doi:10.14720/aas.2021.117.3.1056 Original research article / izvirni znanstveni članek

The role of exogenous glycinebetaine on some antioxidant activity of non-T and T tobacco (*Nicotiana tabacum* L.) under *in vitro* salt stress

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Received February 19, 2019; accepted July 15, 2021.
Delo je prispelo 19. februarja 2019, sprejeto 15. julija 2021

The role of exogenous glycinebetaine on some antioxidant activity of non-T and T tobacco (*Nicotiana tabacum* L.) under *in vitro* salt stress

Abstract: Glycine betaine is an osmoprotectant compound which enhances cell tolerance in plant species in response to environmental stresses. This study aimed to investigate the effect of exogenous application of glycine betaine on some antioxidant activities of tobacco plants overexpressing *P5CS* gene. Sterile tobacco seedlings with four to six leaves were transferred to MS medium containing 0, 100, and 200 mM NaCl, after which glycine betaine (20 and 40 mg l^{-1}) were foliar sprayed on the surface of the plants. After four weeks, glycine betaine treatment enhanced the antioxidant capacity of the plant through activation of catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX). In contrast, H_2O_2 content and MDA level were reduced by glycine betaine under similar conditions. Therefore, application of exogenous glycine betaine under salt stress improved stress tolerance in T and non-T plants. Meanwhile, our results indicated the positive effect of glycine betaine in T plants was greater than in non-T plants. On the other hand, this result suggested that the synergistic effects of glycine betaine and proline in plants enhanced the antioxidant defense system in T plants overexpressing *P5CS* gene.

Key words: glycine betaine; proline; antioxidant enzyme; tobacco plants; salt tolerance

Vloga dodajanja glicin betaina na nekatere antioksidacijske aktivnosti transformiranega in navadnega tobaka (N*icotiana tabacum* L.) v razmerah *in vitro* solnega stresa

Izvleček: Glicin betain je ozmoprotektant, ki vzpodbuja toleranco rastlinskih celic na okoljski stres. V tej raziskavi so bili preučevani učinki dodajanja glicin betaina na nekatere antioksidacijske aktivnosti tobaka, ki ima prekomerno izražanje *P5CS* gena. Sterilne sejanke tobaka s štirimi do šestimi listi so bile premeščene v MS gojišče, ki je vsebovalo 0, 100, in 200 mM NaCl, nakar je bil dodan s pršenjem nadzemnih delov glicin betain (20 in 40 mg l^{-1}). Po štirih tednih je obravnavanje z glicin betainom pospešilo antioksidacijsko sposobnost rastlin z aktivacijo katalaze (CAT), superoksid dizmutaze (SOD) in askorbat peroksidaze (APX). Nasprotno sta se vsebnosti H_2O_2 in MDA zmanjšali po obravnavanju z glicin betainom v podobnih razmerah. Dodajanje glicin betaina lahko v razmerah solnega stresa izboljša toleranco na stres transformiranih in netransformiranih rastlin. Rezultati raziskave so še pokazali, da je bil pozitivni učinek glicin betaina večji pri transformiranih rastlinah. Po drugi strani ti rezultati nakazujejo, da sinergistični učinki glicin betaina in prolina v rastlinah pospešujejo antioksidacijski obrambni sistem v transformiranih rastlinah, ki imajo prekomerno izraženo delovanje *P5CS* gena.

Ključne besede: glicin betain; prolin; antioksidacijski encim; tobak; toleranca na solni stres

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1 INTRODUCTION

Salt stress is one of the most important factors, which limits and reduces the growth and development of plants worldwide. Generally, plant metabolism changes under salinity stress by ion toxicity and osmotic pressure (Mittler, 2002). There are many cellular mechanisms decreasing the adverse effects of environmental stresses such as salinity. Understanding salt tolerance mechanisms is useful to develop novel strategies for salt-tolerant crops. It is known that accumulation of compatible osmolytes such as proline (Pro) and glycine betaine (GB) (Díaz et al., 2005) as well as reactive oxygen species (ROS) detoxification in plants increases salt tolerance. Salinity, drought, and heavy metal stresses induce generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Rajaeian & Ehsanpour, 2015). Under abiotic stresses, plants always improve enzymatic and non-enzymatic antioxidant defense systems to remove ROS content (Hasanuzzaman et al., 2011). The enzymatic antioxidants include superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). In this system, superoxide radical is converted to hydrogen peroxide (H_2O_2) by SOD enzyme (Bowler et al., 1992). Then, H_2O_2 is converted to water by catalase and peroxidase enzymes (Fridovich, 1983).

Further, the accumulation of organic solutes in the cytoplasm is a typical reaction of plants to osmotic stress resulting in osmotic adjustment. Compatible solutes such as Pro and GB are general osmoregulators, which are accumulated during salt stress in plants and play a critical role in osmotic adjustment (Szabados & Savoure, 2010). Pro biosynthesis and signaling contribute to the redox balance of cells under normal and stressful conditions (Per et al., 2017).It seems that Pro has an important role in salinity tolerance by preserving the metabolism and protein synthesis, osmotic balance, protecting cellular enzymes and proteins, protecting intracellular structures, maintaining carbon and nitrogen reserves, and regulating cellular pH. Furthermore, GB and Pro protect the protein structure and cellular membrane integrity under salt stress (Iqbal et al., 2014; Per et al., 2017). It has been well documented that, GB is an osmolyte in plants and bacteria. Nevertheless, recently many studies have indicated its role as a methyl donor in homocysteine metabolism and a protein stabilizer (Figueroa-Soto & Valenzuela-Soto, 2018). It has also been demonstrated that, Pro and GB have also an important role in the removal of free radical by activating antioxidant defense systems (Banu et al., 2010).

The GB content has been increased in many crop plants such as spinach (Parida & Das, 2005), barley (Chen et al., 2007), wheat (Wang et al., 2010), and sorghum (Forghani et al., 2018; Neto et al., 2009) in response to various stresses. However, all plants are not capable to produce enough amounts of GB under abiotic stress. There is extensive evidence suggesting the positive effects of exogenous application of GB on plant growth and crop yield under salt stress (Ashraf & Foolad, 2007; Figueroa-Soto & Valenzuela-Soto, 2018). One of the key enzymes in Pro biosynthesis pathway is 1-pyrroline-5-carboxylate synthase (*P5CS*). This enzyme phosphorylates glutamate to form glutamyl phosphate, which is reduced to an intermediate glutamic-5-semialdehyde. It has been documented that, accumulated Pro by overexpressing the *P5CS* gene can improve salt tolerance in plants under salt stress (Kishor et al., 1995). Although there are several reports about the positive effect of GB and Pro on growth and induction of antioxidant defense systems of plants under salt stress, the effect of GB on salinity tolerance of T plants overexpressing *P5CS* gene under salinity is still unknown. Therefore, it was hypothesized that, increasing proline in transgenic tobacco plants and application of glycine betaine in the medium may offer positive effects on the response of tobacco plants under salinity stress? Accordingly, the present study investigated the protective effect of GB on the antioxidant defense systems of transgenic (T) tobacco plants overexpressing *P5CS* gene to determine the relationship between osmoprotectant properties of GB and its antioxidant capacity under salt stress.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS AND TREATMENTS

The transgenic *Nicotiana tabacum*eeds cv. Wisconsin (T) carrying *P5CS* gene and non-transgenic seeds (NT) were supplied from Laboratory of Plant Physiology, Univerity of Isfahan, Iran. Our previous study confirmed that, regenerated plants from transgenic seeds produced more proline than wild type seeds (Razavizadeh & Ehsanpour, 2009). The seeds were surface sterilized in ethanol 70 % and were then grown on MS medium (Murashige & Skoog, 1962) and kept in the growth room (16/8 h light and dark respectively, with approximately 40 μmol photon $m^{-2}s^{-1}$ light density) at 25 °C. After 20 days, seedlings (T and non-T) were transferred to MS medium containing 0, 100, and 200 mM NaCl. Then, foliar application of glycine betaine (0, 20, and 40 mg. l^{-1}) was sprayed on top of the seedling with four to six leaves. After 4 weeks, plants were harvested with H_2O_2 contents, malondialdehyde (MDA) level, ascorbate peroxidase (APX), catalase (CAT), and superoxide

dismutase (SOD) activities measured in the leaves. The non-T plants not treated with salt and GB were used as control

2.2 DETERMINATION OF H_2O_2 CONCENTRATION

The H_2O_2 content was measured according to the method of Velikova et al. (2000). Fresh leaves (200 mg) were homogenized in trichloroacetic acid (TCA) 0.1 % (w/v) on an ice bath. The homogenates were then centrifuged at 10000 × g for 15 min at 4 °C. Then, 0.5 ml of the supernatant was added to 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide. The absorbance was recorded at 390 nm using a spectrophotometer (Shimadzu UV-160, Japan). The H_2O_2 content was quantified by a calibration curve using H_2O_2 solutions.

2.3 DETERMINATION OF LIPID PEROXIDATION

Fresh leaf samples (200 mg) were homogenized with 5ml of TCA0.1 % (w/v). They were then centrifuged at 10000 × g for 5 min and supernatant was mixed with 0.5 % thiobarbituric acid (TBA) in TCA 20 % (w/v). Next, the samples were heated at 95 C for 30 min in a water bath, and the reaction was stopped in an ice bath. The absorbance of the extract was measured at 532 and 600 nm using a spectrophotometer. The concentration of MDA was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$and expressed as nmol MDA g^{-1} fresh mass (Heath & Packer, 1968).

2.4 ENZYME EXTRACTION

Approximately, 100 mg of fresh leaves was homogenized with 1 ml of 100 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA, 4 mM dithiothreitol (DTT), polyvinylpyrrolidone (PVP)1 % (w/v). The homogenate was centrifuged at 14000 rpm at 4 °C for 20 min. The supernatants were then used for protein and enzyme activity assays. Protein concentration was determined by Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.5 SUPEROXIDE DISMUTASE ACTIVITY (SOD, EC 1.15.1.1)

Total SOD activity was measured using nitro blue tetrazolium (NBT) method with some modifications (Beauchamp & Fridovich, 1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM nitro blue tetrazolium, 2 µM riboflavin, 0.1 mM EDTA, and 50 µl enzyme extract. The samples were shaken and exposed under light intensity (5000 lux) at 25 °C for 30 min, after which they were transferred to the dark room. Absorbance was measured by spectrophotometer at 560 nm. The activity of SOD was recorded as NBT reduction in light compared with the samples in the dark. One unit of SOD activity refers to the amount of protein required to inhibit 50 % of initial reduction of NBT under light.

2.6 CATALASE ACTIVITY (CAT, EC 1.11.1.6)

The CAT activity was determined by 1 ml of the reaction mixture (50 mM potassium phosphate buffer (pH 7), 10 mM H_2O_2, and 0.09 ml of the enzyme extract). The decrease in the absorbance of H_2O_2 was recorded at 240 nm for 1 min. The CAT activity was calculated using the coefficient of absorbance of 0.0394 mM^{-1} cm^{-1} (Aebi, 1984).

2.7 ASCORBATE PEROXIDASE ACTIVITY (APX)

The reaction mixture (1 ml) for the APX activity contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM EDTA, 0.2 mM H_2O_2, and 50 µl of the enzyme extract. The activity was recorded by decreasing the absorbance at 290 nm for 1 min (extinction coefficient 2.8 $mM^{-1}cm^{-1}$) (Nakano & Asada, 1987).

2.8 STATISTICAL ANALYSIS

All experiments were performed with three replicates per treatment. Analysis of the data was carried out by three-way ANOVA and mean data were compared using Duncan's test at the level of $p \leq 0.05$. SPSS software (version 21) was utilized for statistical analysis of the data and the results were expressed as the mean ± standard deviation (SD).

3 RESULTS AND DISCUSSION

3.1 H_2O_2 CONTENT AND LIPID PEROXIDATION

Analysis of variance showed that salt, GB and plant effect were significant on H_2O_2 and MDA content (Table1). Also, there was a significant interaction in two-way analysis between salt and GB on H_2O_2 and MDA content. Furthermore, the two-way interaction between salt and plant were significant for H_2O_2 and MDA. The two-way interaction between GB and lines as well as three-way interaction of salt × GB× lines were only significant for MDA content. The H_2O_2 content of T and non-T plants was increased in response to salinity. The results indicated that GB treatment significantly reduced H_2O_2 content in both T and NT plants under salt stress (Fig.1). Notably, the H_2O_2 content in T plants either with or without GB was significantly lower than in non-T plants in the medium with similar NaCl concentration. Specifically, the H_2O_2 content of T plants treated with 40 mg l^{-1} GB and 0, 100, and 200 mM NaCl was 12, 25, and 13 % lower than in NT plants with the similar treatment of GB and salinity respectively. Therefore, the positive impact of GB for decreasing the H_2O_2 content in T plants was higher than in non-T plants.

Based on the data illustrated in Fig. 2, the MDA content was elevated by increasing the salinity in T and NT plants. The results showed that application of GB significantly reduced lipid peroxidation under salt stress in both types of plants especially in 200 mM salt. Hence, the MDA content in NT plants treated with 200 mM NaCl and 40 mg l^{-1}GB was reduced by about 45 % compared to NT plants treated only with 200 mM NaCl. Furthermore, the MDA content of T plants was lower than that of non-T plant under salt stress. Indeed, the MDA content of T plants treated with 100 and 200 mM salt was 29 and 28% lower than that of non-T plants treated with similar concentrations of NaCl, respectively. The major changes in biochemical and physiological processes such as antioxidant capacity under salt stress trigger generation of ROS, tissue destruction, and oxidative damages. It has been proposed that the MDA content is a reliable indicator for extended oxidative damage. Indeed, an increase in MDA content is correlated with intensified oxidative damage caused by biotic and abiotic stress such as salinity (Garg & Manchanda, 2009). Therefore, accumulation of H_2O_2, which is toxic for the cell, has been suggested as an indicator of oxidative stress (Hasanuzzaman et al., 2011). According to previous reports (Seckin et al., 2009), an increase in both MDA and H_2O_2 content was observed in plants in the research under salt stress. Similar to our findings, the reduction of MDA and H_2O_2 contents were also observed in some plants using GB(El-Samad et al., 2011; Nawaz & Ashraf, 2007; Yamada et al., 2009). It was observed that compatible solutes such as Pro and GB had an important role in osmotic adjustment and protection of proteins, mitochondria, and chloroplast membranes under salt stress (Dawood, 2016; Takabe et al., 2006). Therefore, modification of negative effects of salinity by GB might be due to its protective role and antioxidant capacity against oxidative stress (Park et al., 2004). Further, we found that overexpression of *P5CS* gene and consequently Pro overproduction had a remarkable role in scavenging free radicals and lipid peroxidation under salinity. Our findings indicated that exogenous GB reduced the negative effect of salt stress by decreasing of MDA and H_2O_2 content and improved antioxidant defense system in transgenic tobacco plants.

3.2 ANTIOXIDANT ENZYMES

Analysis of variance showed that salt, GB and lines effect were significant on SOD and APX activity. Also, salt and GB effect were significant on CAT activ-

Table 1: Analysis of variance (mean squares) for the main and interaction effects of salt, GB and line on H_2O_2, MDA, SOD, CAT and APX (* Significant at the 0.05 probability levels. ** Significant at the 0.01 probability levels. ns = not significant)

Source	df	H_2O_2	MDA	SOD	CAT	APX
Salt	2	168.403**	156.448**	.472**	612.870**	.007**
GB	2	103.218**	99.401**	.049**	86.484**	.0003**
line	1	161.317**	75.219**	.032**	450^{ns}	.0003**
Salt * GB	4	11.681**	24.267**	.007**	23.552**	$.00013^{ns}$
Salt * line	2	15.934**	27.015**	.007**	3.45^{n}	.00012**
GB * line	2	$.996^{ns}$	25.964**	$.001^{ns}$	7.190^{ns}	$.000012^{ns}$
Salt * GB * line	4	2.335^{ns}	6.970*	$.00032^{ns}$	2.827^{ns}	$.000002^{ns}$
Error	36	1.370	2.289	.001	2.621	.00001

ity. Moreover, there was a significant two-way interaction between salt and GB on SOD and CAT activity. Furthermore, the two-way interaction between salt and lines were significant for SOD and APX (Table1). The SOD activity of T and NT plants was enhanced by salt stress (Fig.3). Although the application of GB significantly improved SOD activity in both type of plants, the SOD activity of T plants either with or without GB was significantly higher than that of NT plants under salt stress. For instance, the SOD activity of T plants treated with 200 mM NaCl and 40 mg l^{-1} GB grew by 4 times in T plant, while NT plants treated with the same concentration of salt and GB increased 3 times compared to the control. Like SOD activity, the CAT activity of both types of plants was enhanced in response to salinity (Fig.4). When 200 mM NaCl was added to the medium, CAT activity of NT plants treated with 20 and 40 mg l^{-1} GB rose by 44 and 20 % compared to NT plants without GB respectively. On the other hand, only T plants treated with 20 mg l^{-1} GB and 200 mM salt improved CAT activity compared to T plants treated with same salinity and 0 mg l^{-1} GB. Notably, the level of CAT activity in T plants treated only with 200 mM NaCl was higher than in NT plants. Elevation of NaCl concentration enhanced APX activity in both types of plants (Fig. 5). Our results indicated that GB application improved APX activity in NT and T plants under salt stress. Meanwhile, the APX activity in T plants either with or without GB was higher than in NT plants under salinity.

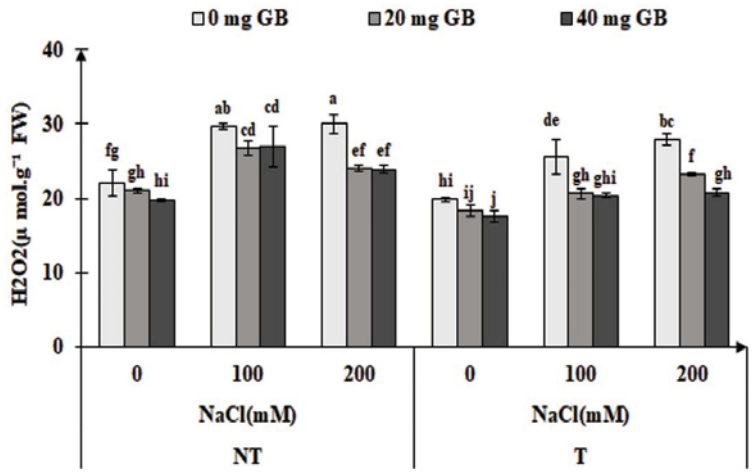

Figure 1: The effect of GB treatment on the H_2O_2 content of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

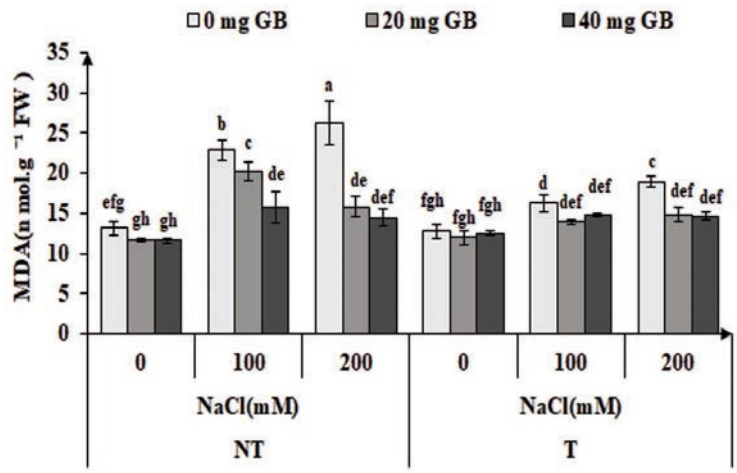

Figure 2: The effect of GB treatment on MDA content of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

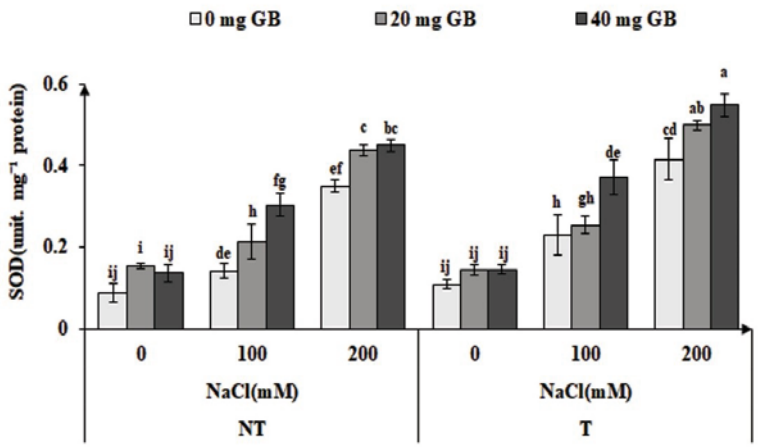

Figure 3: The effect of GB treatment on SOD activity of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

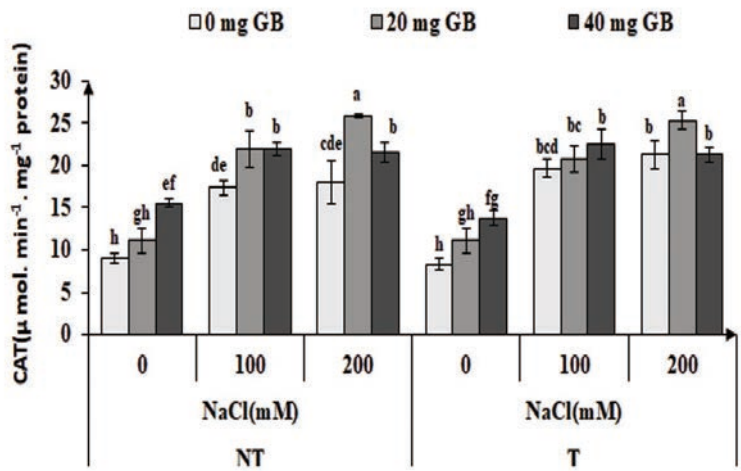

Figure 4: The effect of GB treatment on CAT activity of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

Under salinity stress, balance between production and destruction of ROS is crucial for plant survival. Generation of ROS induced by salt stress is a common response in plants (Fahad et al., 2015). Subsequently, an increase in the activity of many antioxidant enzymes such as SOD, CAT, and APX were supposed to be activated for detoxification of ROS in many plant species (Fahad et al., 2015; Hasanuzzaman et al., 2014). The first enzyme in detoxification pathway is SOD. This enzyme converts superoxide molecules to H_2O_2 (Foyer & Noctor, 2003). The activity of SOD was enhanced by GB in the present study as also reported in rice under salt stress (Raza et al., 2007). Hasanuzzaman et al. (2014) showed that SOD activity grew considerably by GB under salt stress. Then, H_2O_2 as a product of SOD is converted to H_2O and O_2 by CAT and APX enzymes (Gossett et al., 1996). Therefore, the high activity of CAT and APX enzymes in transgenic and non-transgenic tobacco plants treated with GB might be due to the high amount of H_2O_2 produced by SOD under salinity. It is important to note that APX has high affinity for the substrate (H_2O_2), while CAT is known an enzyme with lower affinity for H_2O_2. Thus, CAT is suggested to be involved in mass scavenging of H_2O_2 (Abogadallah, 2010) as we observed in our study. Accordingly, the main H_2O_2 scavenger in plants treated by GB was CAT. Indeed, the high activity of SOD is correlated with high activity of CAT in plants treated with GB. These results were in accordance with the findings obtained by Nounjan et al. (2012) on rice seedlings. It has been suggested that GB might be in-

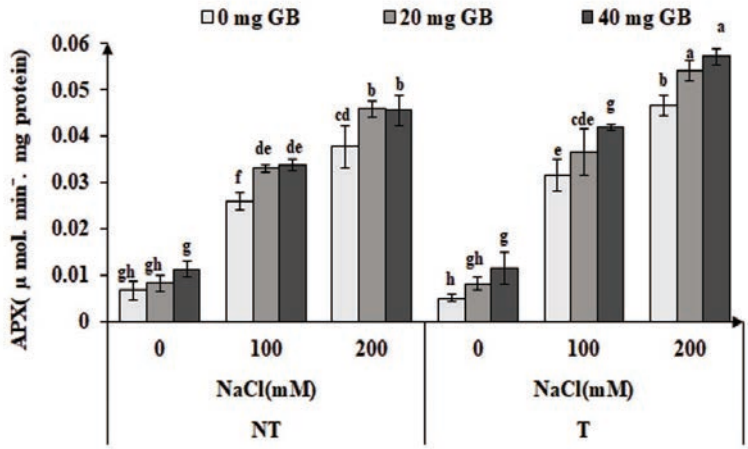

Figure 5: The effect of GB treatment on APX activity of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

volved indirectly in the regulation of gene expression (Al Hassan et al., 2015; Ben Ahmed et al., 2010; Nounjan et al., 2012).

Different functions have been considered for Pro such as protection of enzymes and proteins as well as antioxidant for ROS scavenging (Bellinger, 1987). In accordance with the function of Pro, our findings clearly suggested that transgenic tobacco plants treated by GB maybe had greater scavenging power under salt stress. The previous study on transgenic tobacco plants with overexpression *P5CS* gene indicated improved activity of antioxidant enzymes and proline content (Razavizadeh & Ehsanpour, 2009).

4 CONCLUSION

Although some evidences indicated that use of GB has not been effective to improve salt tolerance in plants (Figueroa-Soto & Valenzuela-Soto, 2018), the present data revealed transgenic (T) plants overexpressing *P5CS* gene treated with GB reduced H_2O_2 and MDA (in particular 100 mM NaCl) more than non-transgene plant (NT) suggesting better adaptation to salinity could be related to proline production due to overexpressing *P5CS* gene and enhanced levels of proline biosynthesis. Moreover, the synergistic effects of glycine betaine and proline in tobacco plants enhanced antioxidant defense system and resulted in increasing salt tolerance of tobacco plants overexpressing *P5CS* gene.

Acknowledgment: Authors thanks University of Isfahan and Plant Antioxidant Center of Excellence (PACE).

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