

## Interaction of nitrogen stress and salicylic acid on physiologic and photosynthetic characteristics of borage (*Borago officinalis* L.)

Narges Heidari<sup>1</sup>, Fraid Shekari\*<sup>1</sup>, Ahmad Golchin<sup>2</sup> and Negar Sehati<sup>3</sup>

<sup>1</sup> Department of Plant Production and Genetics, Faculty of Agriculture, University of Zanjan. Zanjan, Iran

<sup>2</sup> Department of Soil Science, Faculty of Agriculture, University of Zanjan. Zanjan, Iran

<sup>3</sup> Department of Chemistry, Faculty of Science, University of Zanjan. Zanjan, Iran

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### Abstract

Deficiency of nitrogen may lead to decrease in yield and yield potential of plant crops. This experiment was done under glasshouse conditions in order to investigate the nitrogen levels and salicylic acid interaction on some physiologic traits of borage (*Borago officinalis* L.). In this experiment various levels of nitrogen (including; 27.5, 55, 110, 220 and 330 mg/L from ammonium nitrate) and salicylic acid (including; 0, 400, 800 and 1200  $\mu$ M) were applied. The results indicated that stomatal conductance, photosynthesis rate, chlorophyll a, chlorophyll b, carotenoids, proline, and ascorbate peroxidase, were increased by increasing of nitrogen level. However, intercellular CO<sub>2</sub> concentration, anthocyanin, flavonoids, soluble carbohydrates, catalase, and peroxidase were decreased. Among nitrogen levels, the maximum effect was obtained by 220 and 330 ppm treatments. Furthermore, salicylic acid spraying led to promote stomatal conductance, photosynthesis rate, transpiration rate, photosynthetic pigment content, proline, anthocyanin, flavonoids, soluble carbohydrates, catalase, peroxidase and ascorbate peroxidase enzyme, but not intercellular CO<sub>2</sub> concentration. The highest values of stomatal conductance, photosynthesis rate, transpiration rate, photosynthetic pigment content, proline, anthocyanin, flavonoids, soluble carbohydrates, and activities of catalase, peroxidase, and ascorbate peroxidase enzymes were achieved by 800 and 1200  $\mu$ M concentrations of salicylic acid and 330 ppm of nitrogen. The results showed that appropriate concentrations of salicylic acid can recover nitrogen shortage and improve the measured traits. According to data, there was a synergic effect between SA and N concentration. By increasing each factor, the response of treated plants in measured trait was raised especially in higher concentrations of factors.

**Keywords:** Nitrogen, Salicylic acid, Photosynthesis rate, Proline

### Introduction

Nitrogen is an essential element which contributes many plant biomolecules, such as chlorophyll, amino acids, proteins, and enzymes. Not only in natural ecosystems but even in agroecosystems, nitrogen availability is a limiting factor for plant productivity (Connor *et al.*, 2011). Also, nitrogen is necessary for plants growth and development. This element stimulates the growth of the shoots and makes larger and dark green leaves (Fageria, 2009). Nitrogen deficiency delays the phenological development both in the vegetative and reproductive phases and reduces the rate of leaf expansion and duration rate in plants. This leads to a reduction in light use efficiency. On the other hand, with increasing nitrogen concentration in leaf tissue, the rate of carbon assimilation increases too (Barker and Pilbeam, 2007). It is reported that a high portion of leaf nitrogen content is related with photosynthetic proteins, including rubisco and carbonic anhydrase and light harvesting complex proteins (Gastal and Lemaire, 2002). Therefore, the response of leaf photosynthesis to radiation is largely dependent on the leaf nitrogen content (walker, 2001).

The deficiency of this element is mostly seen in arid and semi-arid regions. Because the amount of soil organic matter (SOM), which is the main source of stored soil nitrogen, is very low in these areas if any SOM is available it decompose rapidly (Ankumah *et al.*, 2003). If available nitrogen is less than or greater than the plant requirement, it can cause disturbances in the plant vital processes, which may occur in many ways, such as high rates in growth and expansion, reduction of transpiration or even cessation of reproductive growth (Hasegawa *et al.*, 2008). Chlorophyll biosynthesis does not occur in chloroplast without the presence of nitrogen or its deficiency, and therefore, nitrogen limitation and deficiency decline photosynthesis rate and chlorophyll biosynthesis. Addition of nitrogen to propagate media leads to an increase in leaf appearance rate, leaf number and leaf chlorophyll content (Silva Junior *et al.*, 2013).

Salicylic acid (SA) is a plant hormone and is known as a key messenger component in activating the plants specific defense responses. It has a variety of physiological effects on plants growth (Hayat *et al.*, 2013). The exogenous application of SA in stressed

\*Corresponding Author, Email: shekari@znu.ac.ir

plants leads to proline accumulation (Liang *et al.*, 2013). However much of the scientific evidence suggests that this compound plays a key role in providing systemic acquired resistance and is able to increase the antioxidant capacity of the plant (Szepesi *et al.*, 2005). Another reason to improve the growth parameters under the influence of SA treatment is the effect of this hormone on the photosynthetic device and its protection, photosynthesis rate, Rubisco activity, photosynthetic pigments concentrations, stomatal conductance, antioxidant defense system, reduction of oxidative stress, and ion leakage, increasing the integrity of biological membranes, nitrogen metabolism and mineral nutrition of the plant as referred to in various studies (Hayat *et al.*, 2013; Janda *et al.*, 2012). Mohammadi *et al.* (2017) reported that SA increased the rate of photosynthesis, stomatal conductance and the efficiency of carboxylation in safflower.

Borage is an annual, dicot plant and belongs to the Boraginaceae family. The origin of this plant is Mediterranean regions and grows in Mediterranean countries, Europe and North America (Gupta and Singh, 2010). Borage contains a lot of active ingredients such as tannin, mucilage, phenolic compounds, sugar substances, flavonoids and a lower amount of alkaloids. Also, it is considered as richest sources of gamma linoleic acid (Van-Wyk and Wink, 2004). Akbarpour *et al.* (2014) stated spraying of salicylic acid to borage plants changed some morphological and biochemical traits of treated plants. The photosynthetic pigments such as chlorophyll a, b and carotenoid; and antioxidant activity of sprayed plants increased significantly.

According to our searches, there is not any report about the application of salicylic acid on photosynthetic traits of borage and especially with varied concentration of nitrogen. Therefore, the purpose of this study was to investigate the effect of exogenous salicylic acid on the improvement of growth factors and physiological traits of Borage plants under the nitrogen stress. Also, the possible ameliorative effects of this hormone on the gradient of nitrogen from suboptimal to super-optimal levels were investigated.

## Materials and methods

This research was conducted in the research greenhouse of the Faculty of Agriculture at the University of Zanjan. The experiment was carried out as factorial and in completely randomized design with three replications. In this experiment, nitrogen was used in ammonium nitrate form at five levels (27.5, 55, 110, 220 and 330 mg/L) and salicylic acid sprayed at four levels (0, 400, 800 and 1200  $\mu$ M). The concentration of 220 mg/L N was considered as control and 27.5 mg/L as the lowest level for plant growth. Seeds of borage were planted in the 5 L plastic pots containing perlite as hydroponic cultures.

The first irrigation was carried out immediately after planting. After seedling emergence, a quarter-Hoagland nutrient solution was fed three times and then the half-Hoagland nutrient solution was fed three times too in two days intervals. Subsequently, the Hoagland nutritional solution was regularly given to the plants. At rosette stage when the plants had 4-5 leaves, borage plants sprayed with SA. After seedling establishment, 10 plants per pot stored and the other seedlings were removed.

The composition of used Hoagland solution was as follow:  $\text{KH}_2\text{PO}_4$  (1 mM),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (2 mM),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.0008 mM),  $\text{H}_3\text{BO}_3$  (0.046 mM),  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.009 mM),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.003 mM),  $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$  (0.0001 mM) and Fe (Fe-EDTA) 0.00009 mM (Noggle and Fritz, 1983).

**Photosynthesis and its dependent parameters:** Measurement of this trait was performed at the flowering stage using IRGA, (LCpro, ADC Bioscientific Ltd., Hoddesdon, UK) at 9 o'clock in the morning. The penultimate leaf placed in the chamber and adaxial part of leaf oriented to sunlight. The stomatal conductance ( $\text{CO}_2 \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ), transpiration rate ( $\text{H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ), intercellular  $\text{CO}_2$  concentration and photosynthetic rate ( $\text{CO}_2 \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ) were measured on the penultimate leaf of five plants in each pot which was randomly selected and finally their mean was reported.

**Chlorophyll content:** Determination of chlorophyll a and b was done by the method of Arnon (1949) and carotenoids were done by Linchtenthare method (Porra, 1989). Finally below equations was used for calculation of chlorophyll a, b and carotenoids concentrations.

Chlorophyll a =  $(19.3 \times A_{663} - 0.86 \times A_{645}) V/100W$   
 Chlorophyll b =  $(19.3 \times A_{645} - 3.6 \times A_{663}) V/100W$   
 Carotenoids =  $100 (A_{470}) - 3.27 (\text{mg chl a}) - 104 (\text{mg chl b})/227$

V = volume of filtered solution (upper solution obtained from centrifuges)

A = absorption at 663, 645 and 470 nm

W = fresh weight of sample in grams

**Flavonoid contents:** Flavonoids were measured by the method of Krizek *et al.* (1998). Leaf samples were homogenized in a porcelain mortar with 0.1 gram of leaf tissue and 5 ml of acid ethanol. Then, the extract was centrifuged, and the supernatant was incubated in a water bath for 10 min at 80°C and allowed to cool to room temperature. Then, the concentration of flavonoids was determined from the absorbance at 270 nm.

**Anthocyanin content:** Anthocyanin concentration was measured according to the method of Wagner (1979). Fresh plant samples (0.1 g) were homogenized in a mortar with 5 ml Acidified methanol, and were kept in the dark for 24 hours at 25°C. Then it was centrifuged for 10 min at 4000 rpm and absorption of the supernatant was measured at 550 nm.

**Proline content:** The content of proline was

measured (Bates *et al.*, 1973). Samples (0.02g) were homogenized with 3 mL sulphosalicylic acid (3% w/v) and centrifuged for 10 min, 1 ml of supernatant were mixed with 1 ml of glacial acetic acid and 1 ml of acid ninhydrin and remained on warm bath for 1 h at 100°C. Heating caused a reaction between ninhydrin and proline. Then, test tubes were kept in an ice bath. One ml of the upper part containing proline was separated and the absorption of each sample was read at a wavelength of 520 nm by spectrophotometer system.

**Soluble carbohydrates:** In order to measure soluble sugars, 100 mg of dried and powdered samples were weighed. Then, extraction was performed by using 10 ml of 95% ethanol. Extracts were first shaken for 15 minutes on a shaker with 360 rpm and were then centrifuged for 15 minutes at 3500 rpm. Then 3 ml anthrone reagent was added to the top phase of centrifuged samples. Finally, after applying boiling water temperature for 10 minutes, the amount of light absorption was recorded at a wavelength of 630 nm (Hedge and Hofreitor, 1962).

**Measurement of enzyme activity:** CAT activity was measured by the destruction of  $\text{H}_2\text{O}_2$  by CAT (Aebi, 1984) and at a wavelength of 240 nm was read. APX activity was evaluated by the method of Ranieri *et al.* (2003) and at a wavelength of 290 nm was read. The content of hydrogen peroxide was measured based on Loreto and Velikova (2001) and at wavelength of 390 nm was read.

## Results and discussion

Borage plants showed significant differences in nitrogen and salicylic acid effects and their interactions on all measured traits (Table 1).

**Stomatal conductance:** Increasing levels of nitrogen and salicylic acid increased stomatal conductance. The highest level of stomatal conductance with a mean of 4.25 was related to nitrogen (330 mg/L) and salicylic acid (1200  $\mu\text{M}$ ) treatment, and the lowest level with a mean of 0.1700 was related to 27.5 mg/L N and salicylic acid zero treatment (Fig. 1). The interaction of SA and N showed that increasing the concentration of SA at all levels of nitrogen, increased stomatal conductance. The amount of these changes at low SA concentrations was not significant but a significant synergistic effect was observed between the used N and SA concentrations. So, there was a dramatic increase in the highest levels of SA and N. It is reported that nitrogen deficiency, significantly reduces the leaf photosynthesis, which this reduction, is mainly due to the leaf stomatal conductance reduction (Zhao *et al.*, 2007). Shangguan *et al.* (2000) also reported that a reduction in available nitrogen led to a decrease in photosynthesis and stomatal conductance. Mohammadi *et al.* (2017) also have reported that stomatal conductance has increased with the application of SA in safflower. On the other hand,

Hao *et al.* (2012) reported that application of salicylic acid decreased the stomatal conductance in cucumber leaves. It seems that this reaction could be dependent on salicylic acid concentration, so that, at higher concentrations has a negative effect on stomatal conductance.

**Transpiration rate:** Increasing levels of nitrogen and salicylic acid increased the rate of transpiration. The highest transpiration rate was observed for nitrogen treatment of 220 mg/L and salicylic acid 1200  $\mu\text{M}$  with an average of 6.50 ( $\text{mmol H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ), and the lowest amount was observed in nitrogen treatment of 27.5 mg/L and salicylic acid zero with an average of 3.760 (Fig. 2). If available nitrogen is less than or more than the optimal levels it can disrupt the vital processes of the plant and that may reduce transpiration (Rios *et al.*, 2002). In addition, an increase in nitrogen enhances herbal tissues and leaf area and therefore, increases the transpiration (Hopkins, 2004). Guo *et al.* (2003) reported that  $\text{NO}_3$  has a role in opening the stomata, and because of stomatal aperture, transpiration rate may reduce in the lower concentration of  $\text{NO}_3$ . On the other hand, cytokinins concentration increased by application of SA in plants such as cucumber (Hao *et al.*, 2012). Increasing cytokinin level can lead to increasing stomatal aperture and transpiration. Although, there was relation between stomatal conductance and transpiration rate and it stands to reason that further opening of ostiole may increase transpiration rate. But this relation may not always be seen. For example, in sugarcane Endres *et al.* (2010) stated there was a linear regression between  $g_s$  and  $T$  till to 0.3  $\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . But over this value increment on  $g_s$  had not any effects on  $T$  values. On the other hand, Xu and Zhou (2008) reported on addition of plant water status, stomatal density and size had significant effect on  $g_s$  and  $T$ . As shown in figs 1 and 2 by increasing N levels gradually  $g_s$  and  $T$  increased. But the effect on  $T$  was greater than  $g_s$ . In addition, spraying of SA intensified the  $g_s$  or  $T$  in each level of N. It is possible there is an interaction on N and SA application on stomata density and size which influences  $g_s$  and  $T$ .

**The photosynthesis rate:** At the lowest level of N and without SA, the lowest rate of photosynthesis was observed (fig. 3). Spraying of SA increased the rate of photosynthesis. The major effect of SA was found in lowest and highest concentration of N. It seems that spraying of SA has been able to increase the nitrogen uptake and/or assimilation efficiency. Like the previous attributes, the synergistic effect between SA and N was such that, with increasing levels of both factors, the rate of photosynthesis increased significantly. Thus, the shape of the curve in SA 1200  $\mu\text{M}$  concentration and different levels of N was completely different from the lower treatments. The comparison of  $g_s$  and  $T$  with photosynthesis rate shows that photosynthesis response to N and SA was closer to  $T$  than  $g_s$ . Photosynthesis has a high

**Table 1- Analysis Variance of nitrogen stress and salicylic acid on physiological and photosynthetic characteristics of borage (*Borago officinalis* L.)**

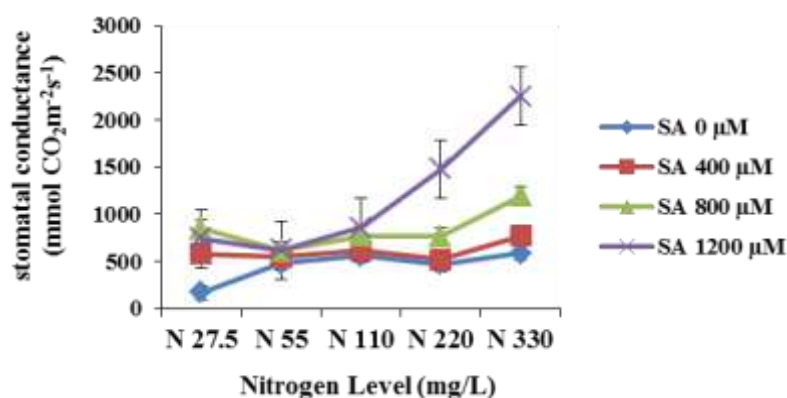
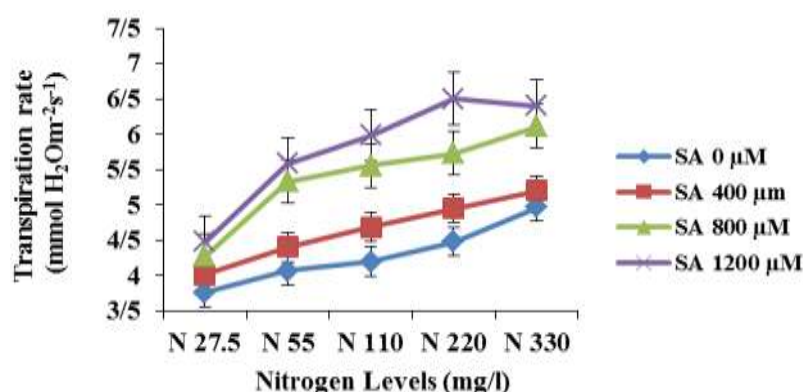
SoV	d.f	Transpiration rate	Stomatal conductance	Photosynthesis rate	Intercellular CO <sub>2</sub> concentration	Chlorophyll a	Chlorophyll b	Cartooned
Nitrogen (N)	4	2.214*	2.693**	16.88*	561.88*	1.043*	0.073*	0.0105*
SA (S)	3	1.119*	2.371**	51.641*	2497.05*	0.050*	0.038*	0.005*
N × S	12	1.951*	1.871*	16.909*	601.08**	0.003*	0.002*	0.001*
Error	40	0.518	0.701	5.840	107.600	0.002	0.001	0.001
CV%		11.50	96.32	29.16	3.15	49.53	35.85	51.70

ns: non significant, \* and \*\*: significant at 5% and 1% probability levels, respectively

**Continue of table 1-**

SoV	d.f	Ascorbate peroxidase	Peroxidase	Catalase	Carbohydrate	Proline	Flavonoid	Anthocyanin
Nitrogen (N)	4	0.028**	0.006*	0.071*	27048.67*	8.465*	0.079*	0.066*
SA (S)	3	0.020*	0.002*	0.091*	86403.96*	2.376*	0.008**	0.020*
N × S	12	0.018*	0.002*	0.038*	137324.56*	0.314*	0.041*	0.012*
Error	40	0.003	0.001	0.009	31352.82	0.007	0.016	0.007
CV%		69.17	47.16	59.80	14.13	7.67	36.93	58.65

ns: non significant, \* and \*\*: significant at 5% and 1% probability levels, respectively

**Fig. 1- Interaction effects of nitrogen and salicylic acid levels on stomatal conductance ( $\text{mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).****Fig. 2- Interaction effects of different nitrogen and salicylic acid levels on transpiration rate ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).**

correlation with leaf nitrogen content. Because photosynthesis is associated with Rubisco, carbonic anhydrase and other proteins that exist in the mesophyll, these enzymes and proteins account for

about 75% of the total nitrogen in the cell. Therefore, more nitrogen in plants results in an increase of Rubisco activity and the electron transfer reaction (Shangguan *et al.*, 2000). Also, nitrogen deficiency

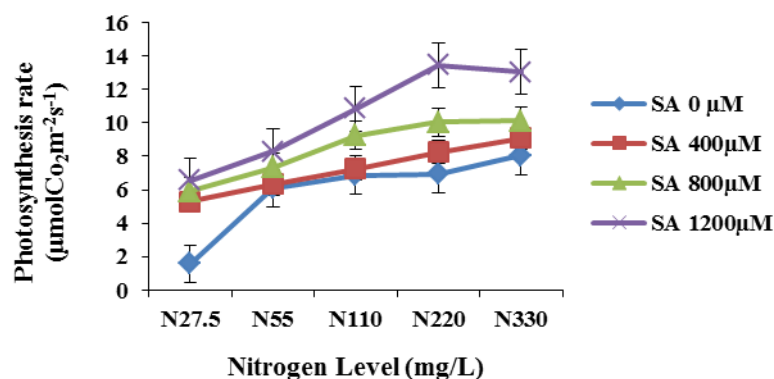


Fig. 3- Interaction effects of different nitrogen and salicylic acid levels on photosynthesis rate ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).

due to the reduction of size and surface area and leaf area duration led to a reduction in the total light interception and amount of absorbed radiation, therefore, the decline in radiation use efficiency and photosynthesis rate (Lak *et al.*, 2008). Application of nitrogen fertilizer in safflower plants significantly increased the photosynthetic rate at pollination stage (Dordas and Sioulas, 2008). Elizabeth *et al.* (2008) reported it is possible that salicylic acid prevents chlorophyll decomposition by inhibition of the activity of chlorophyll oxidase enzymes and therefore increases photosynthesis. According to Shakirova *et al.* (2003), salicylic acid increases the photosynthesis rate by preventing the destruction of chloroplasts and improving the electron transfer capacity by photosystem II. It has been reported that seed priming with salicylic acid increased chlorophyll a, b and total in wheat plants which leads to significant changes in photosynthesis rate (Abdolahi *et al.*, 2018).

**Intercellular CO<sub>2</sub> concentration:** The reduction in nitrogen concentration increased the intercellular CO<sub>2</sub> concentration. Nitrogen in 27.5 mg/L concentration and non-salicylic acid treatment had the highest concentration of CO<sub>2</sub> than high levels of nitrogen and salicylic acid treatments (fig. 4). Also, the use of salicylic acid leads to increase photosynthesis rate, water use efficiency, stomatal conductance, and transpiration rate in safflower (Mohammadi *et al.*, 2017). The high concentration of intercellular CO<sub>2</sub> indicates that the entered CO<sub>2</sub> into the leaf is not efficiently assimilated in photosynthesis (Anyia and Herzog, 2004). In wheat, it was observed that increasing nitrogen level led to a reduction of substomatal carbon concentration (Yang *et al.*, 2007). Possibly the ineffectiveness of the photosynthetic apparatus and its damage due to nitrogen deficiency led to injury to consumption of CO<sub>2</sub> and increasing its concentration. Also, a reduction in stomatal conductance leads to a reduction of entered carbon dioxide into the leaves and limits the photosynthesis. Under the stress conditions, the stomatal conductance decreases but, the concentration of CO<sub>2</sub> on the substomatal chamber increases. Mohammadi *et al.*

(2017) reported the amount of intercellular CO<sub>2</sub> in primed seeds of safflower with salicylic acid was lower, which can be because of efficient use of CO<sub>2</sub> entered in leaf. This can be because of the high rate of photosynthesis in treated plants with salicylic acid (Mohammadi *et al.*, 2017).

**Photosynthetic pigments content:** Increasing nitrogen and salicylic acid level led to increasing chlorophyll a, b and carotenoids. The results showed that the highest level of chlorophyll a, b and carotenoid was in nitrogen treatment 330 mg/L and salicylic acid 1200  $\mu\text{M}$  with the average of 0.9582, 0.4435 and 0.4301 mg/g; and the lowest was in nitrogen treatment 27.5 mg/L and salicylic acid zero with the average of 0.1551, 0.09451 and 0.03338 mg/g (figs 5, 6, 7). Most of the photosynthetic pigments have nitrogen atom on their structure. Hence, fertilizing plants with nitrogen can greatly increase the number of photosynthetic pigments. Leaf chlorophyll content can be used to determine the plant nitrogen content and to adjust the amount of required nitrogen fertilizer. This increases the efficiency of nitrogen consumption in plants by achieving the maximum yield (Zgallai *et al.*, 2006). It has been reported that a relationship existed between chlorophyll, photosynthesis rate and biomass production (Lemon, 2007). Higher chlorophyll content by increasing salicylic acid concentration can lead to an increase in the photosynthesis rate (Ghani *et al.*, 2002).

It has been reported that the application of salicylic acid in some plants increased the chlorophyll content (Abdolahi *et al.*, 2018; Mohammadi *et al.*, 2017). On the other hand, researches have shown that salicylic acid activated the synthesis of carotenoids and xanthophylls in Wheat (Moharekar *et al.*, 2003). Salicylic acid inhibited Aminocyclopropane-1-carboxylic acid (ACC) activity and prevented the formation of ethylene followed by the reduction and degradation of chlorophyll (Ghani *et al.*, 2002).

**Proline:** Increasing nitrogen level increased the proline content. The highest content of proline was related to 330 mg/L N and salicylic acid 1200  $\mu\text{M}$  (fig. 8) and the lowest content was in 27.5 ppm N and

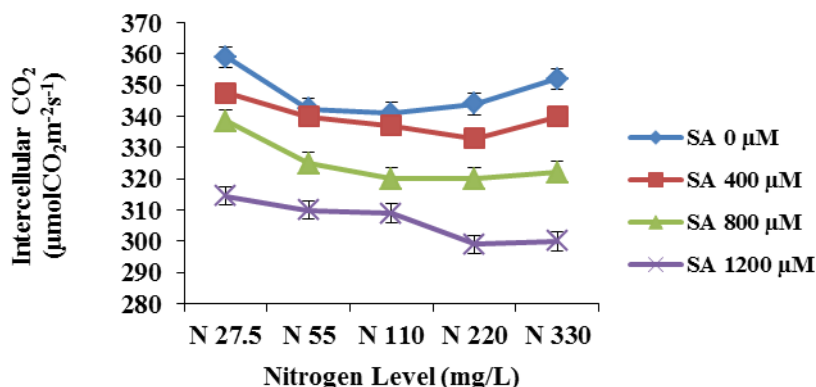


Fig. 4- Interaction effects of different nitrogen and salicylic acid levels on intercellular CO<sub>2</sub> concentration ( $\mu\text{mol CO}_2\text{m}^{-2}\text{s}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).

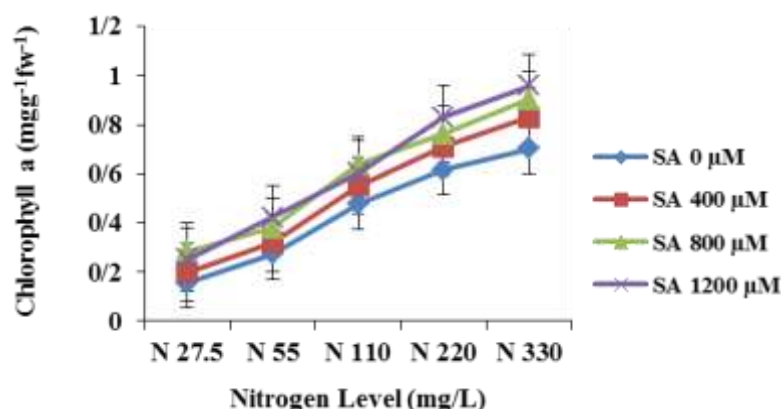


Fig. 5- Interaction effects of different nitrogen and salicylic acid levels on chlorophyll a ( $\text{mg}\cdot\text{g}^{-1}\text{fw}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).

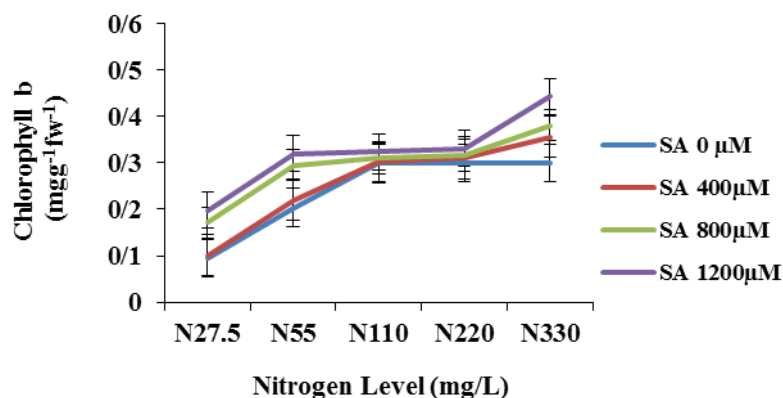


Fig. 6- Interaction effects of different nitrogen and salicylic acid levels on chlorophyll b ( $\text{mg}\cdot\text{g}^{-1}\text{fw}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).

salicylic acid zero. Nitrogen plays an essential role in the structure of amino acids, such as proline, and with increasing concentration of this element proline production increases. Since proline acts as a stabilizer of the cell structure, energy source, and even as a stress-inducing signal, this compound has nitrogen on its structure. So the application of nitrogen can increase the amount of these compounds in the plant (Vendruscolo *et al.*, 2007). Reports have also revealed that the reduction of available nitrogen significantly

reduced the proline accumulation in bean leaves and roots (Sanchez *et al.*, 2002). The amount of proline increased by adding nitrogen in nutritional solution in the culture medium of tomato and cucumber plants (Vendruscolo *et al.*, 2007). In addition, Abdolahi *et al.* (2018) indicated that salicylic acid increased the growth indices and also increased the percentage of proline in the grown germinated lentil.

**Anthocyanin content:** Reduction of nitrogen level increased the anthocyanin content. The highest level

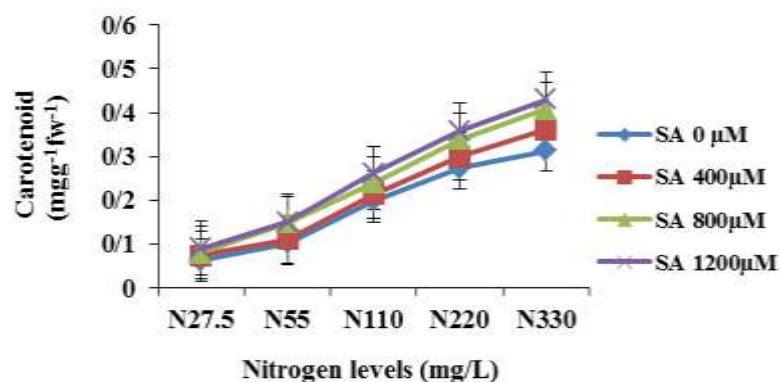


Fig. 7- Interaction effects of different nitrogen and salicylic acid levels on carotenoids content ( $\text{mg.g}^{-1}\text{fw}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).

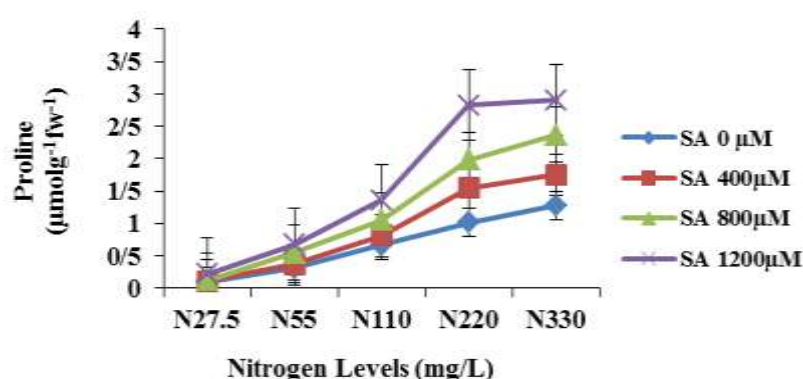


Fig. 8- Interaction effects of different nitrogen and salicylic acid concentrations on proline content ( $\mu\text{mol.g}^{-1}\text{fw}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).

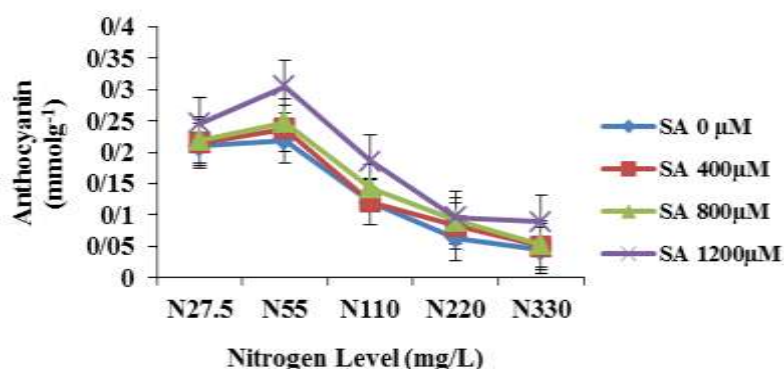
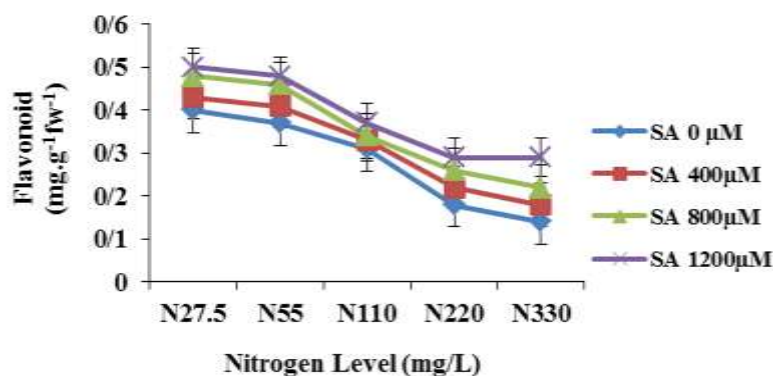


Fig. 9- Interaction effect of different nitrogen and salicylic acid levels on anthocyanin content ( $\text{mmol.g}^{-1}$ ) of borage plants (Error bars shows the difference between treatments).

of anthocyanin with a mean of  $0.3650 \text{ mmol.g}^{-1}$  was about the nitrogen treatment  $55 \text{ mg/L}$  and salicylic acid  $1200 \mu\text{M}$  and the lowest one with the average of  $0.04300 \text{ mmol.g}^{-1}$  was responsive to nitrogen treatment  $220 \text{ ppm}$  and zero concentration of salicylic acid, which had no significant difference with nitrogen treatment at  $330 \text{ ppm}$  and all salicylic acid concentrations, except  $1200 \mu\text{M}$  (fig. 9). Anthocyanins are water-soluble vacuolar pigments which belong to flavonoids. They synthesized via the phenylpropanoid pathway. The anthocyanin in the

plant acts as a free radicals receptor and protects plants against oxidative stresses (Archetti *et al.*, 2011). There is a correlation between carbohydrate and anthocyanin content. For instance, Hilbert *et al.* (2003) reported anthocyanin concentration peaked as sugar continued to increase in grape berries. Also, Larronde *et al.* (1998) stated that in grape cell suspension cultures anthocyanin synthesis increased when intracellular sugar levels were high. In Hilbert *et al.* experiment by increasing available N in medium, anthocyanin concentration was declined. It seems that



**Fig. 10- Interaction effect of different nitrogen and salicylic acid levels on flavonoids (mg.g<sup>-1</sup>fw<sup>-1</sup>) of borage plants (Error bars shows the difference between treatments).**

high nitrogen supply inhibited the synthesis of anthocyanins (Hilbert *et al.*, 2003). The effect of salicylic acid has been investigated on flavonoids, anthocyanin in the *Matricaria chamomilla* L. and it is reported that the anthocyanin level increased by the concentration of 0.125 mM salicylic acid (Zarrinkamar *et al.*, 2013). There are reports on the effect of salicylate on the anthocyanin accumulation which was achieved from the treatment of chervil and cowpea with salicylate (Akinwunmi, 2001). In our experiment the shape of soluble carbohydrates, anthocyanins and flavonoids was similar.

**Flavonoids:** Increasing nitrogen level decreased the flavonoids concentration. The range of variations was from 0.135 to 0.497 mg.g<sup>-1</sup>. The highest amount of flavonoids was related to lowest nitrogen treatment, 27.5 mg/L, and salicylic acid 1200 μM and the lowest one was for nitrogen treatment 330 ppm with no salicylic acid (fig. 10). Flavonoids are a class of natural products and belong to a class of plant secondary metabolites having a polyphenolic structure (Panche *et al.*, 2016). They have numerous functions under some environmental conditions such as temperature, UV, nutrient stress and pathogen attack (Kliebenstein, 2004). Lea *et al.* (2007) stated that in *Arabidopsis* plants grown in different mediums, nitrogen deficiency induced flavonoids and anthocyanins. They showed that nitrogen deficiency up-regulated *PAP1* and *PAP2* which paved the way for flavonoid synthesis. Similarly, Stewart *et al.* (2001) showed nitrogen starvation lead to accumulation of flavonoid on leaf and fruit tissues of tomato plants. Salicylic acid as an elicitor leads to excite a wide range of flavonoids (Pastirova *et al.*, 2004). Observations provided that 20 mM salicylic acid significantly increased flavonoids in *Panax ginseng* (Ali *et al.*, 2007).

**Soluble carbohydrate:** Increasing nitrogen concentration reduced the carbohydrate content. The highest amount of soluble carbohydrate was in the 27.5 mg/L N and salicylic acid 800 μM, and the lowest was in 330 ppm N with no salicylic acid treatments (fig. 11). When nitrogen is available to the

plant in large quantities, the concentration of carbohydrates will be reduced. But when nitrogen is in low level, carbohydrates content will increase. Therefore, a clear negative relationship exists between plant nitrogen content and carbohydrate concentration (Yang *et al.*, 2007). The results of other studies also indicate a negative correlation between carbohydrate percentages with the amount of applied nitrogen fertilizer. This may be due to the main role of nitrogen in the stabilization of amino acids (Taiz and Ziger, 2010). Furthermore, the reduction of nitrate and nitrite requires large amounts of ATP and NADPH that is provided through the respiration and photosynthesis. If the energy is provided through the respiration, this may reduce the carbohydrates content, and if provided by photosynthesis, the lower amounts of carbon dioxide will be reduced and change to the carbohydrates (Yang *et al.*, 2007). Since salicylic acid increased the content of photosynthetic pigments as well as photosynthesis rate, it is reasonable increasing of soluble carbohydrates by application of SA. In corns during the oxidative stress, the sugar accumulation was found with salicylic acid treatment (Kim *et al.*, 2006).

**Anti-oxidant enzymes activity:** Increasing nitrogen levels reduced catalase (CAT) activity. The highest CAT activity was found in 27.5 mg/L nitrogen and salicylic acid 1200 μM treatment (fig. 12). With respect to the mentioned N treatment, the lowest CAT activity was found in non-sprayed plants. Above 110 mg/L N in growth solution, there are not any differences between SA concentrations. Although, a slight increase was observed in activity of peroxidase (POX) in 55 mg/L N compared with 27.5 mg/L N in sprayed plants, but trend of response of this enzyme's activity to N increment was downward (fig. 13). Nitrogen and salicylic acid had no significant effect on ascorbate peroxidase (APX) activity till 110 mg/L N treatment. In addition, the activity of ascorbate peroxidase showed upward trend. So, in 330 mg/L N and salicylic acid 800 μM the highest activity of ascorbate peroxidase was found (fig. 14). According to data, it seems that at lower concentrations of

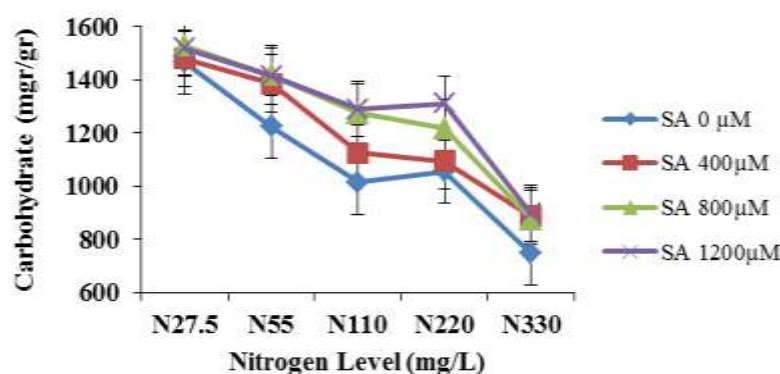


Fig. 11- Interaction effect of different nitrogen and salicylic acid levels soluble carbohydrate content (mg/g) of borage plants (Error bars shows the difference between treatments).

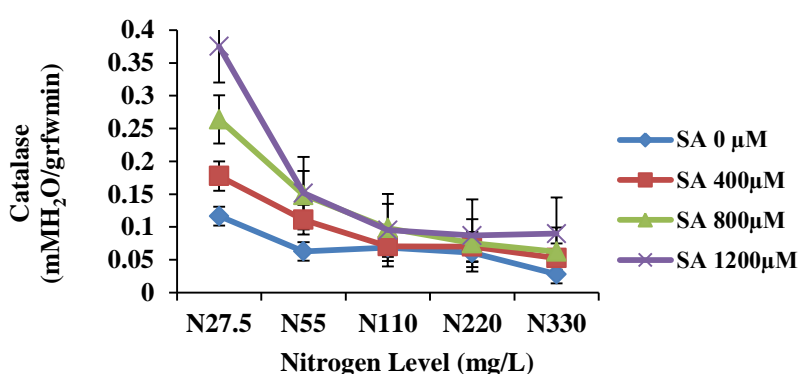


Fig. 12- Interaction effect of different nitrogen and salicylic acid levels on catalase activity of borage plants (Error bars shows the difference between treatments).

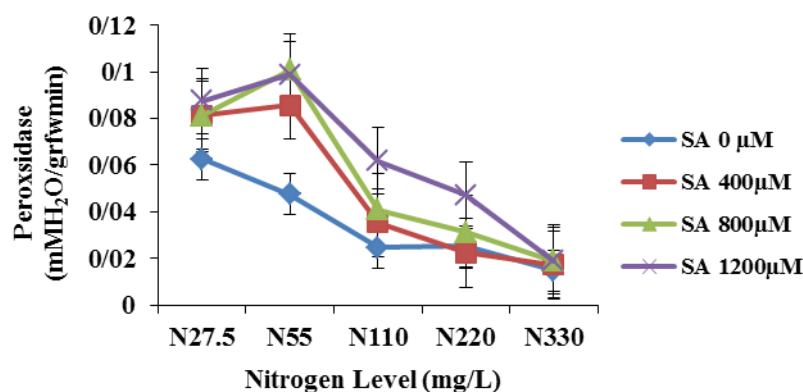


Fig. 13- Interaction effect of different nitrogen and salicylic acid levels on peroxidase activity of borage plants (Error bars shows the difference between treatments).

nitrogen the main role for scavenging of reactive oxygen species is obtained by CAT and POX enzymes.

Under normal growth conditions, many metabolic processes in plants produce reactivated oxygen species. But plants have effective antioxidant mechanisms to eliminate reactive oxygen species. Under the stress conditions, this balance is disrupted, and the amount of reactive oxygen species increases. The presence of these reactive species is harmful to the plants and damages the cellular functions such as membrane integrity, proteins and nucleic acids

structures (Laspina *et al.*, 2005). In the absence of sufficient nitrogen, the activity of ribulose biphosphate carboxylase is reduced and therefore, electrons transferred to the oxidation pathway through the Mehler reaction within the chloroplast. This increases the production of ROSs. In these conditions, it is expected that enzymatic and non-enzymatic antioxidant activities increase in the leaves (Hasegawa *et al.*, 2008). Salicylates by changing the activity of the catalase enzyme which is an H<sub>2</sub>O<sub>2</sub> removal enzyme, affect the amount and the rate of reactive oxygen species production (Horvath *et al.*,

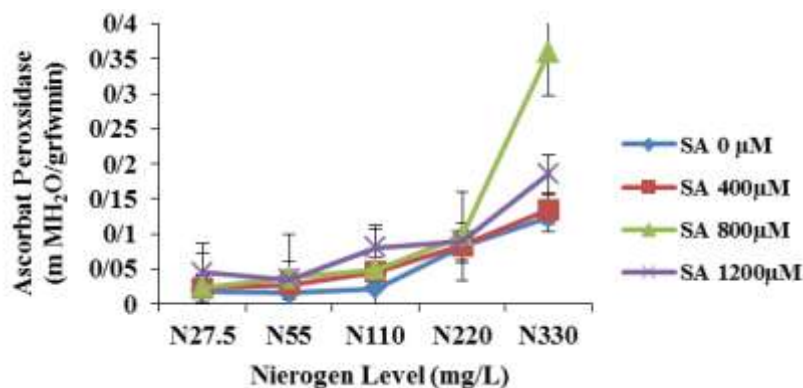


Fig. 14- Interaction effect of different nitrogen and salicylic acid levels on ascorbate peroxidase activity of borage plants (Error bars shows the difference between treatments).

2002). The evidence suggests that salicylic acid plays a key role in providing resistance, compatibility and also can increase the antioxidant capacity of the plant (Szepesi *et al.*, 2005). The activities of antioxidant enzymes while improving the membrane's stability helps to keep the growth under stress (Sairam *et al.*, 2006). It has been reported that nitrogen availability reduces the peroxidase activity (Diego *et al.*, 2003). The exogenous application of salicylic acid on the beans and tomatoes indicated an increase in the activity of antioxidant enzymes including superoxide dismutase and peroxidase (Senaranta *et al.*, 2000). In a study on chickpea, the exogenous use of salicylic acid increased the activity of ascorbate and ascorbate peroxidase (Chakraborty and Tongden, 2005). Reports confirm that salicylic acid controls the activity of antioxidant enzymes through a temporary accumulation of ABA (Hayat and Ahmad, 2007). It seems that nitrogen may play a role in reducing the production of reactive oxygen species, and therefore, reduce needs of cells for scavenging of ROSs by antioxidant enzymes such as peroxidase. The results of this experiment are consistent with the results on alfalfa (Lopez *et al.*, 2007) and sunflower (Lobato *et al.*, 2009), which increased the activity of ascorbate, catalase and peroxidase enzymes with gibberellic acid and salicylic acid treatments. Salicylic acid reduces the effects of stress with affecting on metabolites such as ascorbic acid, glutathione, and antioxidant enzymes including; superoxide dismutase, catalase, polyphenol oxidase and peroxidase (Horvath *et al.*, 2002).

Lin *et al.* (2012) reported that increase of available nitrogen concentration increased the production of antioxidant enzymes SOD and POD, APX in *Populus yunnanensis* plant. Also, Wang *et al.* (2009) reported that salicylic acid have increased ascorbate peroxidase activity in corn.

### Conclusion

The results of this study showed that nitrogen stress caused a significant reduction in physiological characteristics in borage. Application of salicylic acid resulted in an improvement of growth conditions in borage plant with effect on photosynthetic traits and activity of antioxidant enzymes. In general, among treatments 1200 mM SA was less effective than other SA concentrations. Depending on the case application of SA may ameliorate the nitrogen stress had not significant effect on the trait. For instance, in photosynthesis rate, transpiration and activity of CAT and POX in lowest nitrogen concentration spraying of SA improved the studied traits. On the other hand, in some cases such as chlorophyll, carotenoids, proline, anthocyanin and soluble carbohydrates in 27.5 mg/L N SA had not any significant effect on these traits. By increasing available nitrogen in growing media the effects of SA was more impressive. The dramatic and efficient effects of nitrogen and SA were found in highest levels of nitrogen and SA. Finally, our data shows application of SA on borage plants induced nitrogen uptake and its assimilation.

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