spectrophotometer. Total RNA (3 µg) was reverse-transcribed by First-Strand cDNA synthesis Kit (Fermentas, Canada). The primer sequences corresponding to the genes under study were shown in Table 1. Actin expression was used as an internal control. PCR reaction contained 0.5 U Taq DNA polymerase, 0.3 µL of cDNA, 200 µM of deoxyribonucleoside triphosphates (dNTPs), 50 mM KCl, 1.5 mM MgCl₂ and 4 µM of forward and reverse primers in a total volume of 20 µL. The PCR conditions were as follows: initial denaturation at 94 °C for 1 min and 30 cycles of 94 °C: 30 sec; 52 to 58 °C (depending on gene type): 25 sec; and extension for 10 min at 72 °C. The products were electrophoresed in 1.2% (w/v) agarose gels, stained with ethidium bromide under ultraviolet light. The band intensity was measured

| Genes | Sequences |
|-------|----------|
| ACT   | F- primer 5'-GCAGGGATCCACGAGACACCC-3'  |
|       | R- primer 5'-CCCCACCGTGAAGCAGTCATCC-3'  |
| ODC   | F- primer 5'-TTCGCAGAAAGTCGACTGCGG-3'  |
|       | R- primer 5'-CACTGTCGTGATACGGGTCG-3'  |
| ADC   | F- primer 5'-ATGCGTGACCGCCAGGCTC-3'  |
|       | R- primer 5'-CACTGGGAATGACCCATCC-3'  |
| PAO   | F- primer 5'-GACTCGGCAATTCTAGAGCTG-3'  |
|       | R- primer 5'-ACTGCTGTGAATGACCCATCA-3'  |
| CAT   | F- primer 5'-GGATGTCGACCGCCAGGCTC-3'  |
|       | R- primer 5'-CGAGGGAATGACCCATCC-3'  |
| SAMDC | F- primer 5'-TGACCCCTGTTACTCTTCTT-3'  |

Table 1. Sequences of primers used in this study.

Iran J Biotech. 2018;16(1):e1835
by UV Documentation Luminescent Image Analysis software (England). The band quantity was determined with Image Guage software.

4. Results

4.1. Effect of Orn on Cell Viability, Growth and \( \text{H}_2\text{O}_2 \) Content of Tobacco Cells

No changes in cell viability were observed in D-Orn treated cells compared to the control (Figs. 1a and c). Conversely, treatments of cells with different concentrations of L-Orn inhibited cell viability (Fig. 1b). Effect of 1 mM concentration of L- and D-Orn on cell growth was evaluated. The results showed that cell growth was increased by L- and D-Orn (Table 2). Concurrent measurement of \( \text{H}_2\text{O}_2 \) levels showed that \( \text{H}_2\text{O}_2 \) concentration in the D-Orn treated cells is similar to the control condition, while L-Orn led to increasing \( \text{H}_2\text{O}_2 \) accumulation. \( \text{H}_2\text{O}_2 \) increment led to cell viability reduction (Fig. 1d). The key observation from D-Orn treated cells (up to 10 mM) was that these cells did not suffer from any damage. Moreover, they did not accumulate \( \text{H}_2\text{O}_2 \) or showed any lowered viability. Whereas 5 mM L-Orn resulted in elevated \( \text{H}_2\text{O}_2 \) that consequently lowered cell viability.

### Table 2. Effect of D-Orn and L-Orn on growth of tobacco cells.

|          | Growth (g) |
|----------|------------|
| Control  | 11 ± 0.5   |
| D-Orn    | 18 ± 0.4   |
| L-Orn    | 16 ± 0.3   |

4.2. Effect of Orn on PA Biosynthetic Gene Expression

D-Orn showed a promotional effect on the expression of \( ODC \) and \( ADC \) genes, in a concentration dependent manner (Figs. 2 and 3). Conversely, 5 and 10 mM L-Orn treatment did not result in considerable changes of \( ODC \) expression (Fig. 3). In comparison with L-Orn, which highly decreased \( ADC \) and \( SAMDC \) transcript expressions, D-Orn enhanced \( ADC \) and \( SAMDC \) transcript levels. Biosynthesis of nicotine from Put competes with the production of Spd in tobacco. Results showed that the \( PMT \) transcript, representing the key enzyme involved in mPut and nicotine biosynthesis, is highly expressed in L-Orn treated cells (Fig. 3).
5. Discussion

Our results showed that the application of D-Orn caused a sustained viability. Moreover, H$_2$O$_2$ content of cells were quite similar to the cells under normal condition. Therefore, it can be speculated that the observed upregulated levels of genes are not a sign of any stress induced by the D-Orn treatments. While certain D-AAs, e.g. D-Ala and D-Ser (at 3 mM) were shown to be very toxic and limit plant growth (15, 16), D-Orn showed no negative effect on cell suspension culture of tobacco, even at 10 mM. Based on our results, both L-Orn and D-Orn might be involved in the regulation of respective pathways. Upregulation of all the necessary genes for Orn metabolism and PAs biosynthesis by D-Orn revealed its unique effects; the same effects have not been reported for L-Orn or any other D-AAs. While D-Orn emerged as a compound with stimulatory effects on transcript expressions of most associated genes, results for L-Orn implied a suppressive effect on their expression. In our previous paper, it was shown that L-Orn decreased Spd and Spn while increased nicotine. (14). This is in agreement with our result that

Figure 3. Expression level of the gene encoding for a) ODC, b) ADC, c) CAT, d) PMT, e) SAMDC, f) PAO in tobacco cell cultures after L-Orn and D-Orn treatments with 0, 1, 5 and 10 mM concentrations. Data represent average values from 3 separate experiments ± SD.
L-Orn enhanced transcript of PMT as the key enzyme of nicotine biosynthesis. This observation shows that each enantiomer of Orn is different, if not opposing effects on the same pathways actually. Oxidation of PAs by diamine oxidase (DAO) and polyamine oxidases (PAO) produces H$_2$O$_2$. Apparently, these oxidizing enzymes act in opposite direction to catalase (CAT), as the main H$_2$O$_2$ scavenger. $P$AO expression depicts a higher activity in the presence of D-Orn, which might be a reason to enhance the expression of $C$AT. The transcript level of $C$AT was highly enhanced in the presence of D-Orn while the L-enantiomer showed no positive effect on $C$AT transcription level.

D-Ornithine inhibits ODC in yeast (22). In addition, ODC activity in tobacco cells was shown to be inhibited by putrescine and other PAs (23). We have previously reported that the application of D-orn enhanced the levels of spermine and spermidine, but not putrescine (14). It seems that D-ornithine may increase the levels of spermidine and spermine through the activation of ADC and probably other genes including $S$AMDC, $S$PDS and $S$PMS. The upregulated levels of ODC and ADC suggests that D-Orn has a potential effect on the transcript of all the related genes to PAs metabolism. Higher enhancement of $P$AO transcript, implies that PAs catabolism should be considered in addition to their production via ODC/ADC action. When examining different AAs for cell growth in suspension cultures, Behrend and Mateles (20) used a racemic mixture of Orn (at similar concentrations as in this study) and found that it acts as nitrogen source. An investigation into the effects of L-Orn on plant cell viability was reported in Aechmea fasciata. It was shown that L-Orn has inhibitory effects on growth of cultured pollen tubes (24). This data was in parallel to ours. Another study showed that L-Orn feeding to transgenic tobacco cell cultures (overexpressing an ODC) did not result in the expected accumulation of Put in cells, suggesting that Put production was limited partly by L-Orn availability (25). The results demonstrated that increasing capacity of cells to decarboxylate L-Orn increased the content of free or conjugated PAs.

Qu et al. showed that in comparison with the D-enantiomer, the L-enantiomer, is a more effective inhibitor of ODC (26). It has also been shown that ODCs extracted from different animal cells can be inhibited by both enantiomers. However, D-Orn is a very weak inhibitor of ODC (27), while L-Orn is a strong type. Erçal et al. showed that, treatments with most L-AAs are associated with a production of bulk of H$_2$O$_2$, which was not observed upon treatment with D-AAs. This is due to interconversion of AAs to each other through various cycles and catabolic reactions (28).

As expected, L-Orn can enter the amino acid cycles and pathways: resulting in increased H$_2$O$_2$ and other AAs while the D-enantiomer cannot. It can be safely argued that the constant level of H$_2$O$_2$ in D-Orn treated cells is a result of upregulation of CAT, which is the main scavenger of H$_2$O$_2$.

CAT and PAO, H$_2$O$_2$ producers via catabolism of PAs, are acting on opposite directions. Moreover, D-Orn is shown to have a stimulatory effect on the transcripts of both producing and recycling of PA enzymes. Results of our study suggest that a combination of accumulation of H$_2$O$_2$, driven from L-AAs conversions, and inhibition of gene transcripts determine the observed effects of L-Orn on tobacco cells. Current study indicates that stereospecific regulation of PAs biosynthesis might be a novel approach to enhance plant cells’ metabolism, growth and development. To fully determine the role of D-Orn will demand further metabolic examination such as stable isotope labeling.

Acknowledgements

We are thankful to Department of Plant sciences, Faculty of Biological Sciences, Tarbiat Modares University, Tehran- Iran for PCR analyses.

References

1. Alcázar R, Altabella T, Marco F, et al. Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta*. 2010;231:1237-1249. doi: 10.1007/s00425-010-1130-0
2. Roussos PA, Pontikis CA. Changes of free, soluble conjugated and bound polyamine titer of jojoba explants under sodium chloride salinity in vitro. *J Plant Physiol*. 2007;164:895-903. doi:10.1016/j.jplph.2006.05.003
3. Yang H, Shi G, Wang H, Xu Q. Implication of polyamines in adaptation of *Potamogeton crispus* L. to cadmium stress. *Aquat Toxicol*. 2010;100:282-288. doi:10.1016/j.aquatox.2010.07.026
4. Mapelli S, Brambilla IM, Radyukina NL, Ivanov YV, Kartashov AV. Free and bound polyamine changes in different plants as a consequence of UV-B light irradiation. *Gen Appl Plant Physiol*. 2008;34:55-66.
5. Shevyakova NI, Rakitin VY, Stetsenko LA, Aronova EE, Kuznetsov VV. Oxidative stress and flV. Oxidat of free and conjugated polyamines in the halophyte Mesembryanthemum crystallinum L. under NaCl salinity. *Plant Growth Regul*. 2006;50:69-78. doi: 10.1007/s10725-006-9127-1
6. Wallace HM, Fraser AV, Hughes A. A perspective of polyamine metabolism. *Biochem J*. 2003;376:1-14. doi: 10.1042/bj20031327
7. Katoh A, Ohki H, Inai K, Hashimoto T. Molecular regulation of nicotine biosynthesis. *Plant Biotech*. 2005;22:389-392. doi: 10.5511/plantbiotechnology.22.389
8. Kwak SH, Lee SH. The transcript level independent activation of ornithine decarboxylase in suspension-cultured BY2 cells entering the cell cycle. *Plant Cell Physiol*. 2002;43:1165-1170. doi: 10.1093/pcp/pcf132
9. Yoo TH, Park CJ, Ham BK, Kim KJ, Paek KH. Ornithine
decarboxylase gene (CaODC1) is specifically induced during TMV-mediated but salicylate-independent resistant response in hot Pepper. *Plant Cell Physiol.* 2004;45:1537–1542. doi: 10.1093/pcp/pch176

10. Jang SJ, Cho HW, Park KY, Kim YB. Changes in cellular polyamine contents and activities of their biosynthetic enzymes at each phase of the cell cycle in BY-2 cells. *J Plant Biol.* 2006;49:153-159. doi: 10.1007/s10037-006-0322-z

11. Gholami M, Boughton BA, Fakhari AR, Ghanati F, Mirzaei HH, Yousefzaei L, et al. Metabolomic study reveals a selective accumulation of L-arginine in the D-ornithine treated tobacco cell suspension culture. *Process Biochem.* 2014;49(1):140-147. doi:10.1016/j.procbio.2013.09.010

12. Mohapatra S, Minocha R, Long S, Minocha SC. Transgenic manipulation of a single polyamine in poplar cells affects the accumulation of all amino acids. *Amino Acids.* 2010;38:1117-1129. doi: 10.1007/s00726-009-0322-z

13. Moschou PN, Wu J, Cona A, Tavladoraki P, Angelini R, Roubelakis-Angelakis KA. The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. *J Exp Bot.* 2012;63(14):5003-5015. doi:10.1093/jxb/ers202

14. Gholami M, Fakhari AR, Ghanati F. Selective regulation of nicotine and polyamines biosynthesis in tobacco cells by ornithine enantiomers. *Chirality.* 2013;25:22-27. doi: 10.1002/chr.22107

15. Erikson O, Hertzberg M, Näsholm T. A conditional marker gene allowing both negative and positive selection in plant. *Nat Biotechnol.* 2004;22:455-458. doi:10.1038/nbt946

16. Gordes D, Kolukisaoglu U, Thurow K. Uptake and conversion of D-amino acids in *Arabidopsis thaliana*. *Amino Acids.* 2011;40:553-563. doi:10.1007/s00726-010-0674-4

17. Näsholm T, Kielland K, Ganeteg U. Uptake of organic nitrogen by plants. *New Phytol.* 2009;182:31-48. doi: 10.1111/j.1469-8137.2008.02751.x

18. Ghanati F, Morita A, Yokota H. Effects of aluminum on the growth of tea plant and activation of antioxidiant system. *Plant Soil.* 2005;276:133-141. doi: 10.1007/s11104-005-3697-y

19. Hao YJ, Kitashiba H, Honda C, Nada K, Moriguchi T. Expression of arginine decarboxylase and ornithine decarboxylase genes in apple cells and stressed shoots. *J Ex Bot.* 2005;56:1105-1115. doi: 10.1093/jxb/erl102

20. Behrend J, Mateles RI. Nitrogen metabolism in plant cell suspension cultures. *Plant Physiol.* 1975;56:584-589. doi: 10.1104/pp.56.5.584