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Effects of silver nitrate ($AgNO_3$) on growth and anatomical structure of vegetative organs of liquorice (*Glycyrrhiza glabra* L.) under *in vitro* condition

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Abstract

Liquorice (*Glycyrrhiza glabra* L.) has been used worldwide as a medicine for a long time. In this research, the effect of silver nitrate ($AgNO_3$) as a growth regulator and anti-ethylene in *in vitro* culture was investigated on growth and anatomical structure of vegetative organs (root, hypocotyl, shoot, leaf) as well as the number of stomata and trichomes in the leaves of liquorice under vitro culture condition. The seeds were cultured in MS culture media containing different concentrations of $AgNO_3$ (0, 2, 4, 8, and 10 mg L^{-1}). Investigations on 20-day seedlings after three replications showed a significant increase in length and growth of roots, hypocotyls and shoots, and decreased number of stomata and trichomes in the samples treated with $AgNO_3$ ($P \leq 0.05$). The effects of $AgNO_3$ on anatomical structures of the organs included the increased cell division in root and shoot tips, reduced vascular tissues and sclerenchyma-fiber (with lignified cell walls), increased thickness of Casparian strip and cell walls of endodermis, reduced thickness of epidermis and increased intercellular spaces in mesophyll. The leaf area was measured in the 4-month plantlets, showing a significant increase in the samples treated with $AgNO_3$. Furthermore, there was significant difference in increased leaf area applying 10 mg L^{-1} treatment and other concentrations as well as between the concentrations of 2 and 8 mg L^{-1}. It seems that these results are due to the inhibitory effects of $AgNO_3$ on the production and function of ethylene and the plant strategy to increase the tolerance against silver metal.

Keywords: $AgNO_3$, anatomical structure, *Glycyrrhiza glabra* L., growth, *in vitro* culture, vegetative organs.
Abbreviations: AgNO3_silver nitrate; ANOVA_analysis of variance; F.A.A_formaldehyde-acetic acid-ethanol; HIV_human immunodeficiency virus; MS_murashige and skoog; SAM_S-adenosylmethionine.

Introduction

Liquorice (*Glycyrrhiza glabra* L.), belonging to Fabaceae, is a perennial herb. Its height reaches up to 1 to 2 meters. The leaves are pinnately compound and the flowers are purple blue in a panicle inflorescence. The root and rhizome have a brownish skin, below which is yellowish with sweet taste (Chaudhary et al., 2010; Bode and Dong, 2015). This plant grows in tropical areas of the world (Badkhane et al., 2014) and nowadays is cultivated throughout Europe, the Middle East and Asia (Nitalikar et al., 2010; Thakur and Raj, 2017; Asnaashari et al., 2018). Commercially, the roots and rhizomes of this plant are desirable parts, containing important biochemical or secondary metabolites, which have been used as medicine and natural sweetener for a long time throughout the world (Nomura et al., 2002; Makky et al., 2012; Patel and Krishnamurthy, 2013; Li et al., 2014; Kozhuharova and Stanilova, 2017). Glycyrrhizin is one of the secondary metabolites in this plant with anticarcinogenic and antihistamine properties. It is also used to treat liver disease, gastric ulcer, HIV infection, throat infection, eye diseases, tuberculosis, diabetes, herpes and immune system regulation (Rao, 1993; Harwansh et al., 2011; Karahan et al., 2016). Besides the usage of secondary metabolites of Liquorice in pharmaceutical industry, these compounds are used in cosmetic products and food industries (Nassiri and Hosseinzadeh, 2008; Karaoğul et al., 2016). Consequently, due to the importance of liquorice, the use of methods and compounds that can improve plant growth and reproduction over a short period are beneficial. Due to plant conservation against environmental factors such as pests, microbial diseases and seasonal and geographical stresses, the *in vitro* culture could be an appropriate method for rapid propagation of plants. $AgNO_3$ is a compound that has been widely used in *in vitro* culture of plants due to its inhibitory effect on the function of the ethylene hormone and as a result interference in organogenesis, morphogenesis, somatic embryogenesis, growth and development, aging delay, plant adaptation to environmental conditions, as well as ease of access and dissolution in water (Turhan, 2004; Mohiuddin et al., 2005; Kumar et al., 2009; Chae and Park, 2012; Alva Ticona and Oropeza, 2013; Mookkan and Andy, 2014; Geetha et al., 2016). Therefore, this research was aimed to investigate the effects of $AgNO_3$ (as an anti-

ethylene and growth regulator) at concentrations of 0, 2, 4, 8, 10 mg L^{-1} in *in vitro* culture condition on the growth and anatomical structure of vegetative organs such as roots, hypocotyls, shoots and leaves.

Results

Growth and development parameters

Investigations on 20-day seedlings showed that longitudinal growth of roots (Fig 1A), hypocotyls (Fig 1B) and shoots (Fig 1C) increased significantly ($P \leq 0.05$) at different concentrations of $AgNO_3$ (2, 4, 8, 10 mg L^{-1}) as compared with control samples. In different concentrations of $AgNO_3$, no significant difference was found among the average longitudinal growth of roots, hypocotyls and shoots. The average number of stomata (Fig 1D) and trichomes (Fig 1E) in lower epidermis of leaves in the seedlings treated with $AgNO_3$ showed significant decrease in comparison with control samples. The difference between the average number of stomata and trichomes in the leaves treated with different concentrations of $AgNO_3$ was not significant. The mean comparison of leaf area in the 4-month plantlets treated with $AgNO_3$ showed a significant increase in comparison with control samples. Moreover, significant differences were found between the treatment of 10 mg L^{-1} and other treatments as well as between the treatment of 2 mg L^{-1} and 8 mg L^{-1} (Fig 1F).

Anatomical structures

Longitudinal and microtome sections of apical meristems of shoot (Fig 2) and root (Fig 3) in 20-day seedlings showed that different concentrations of $AgNO_3$ can increase the cell color intensity and homogenization of regions, compared to the control.

The microscopic observation of transverse section of vegetative organs such as root (Fig 4), hypocotyl (Fig 5) and shoot (Fig 6) of 20-day seedlings after treatment showed the decrease of vascular, sclerenchyma-fiber (with lignified cell walls) tissues in all concentrations of $AgNO_3$. In addition, different concentrations of $AgNO_3$ in comparison with the control group led to thickening of Casparian strip and cell walls of endodermis in the roots (Fig 4) and decrease of epidermis thickness and increased intercellular spaces of mesophilic tissues in leaves (Fig 7).

Discussion

Effect of AgNO3 on growth parameters

There are several reports indicating that *in in vitro* culture of plants, the production of ethylene and its accumulation in culture media is associated with reduced growth and morphological changes (Apelbaum and Burg, 1972; Burg, 1973; Schwarzbach et al., 1992; Tamimi, 2015; Harathi and Naidu, 2016; Roshanfekrrad et al., 2017). In such conditions, the use of compounds that can control the growth of plant by regulating the production and function of ethylene would be useful. $AgNO_3$ is a salt of silver and nowadays is used widely as an anti-ethylene compound in *in vitro* cultures (Nomura et al., 2002; Shah et al., 2014; Mookkan and Andy, 2014; Ghobeishavi et al., 2015; Sarropoulou et al., 2016; Jaberi et al., 2018). Ethylene hormone attaches to its receptors in the presence of copper ions. It has been proved that silver ions could be substituted by copper ions because of similarity in size and thus blocks the receptors and prevent the response from ethylene (Kumar et al., 2009; McDaniel and Binder, 2012). The inhibitory effect of silver ions on ethylene receptors and preventing its function is also reported by other researchers (Curtis, 1981; Alva Ticona and Oropeza, 2013; Moniuszko, 2015; Lima et al., 2017). Moreover, silver nitrate reduces its production by inhibiting aminocyclopropane-1-carboxylic acid, which exists in the ethylene biosynthesis pathway (Kumar et al., 2016). On the one hand, $AgNO_3$, by reducing the production and function of ethylene and on the other hand by increasing the production of polyamines, having a common precursor with ethylene (SAM) (Asgher et al., 2018) stimulates cell division and growth (Shyamali and Hattori, 2007; Moschou et al., 2012; Park et al., 2012; Kumar et al., 2016). It also can cause increased cell division. Overall, longitudinal growth of roots and shoots as well as increased leaf area could be attributed to the effect of $AgNO_3$. These results are consistent with those reported by other researchers regarding increased shoot and root length and leaf area (Park et al., 2012; Alva Ticona and Oropeza, 2013; Tamimi, 2015; Taha and Hassan, 2016; Ejaz et al., 2018). In addition to the inhibitory effect of silver ions on ethylene and growth stimulation, nitrate as the main source of nitrogen and interference in the structure of amino acids and nucleic acids, is one of the growth factors in plants (O'Brien et al., 2016; Hachiya and Sakakibara, 2016; Sun et al., 2017) leading to longitudinal growth of roots and shoots and increased leaf area. There are some reports indicating the increased longitudinal growth of hypocotyls by $AgNO_3$, which are consistent with our results (Zheng et al., 1997; McDaniel and Binder, 2012). In some plants such as *Solanum tuberrosum*, $AgNO_3$ (2 mg L^{-1}) has increased the root length and leaf area and reduced the length of shoot (Homaee and Ehsanpour, 2015). Another report showed that the use of $AgNO_3$ (50 µM) in the culture medium of cherry rootstocks Gisela 6, caused a decrease in root length (Sarropoulou et al., 2016). It seems that the reaction of the plants varies according to their species and the concentration of $AgNO_3$.

Microscopic observations

Observation of root and shoot tips anatomy

Ethylene inhibits growth by restricting cell division in apical meristem of roots and shoots (Kazama et al., 2004; Vaseva et al., 2018) and polyamines are organic compounds that have important roles in cellular processes such as cell division and proliferation (Michael, 2016; Masson et al., 2017). Earlier, we mentioned that $AgNO_3$ can increase cell division and longitudinal growth by inhibiting ethylene and increasing the production of polyamines. As a result, homogeneous colors are observed in different sections of root and shoot apical meristem of treated plants as compared with control samples.

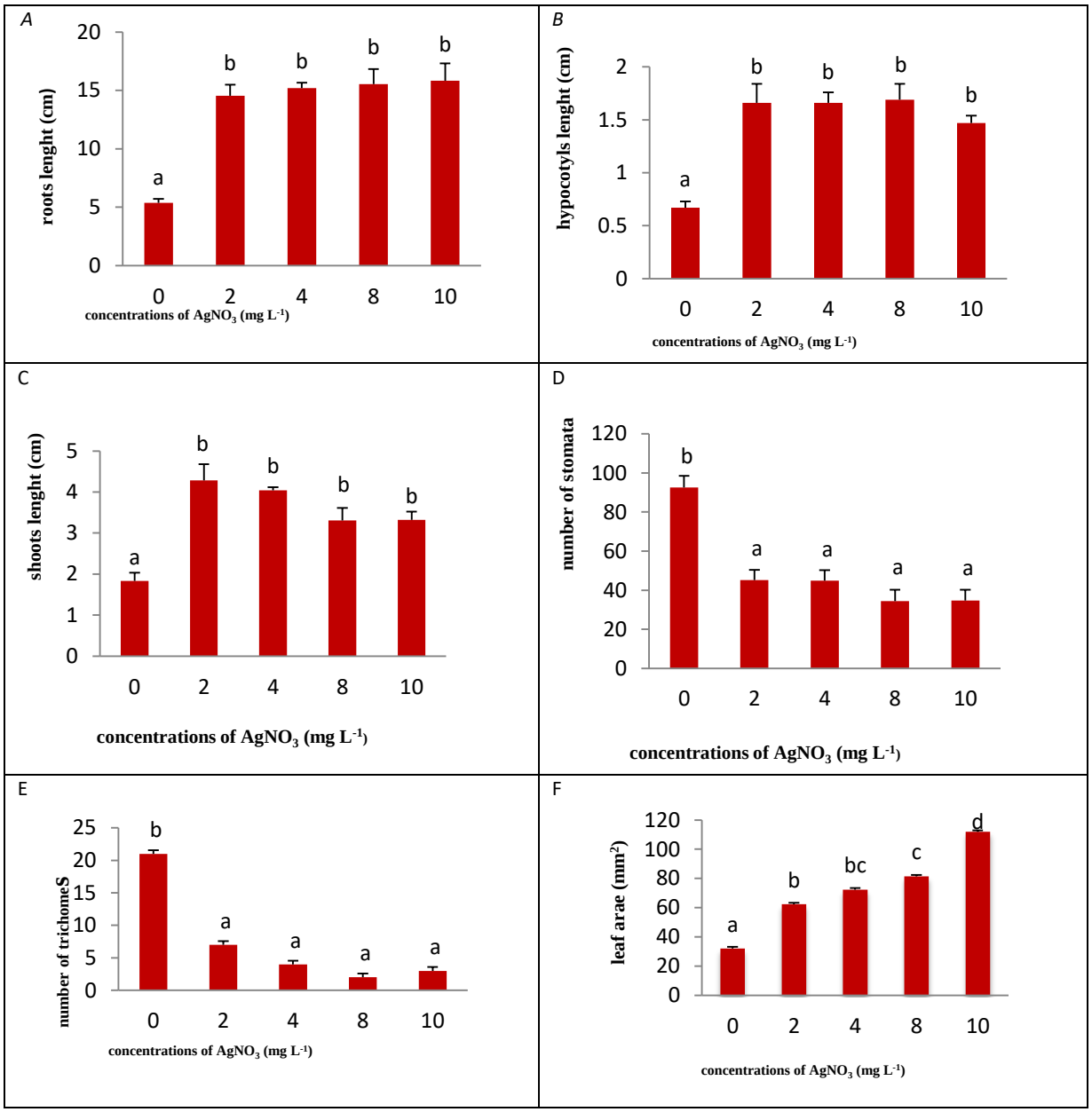

Fig 1. Mean comparison of longitudinal growth of roots (A) hypocotyls (B) and shoots (C) of 20-day seedlings treated with different concentrations of $AgNO_3$. Mean comparison of the number of stomata (D) and trichomes (E) of 20-day seedlings treated with different concentrations of $AgNO_3$. Mean comparison of leaf area of 4-month plantlets treated with different concentrations of $AgNO_3$ (F). Values are the mean ± standard error and the different letters are significantly different at $P \leq 0.05$ using Tukey's multiple range test.

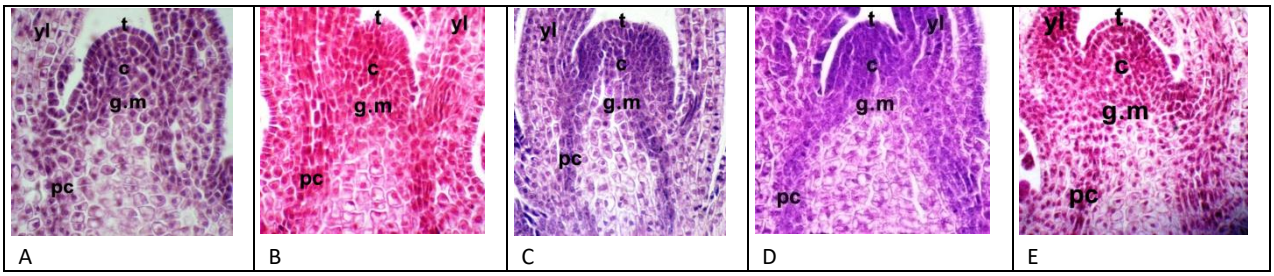

Fig 2. Longitudinal and microtome sections of shoot apical meristem of 20-day seedlings treated with different concentrations of $AgNO_3$. A: control, B: 2 mg L^{-1}, C: 4 mg L^{-1}, D: 8 mg L^{-1}, E: 10 mg L^{-1}. t: Tunica, c: Corpus, g.m: Ground meristem, pc: Procambium, yl: Young leaf. Obj.: 40X. Figures B, C, D, E, show an increase in cell color intensity and homogenization, which indicate more activity and cell division in the root apical meristem of the samples treated with $AgNO_3$, compared to the control sample.

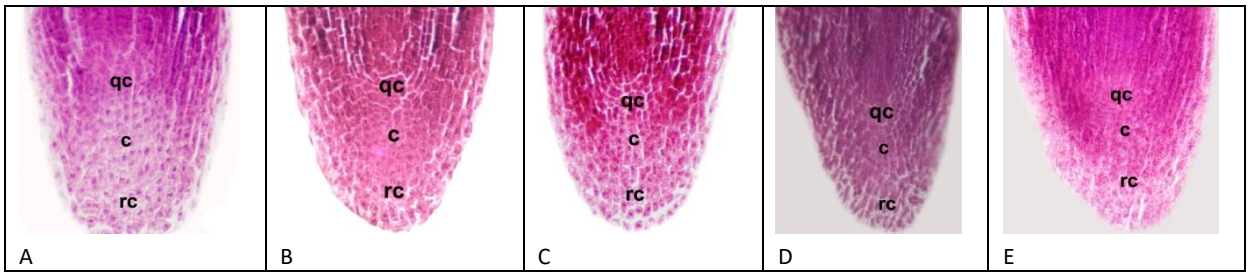

Fig 3. Longitudinal and microtome sections of root apical meristem of 20-day seedlings treated with different concentrations of $AgNO_3$. A: control, B: 2 mg L^{-1}, C: 4 mg L^{-1}, D: 8 mg L^{-1}, E: 10 mg L^{-1}. rc: Root cap, c: Calyptrogen, qc: Quiescent center. Obj.: 40X. Figures B-E show an increase in cell color intensity, which indicate more activity and cell division in the root apical meristem of the samples treated with $AgNO_3$, compared to the control sample.

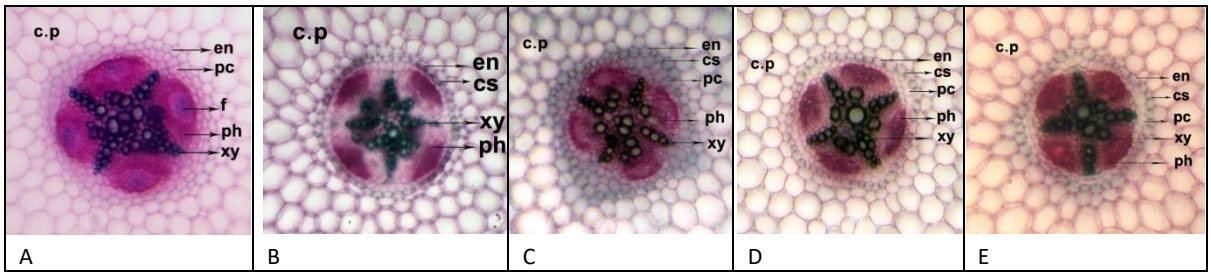

Fig 4. Transverse section of a part of root of 20-day seedlings treated with different concentrations of $AgNO_3$. A: control, B: 2 mg L^{-1}, C: 4 mg L^{-1}, D: 8 mg L^{-1}, E: 10 mg L^{-1}. en: Endodermis, pc: Pericycle, f: Fiber, ph: Phloem, xy: Xylem, c.p: Cortex parenchyma, cs: Casparian strip. Obj: 10X. Note the reduction in vascular and fiber (with lignified cell walls) tissues and increase in thickness of Casparian strip and cell walls of endodermis in figures B-E as compared with figure A.

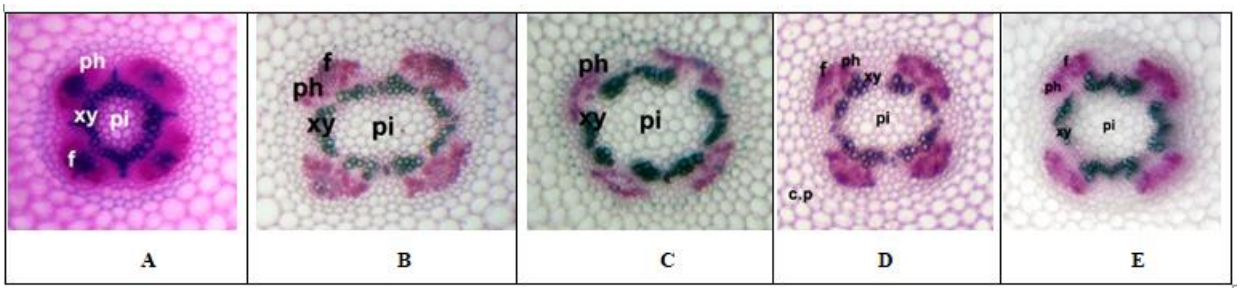

Fig 5. Transverse section of a part of hypocotyl of 20-day seedlings treated with different concentrations of AgNO3. A: control, B: 2 mg L-1, C: 4 mg L-1, D: 8 mg L-1, E: 10 mg L-1. ph: Phloem, xy: Xylem, pi: Pith, f: Fiber, c.p: Cortex parenchyma. Obj: 10X. Note the reduction in vascular and fiber (with lignified cell walls) tissues in figures B-E as compared with figure A.

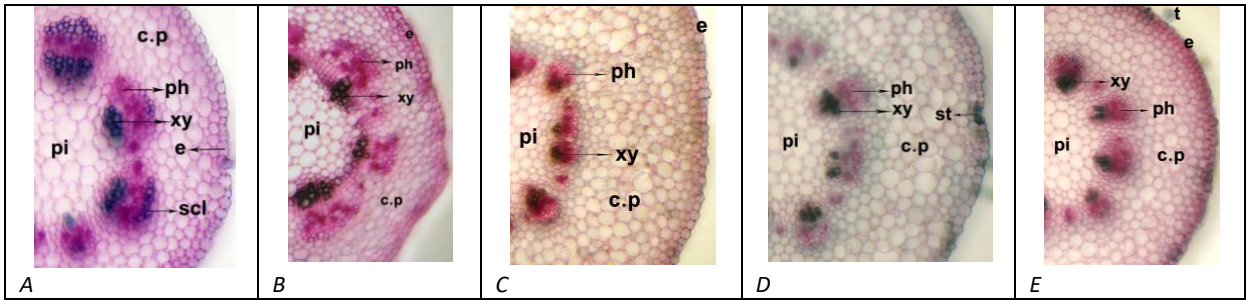

Fig 6. Transverse section of a part of shoot of 20-day seedlings treated with different concentrations of $AgNO_3$. A: control, B: 2 mg L^{-1}, C: 4 mg L^{-1}, D: 8 mg L^{-1}, E: 10 mg L^{-1}. ph: Phloem, xy: Xylem, c.p: Cortex parenchyma, e: Epidermis, pi: Pith, scl: Sclerenchyma, st: Stoma, t: trichome. Obj: 10X. Note the reduction in vascular and sclerenchyma (with lignified cell walls) tissues in figures B-E as compared with figure A.

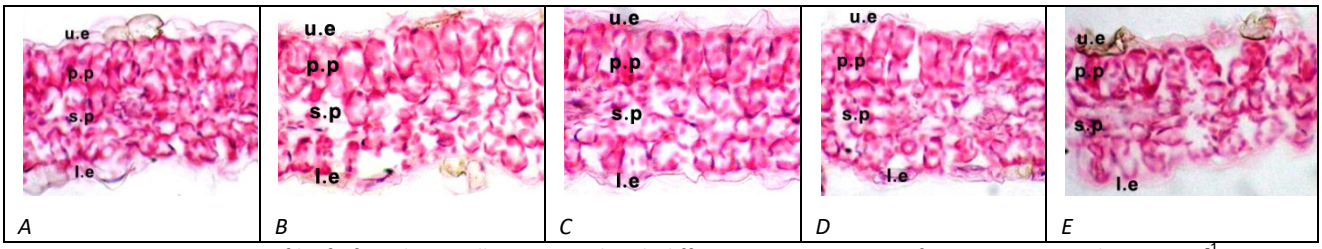

Fig 7. Transverse section of leaf of 20-day seedlings treated with different concentrations of $AgNO_3$. A: control, B: 2 mg L^{-1}, C: 4 mg L^{-1}, D: 8 mg L^{-1}, E: 10 mg L^{-1}. u.e: Upper epidermis, l.e: Lower epidermis, p.p: Palisade parenchyma, s.p: Spongy parenchyma. Obj: 10X. Figures B, C, D and E show the reduction in thickness of epidermis (u.p) and increase intercellular spaces in mesophyll as compared with figure A.

Observation of roots, hypocotyls and shoots anatomy

In this research, the study of anatomical structure of vegetative organs (roots, hypocotyls and shoots) of the seedlings treated with $AgNO_3$ indicated the thickening of endodermis cell walls and Casparian strip. $AgNO_3$ is the salt of silver. As a heavy metal, it reportedly limits the apoplastic pathway and prevents the transfer of water into xylem, which is the first line of defense in plants to maintain survival against heavy metal stress (Chen et al., 2011; Gomes et al., 2012; Rucińska-Sobkowiak, 2016; Brunetto et al., 2017; Ricachenevsky et al., 2018; Nakamura and Grebe, 2018). As a result, thickening of Casparian strip and endodermis cell walls reduce the absorption and transfer of heavy metals to the shoots by limiting apoplastic pathway as well as providing a place for accumulation of these metals to avoid the transfer to the xylem and shoots (Gomes et al., 2011; Liu et al., 2018). The decreased xylem and phloem in plantlets treated with $AgNO_3$ could be also because of reduced transfer of water and soluble materials and preventing the transfer of heavy metals to leaves, mentioned in some other reports (Pokhrel and Dubey, 2013; Chaudhary, 2014). In this research, decreased sclerenchyma and fiber tissue with lignin cell walls is probably related to anti-ethylene effect of $AgNO_3$ and the inhibition of lignin biosynthesis and senescence (Kumar et al., 2009; Huang et al., 2013; Kim et al., 2015).

Observation of leaves anatomy and stomata and trichomes number

Reagrding the anatomical structure of leaves, the effect of $AgNO_3$ on *Potamogeton* showed a kind of hydrophytic adaptation including the decreased thickness of epidermal cells, trichomes, stomata and vascular tissues, as well as increased intercellular spaces in mesophyll (Al-Saadi et al., 2013). These results are consistent with the present study.

Materials and methods

Plant materials and culture conditions

For the preparation of the seeds, the seeds, produced by Pakan Bazr Co (Isfahan, Iran), were soaked in hypochlorite 3% for 3 minutes for surface sterilization. After 3 to 4 times washing with distilled water, the seeds were cultivated in containers containing MS medium (Murashige and Skoog, 1962) with different concentrations of silver nitrate (0, 2, 4, 8 and 10 mg L^{-1}). All culture media contained saccharose 3%, agar 0.8% in pH 5.8. Culture media sterilization was performed at a pressure of one atmosphere and a temperature of 121°c for 20 minutes (121°c, 1 atm). The photoperiod was adjusted to 16:8-h light (using 36-watt fluorescent lamps at a distance of 30 cm from the samples): dark. We prepared vegetative organs including root, hypocotyl, shoot and leaf from 20-day seedlings. In order to measure the leaf area, we used 4-month plantlets.

Measuring growth and development parameters

The longitudinal growth of roots, hypocotyls and shoots in 20-day seedlings were measured by a ruler. The number of stoma and trichome in different areas of the lower epidermis of leaves in 20-day seedlings was counted with a light microscope. The leaf area was measured with a leaf area meter (ADC BioScientific Ltd., Area meter AM 200) after 4 months.

Preparation of longitudinal and transverse sections for anatomical study

The analysis of anatomical structure of root and shoot apex as well as the blade of leaves in 20-day seedlings was performed by the common cell histology. Following the stabilization with F.A.A (Formaldehyde: Acetic acid: Ethanol, 2:1:17, v/v/v), washing, dehydration with ethanol, replacement of ethanol with toluene as the solvent of paraffin, the samples were saturated in paraffin. Series of cross-sections were made by Microtome (Cut 4060 SLEE) at a thickness of 8µm. After removal paraffin and hydration, the samples were dyed by Hematoxylin-Eosin. After dehydration and final clarification, coverslips fixation was performed by Entalen glue.

The anatomical structure analysis of vegetative tissues including roots, hypocotyls and shoots in 20-day seedlings was carried out by hand sections from the same areas of tissues which followed by staining with carmine (for non-lignified cell walls) and methylene green (for lignified cell walls). The analysis of sections was done with a light microscope (Leica BME) and photos were taken from the suitable samples by a digital camera (Canon IXUS 75).

Experimental design and Statistical analysis

Preparation of culture media containing various concentrations of $AgNO_3$ (0, 2, 4, 8, 10 mg L^{-1}) and seed culture with 6-7 seeds in each container were performed three times with three replications for each group. Data analysis was performed by SPSS software $_{V.16}$. The statistical

significance was determined by ANOVA, Tukey's test, ($P \leq 0.05$). Charts were drawn in the EXCEL 2007 software.

Conclusion

It seems that increasing the longitudinal growth of roots, hypocotyls and shoots, as well as increasing leaf area are due to the inhibitory effects of $AgNO_3$ on the production and function of ethylene. In these conditions, the measurement of ethylene production is suggested. The changes in anatomical structure of vegetative organs of plants treated with $AgNO_3$ are probably related to the plant strategy to increase the tolerance against $AgNO_3$ toxicity. Therefore, in order to obtain optimal growth with minimal anatomical changes, the use of lower concentrations of $AgNO_3$ is recommended.

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