Physiol Mol Biol Plants (March–April 2018) 24(2):335–341
https://doi.org/10.1007/s12298-017-0498-8

SHORT COMMUNICATION

# Antioxidant potential in *Stevia rebaudiana* callus in response to polyethylene glycol, paclobutrazol and gibberellin treatments

**Shokoofeh Hajihashemi**[1] · **Shakiba Rajabpoor**[2] · **Ivica Djalovic**[3]

Received: 12 July 2017 / Revised: 13 November 2017 / Accepted: 22 December 2017 / Published online: 17 January 2018


**Abstract** The study on *Stevia* callus has the potential to advance the knowledge of antioxidant mechanisms involved in unorganized cells response to drought stress. The effects of polyethylene glycol (PEG; 0 and 4% w/v) in combination with paclobutrazol (PBZ; 0 and 2 mg $l^{-1}$) and gibberellin (GA; 0 and 2 mg $l^{-1}$) were studied on *Stevia rebaudiana* callus. PEG treatment led to an oxidative stress, as indicated by increased $H_2O_2$ content whose accumulation was prevented with PBZ and GA treatments. All treatments of PEG, PBZ and GA increased the total antioxidant capacity, with the highest antioxidant power in PBZ and GA treatments without PEG. The activity of superoxide dismutase, catalase and ascorbate peroxidase significantly increased in PEG treatment alone or in combination with PBZ and GA. All treatments of PEG, PBZ and GA significantly increased proteins, amino acids and proline contents, with the highest increase in presence of PBZ in medium culture. In contrary to proline, the activity of pyrroline-5-carboxylate synthetase and proline dehydrogenase did not change in response to any of the treatments. Collectively, our results demonstrated that PBZ and GA increased reactive oxygen species scavenging and osmolytes in PEG-treated calli more than PEG treatment alone to alleviate negative effects of PEG on *Stevia* calli. These findings will enable us to design effective genetic engineering strategies in callus culture to generate some somaclonal variation that may be useful in enhancing drought resistance in *Stevia*.

**Keywords** Callus · Gibberellin · Paclobutrazol · Polyethylene glycol · *Stevia rebaudiana*

**Abbreviations**

| | |
|---|---|
| APX | Ascorbate peroxidase |
| CAT | Catalase |
| FRAP | Ferric reducing antioxidant power |
| $H_2O_2$ | Hydrogen peroxide |
| KO | Kaurene oxidase |
| KS | Kaurene synthase |
| MS | Murashige and Skoog |
| P5CS | Pyrroline-5-carboxylate synthetase |
| PDH | Proline dehydrogenase |
| PEG | Polyethylene glycol |
| ROS | Reactive oxygen species |
| SVglys | Steviol glycosides |
| SOD | Superoxide dismutase |
| SV | Steviol |

## Introduction

Water scarcity is one of the main limiting factors in crop cultivation worldwide (Anjum et al. 2012). The drought causes both osmotic stress, by reducing water availability and enhancing turgor pressure, and oxidative stress due to

✉ Shokoofeh Hajihashemi
hajihashemi@bkatu.ac.ir

Shakiba Rajabpoor
shakiba_rajabpoor@yahoo.com

Ivica Djalovic
ivica.djalovic@ifvcns.ns.ac.rs

[1] Plant Biology Department, Faculty of Science, Behbahan Khatam Alanbia University of Technology, Behbahan, Khuzestan, Iran

[2] Department of Biology, Payame Noor University, PO Box 19395-3697, Tehran, Iran

[3] Department for Maize, Institute of Field and Vegetable Crops, M. Gorki 30, 21000 Novi Sad, Serbia

high accumulation of reactive oxygen species (ROS). ROS reaction with lipid membranes, proteins and DNA results in cell damage, followed by cell death (Torres-Franklin et al. 2008). Enhancement of antioxidant defense system is an important strategy to scavenge ROS with enzymatic and non-enzymatic antioxidants (Gupta and Prakash 2009; Anjum et al. 2012). In enzymatic system, superoxide dismutase (SOD) catalyze the dismutation of $O^{2-}$ into $H_2O_2$, whereas peroxidase, catalase (CAT) and ascorbate peroxidase (APX) decompose $H_2O_2$ to $H_2O$ at different cellular locations (Noctor and Foyer 1998). In response to osmotic stress, the accumulation of compatible solutes such as proline, proteins and amino acids protects the cell by balancing the osmotic pressure of cytosol, which prevents some injuries caused by water deficiency (Anjum et al. 2012).

*Stevia rebaudiana* L. is a valuable medicinal plant originated from Paraguay with a subtropical climate and average rainfall of 1500 mm per year (Hajihashemi and Ehsanpour 2013). *Stevia* has a huge demand in industry of food and beverage as a source of high potency natural sweetener with low calorie. Due to increasing the demand for sweet herb of *Stevia*, its cultivation has been spread in several countries worldwide, including Iran. *Stevia* cultivation in Iran with a hot and dry weather faces some problems associated with drought stress. Previous studies have shown that *S. rebaudiana* was not resistant to water stress under in vitro and greenhouse conditions (Hajihashemi and Ehsanpour 2013; Hajihashemi and Geuns 2016; Hajihashemi and Ehsanpour 2014). Hence, the most practical approach for increasing *Stevia* yield in drought condition is to study on features involved in *Stevia* cell response to drought stress.

Steviol glycosides (SVglys) are sweet compounds mainly biosynthesized in *S. rebaudiana* leaves. Besides their sweet taste, the antioxidant activity has been reported for these molecules (Hajihashemi and Geuns 2013). In SVglys biosynthesis pathway, four consecutive enzymes convert geranylgeranyl-diphosphate into steviol molecule. Then, the glycosyltransferase enzymes produce different SVglys by glycosylation of steviol (Brandle and Telmer 2007). Swanson et al. (1992) reported that callus of *Stevia* is not capable to biosynthesise steviol (SV) and suggested that SVglys biosynthesis is function of tissue differentiation, therefore these sweet compounds can be found mainly in leaves. Gibberellic acid (GA) and SV have similar tetracyclic diterpene skeletons with a common precursor for both of them. The enzymes of kaurene oxidase (KO) and kaurene synthase (KS) involve in biosynthesis of both GA and SV molecules (Brandle and Telmer 2007). It is reported that GA treatment significantly increased the transcription of *ent-KS1*, *ent-KO* in *S. rebaudiana*, while the GA biosynthesis inhibitor, paclobutrazol (PBZ) reduced their transcriptions (Hajihashemi and Geuns 2016; Hajihashemi et al. 2013).

Hence, high crop production under unfavorable growth condition is one major target for researches in 21$^{th}$ century, the knowledge of cell response to drought stress could be used to create new varieties of crops with higher yield under water deficit. Our previous study on *Stevia* plants showed that PEG-induced drought stress had negative effect on SVglys biosynthesis, plant growth and yield (Hajihashemi and Ehsanpour 2013; Hajihashemi and Geuns 2016). Therefore, we decided to investigate callus of *Stevia* to better understand unorganized cell responses to water deficit. In order to identify traits that can decrease the cell sensitivity to dehydration; analysis of antioxidant capacity and osmolytes of callus exposed to PEG-induced drought stress seems to be a promising approach. In order to evaluate antioxidant system and osmolytes, the enzymes SOD, CAT and APX, total antioxidant capacity (FRAP), $H_2O_2$, proteins, proline, total amino acids and activities of proline metabolizing enzymes (pyrroline-5-carboxylate synthetase, proline dehydrogenase) were studied. Study on *Stevia* showed that PBZ and GA treatments could partly alleviate negative effect of PEG treatment on plant (Hajihashemi and Ehsanpour 2013, 2014; Hajihashemi and Geuns 2016). In a follow-up study, the interaction of PBZ or GA with PEG treatments was examined in which we figured out if these treatments can reduce negative effects of PEG-induced drought stress in callus. A comprehensive examination of the antioxidants mechanisms underlying in *Stevia* callus in response to interaction of PEG with GA or PBZ allowed the characterization of some antioxidant factors modulating the negative effects of drought stress and provided evidence that can be used in crop improvement.

## Materials and methods

### Callus culture

*S. rebaudiana* (Bert.) seeds were provided from Prairie Oak Publishing, 221 South Saunders Street, Marville MO 64468. The seeds were cultured in MS (Murashige and Skoog 1962) medium. Then, plants were propagated in vitro. The leaves of *Stevia* accumulate up to 30% of SVglys (Geuns 2003) so the plant leaves were used to generate callus. The plants leaves were cut in 1.0 $cm^2$ pieces. In a preliminary experiment, the explants were placed in MS medium containing 2, 4 D and kinetin (each at 0–5 mg $l^{-1}$). Then, according to the callus weight analysis (data not shown), we decided to use MS medium containing 2, 4 D and kinetin (each at 2 mg $l^{-1}$), as callus medium. According to previous studies on *Stevia* callus

(Gupta et al. 2015; Hajihashemi and Ehsanpour 2013, 2014), some experiments were done on effects of different concentrations of PEG (molecular weight 6000; 0 to 6% w/v) and PBZ (0–4 mg $l^{-1}$) on callus weight (data not shown). The results showed that 4% of PEG and 2 mg $l^{-1}$ of PBZ were the most effective treatments, and GA was used at the same concentration to PBZ. Then, the emerged calli with 1.0 g weight were transferred to callus medium containing PEG (0 and 4% w/v), PBZ (0 and 2 mg $l^{-1}$) and GA (0 and 2 mg $l^{-1}$). In this experiment, there were 8 treatments including: control, PBZ, GA, GA + PBZ, PEG, PEG + PBZ, PEG + GA and PEG + PBZ + GA. All cultures were incubated at 26 ± 1 °C under 2500 lx light under a 16/8 h photoperiod for one month. The treated calli were used for further analysis.

### Total antioxidant capacity (FRAP assay)

One gram of callus was homogenized in 10 ml phosphate buffer (0.1 M; pH 7.6) described by Hajihashemi and Ehsanpour (2014). The callus extract was used to determine the antioxidant power (FRAP) of samples according to Szôllôsi and Varga (2002).

### $H_2O_2$ content measurement

The fresh calli (0.25 g) were extracted with trichloroacetic acid (2.5 ml; 0.1% w/v) and centrifuged at 10,000 rpm for 15 min (Hajihashemi and Ehsanpour 2014). Then, the supernatants were used to measure the $H_2O_2$ content using Velikova et al. (2000) method.

### Protein and antioxidant enzymes measurement

The calli samples were extracted using 50 mM sodium phosphate buffer (pH 7.8) including 1.0 mM EDTA, 4 mM dithiothreitol, 2% w/v polyvinyl polypyrrolidone, and 5 mM magnesium sulfate. The samples were centrifuged at 13,000 rpm at 4 °C for 40 min. They were used for measuring proteins and enzymes activity (Hajihashemi and Ehsanpour 2014). The proteins content was measured according to Bradford (1976) method. The SOD activity assay was based on Beauchamp and Fridovich (1971) method. The CAT activity measurement was done according to Aebi (1984). The APX activity was determined according to Asada (1992).

### Total amino acids, free proline and proline metabolism enzymes

The total amino acids content was determined based on the Yemm et al. (1955) method using the protein extract. Free proline was extracted from 0.5 g of fresh callus using 10 ml of sulfosalicylic acid (3% w/v) and determined according to Bates et al. (1973) method. The enzyme extract was used to determine pyrroline-5-carboxylate synthetase (P5CS; EC 1.2.1.41) activity by Stines et al. (1999) method. The proline dehydrogenase (PDH; EC 1.5.1.2) activity was assayed according to Rena and Splittstoesser (1975) method.

### Statistical analysis

The experimental set-up was designed based on a Randomized Block Designs with 3 biological replicates and each replication comprised up to 12 jars. The data were analyzed by the ANOVA test's SPSS (version 16) statistical package to assess significant differences (at the 5% level) between means. The statistical analysis results are shown by superscripted letters to reveal significant differences.

## Results

The ferric reducing antioxidant power (FRAP) method was used to measure the total antioxidant capacity. The results showed a significant increase in total antioxidant capacity of PBZ, GA and PEG-treated calli (Fig. 1A). FRAP in PBZ and GA treatments was more than PEG (with or without PBZ and GA) treatment. As the results show, the $H_2O_2$ content was not affected by PBZ and PBZ treatments, while PEG (with or without PBZ and GA) significantly increased it (Fig. 1B). The highest $H_2O_2$ content were observed in PEG-treated callus, by about 2-folds more than control. Figure 2A shows that PBZ, GA and PEG treatments significantly enhanced proteins content in calli. The highest and lowest increases in proteins content were observed in PBZ and PEG treatments, respectively. The activity of three key antioxidant enzymes of SOD, CAT and APX were evaluated in *Stevia* callus. PBZ and GA treatments showed no significant effect on SOD activity, while PEG (with or without PBZ and GA) in MS medium significantly increased SOD activity (Fig. 2B). The SOD activity increased in *Stevia* callus exposed to PEG by about 2.5-folds more than control. Similar to SOD enzyme, PEG (with or without PBZ and GA) treatment significantly increased CAT and APX activity, while no significant changes in their activity was observed in PBZ and GA (without PEG) treatments (Fig. 2C, D).

The results showed that the total amino acids content significantly increased in PBZ, GA and PEG-treated calli (Fig. 3A). The increase in amino acids in PBZ treatment alone or with GA and/or PEG was more than GA and/or PEG treatments without PBZ. In presence of PBZ in the

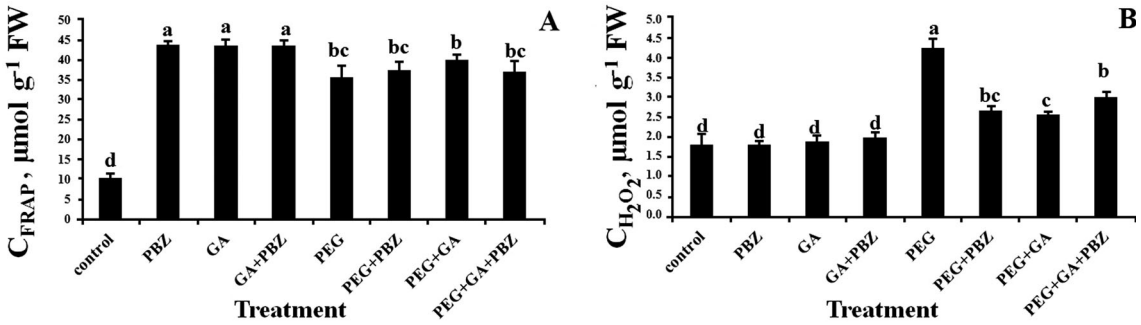


**Fig. 1** The effect of (PEG; 0 and 4% w/v), paclobutrazol (PBZ; 0 and 2 mg $l^{-1}$) and gibberellin (GA; 0 and 2 mg $l^{-1}$) treatments on ferric reducing antioxidant power (FRAP; **A**) and $H_2O_2$ (**B**) in *S. rebaudiana* callus. Values are expressed as mean ± SEM of three independent experiments. Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's test at $p < 0.05$

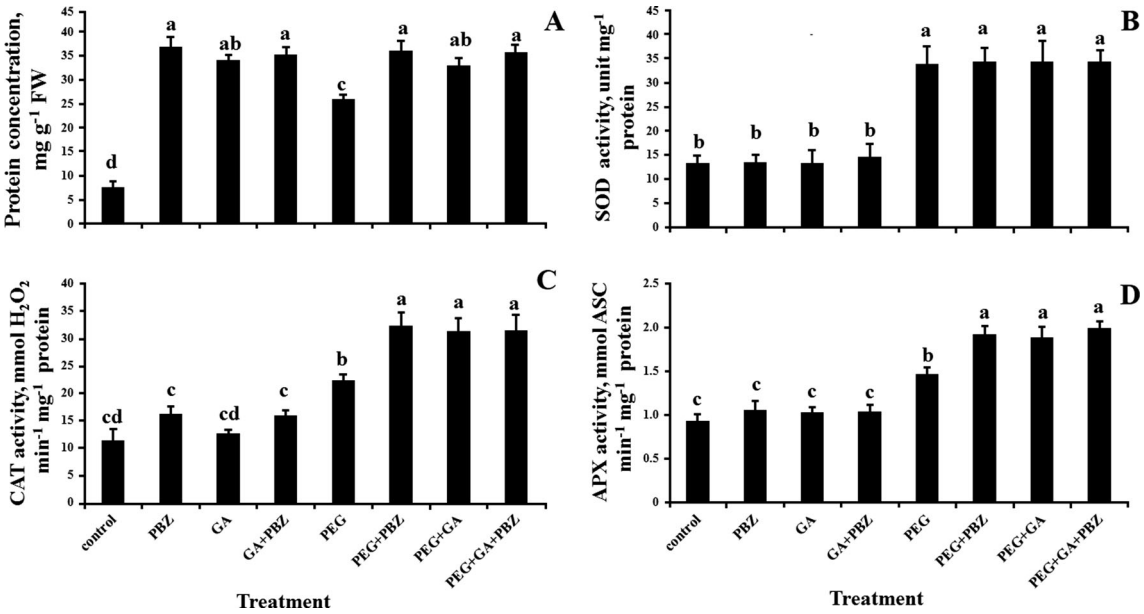


**Fig. 2** The effect of (PEG; 0 and 4% w/v), paclobutrazol (PBZ; 0 and 2 mg $l^{-1}$) and gibberellin (GA; 0 and 2 mg $l^{-1}$) treatments on the proteins content (**A**), activity of superoxide dismutase (SOD; **B**), catalase (CAT; **C**), ascorbate peroxidase (APX; **D**) in *S. rebaudiana* callus. Values are expressed as mean ± SEM of three independent experiments. Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's test at $p < 0.05$

medium culture, the total amino acids content was almost 5-folds more than control. Proline accumulation in PBZ, GA and PEG-treated calli was significantly more than control callus (Fig. 3B). The data showed that the effect of PBZ treatment on the proline content was similar to the total amino acids content, with the highest accumulation in presence of PBZ in MS medium. As shown in Fig. 3C, D, PBZ, GA and PEG treatments had no significant effect on the activity of P5CS and PDH enzymes which are involved in proline metabolism.

## Discussion

The first objective of present study was to evaluate the effects of PEG-induced drought stress on the leaves-derived callus of *S. rebaudiana*. The second aim of this study was to understand if PBZ (a GA biosynthesis inhibitor) and GA treatments could reduce negative effects of PEG on *Stevia* calli. Our previous study showed that PBZ treatment reduced the negative effects of PEG on physiology and growth of *Stevia* plants but it could not reduce negative effect of PEG on the transcription of SVglys biosynthetic genes and SVglys content. In contrast to PBZ, GA treatment reduced the adverse effects of PEG on the transcription of SVglys biosynthetic genes and SVglys

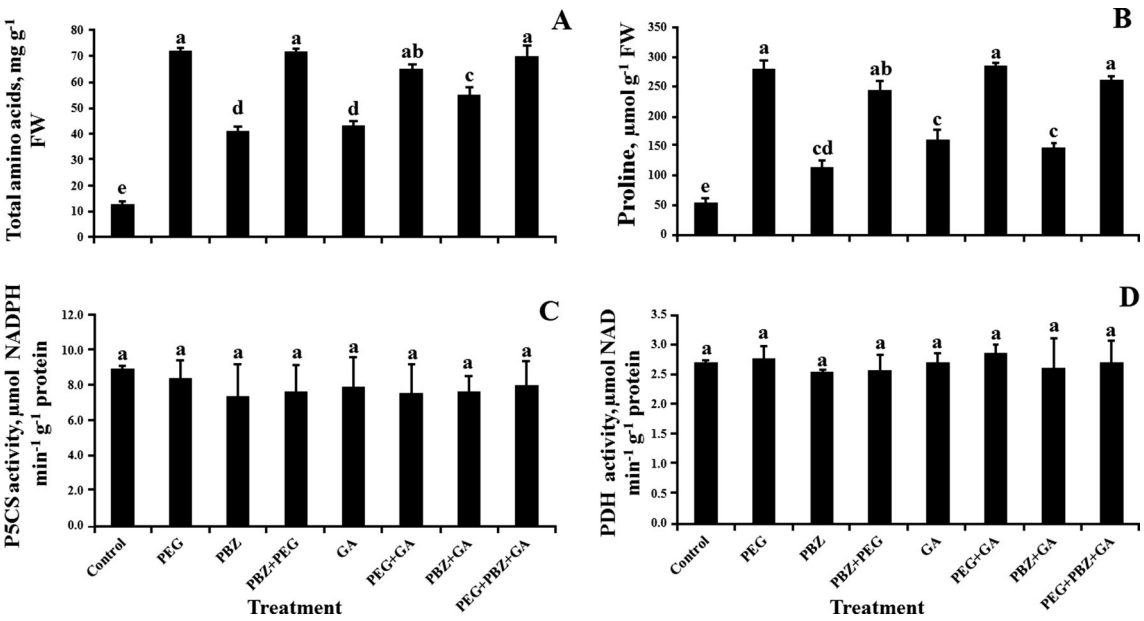


**Fig. 3** The effect of (PEG; 0 and 4% w/v), paclobutrazol (PBZ; 0 and 2 mg $l^{-1}$) and gibberellin (GA; 0 and 2 mg $l^{-1}$) treatments on the total amino acids (**A**), proline (**B**), pyrroline-5-carboxylate synthetase (P5CS; **C**), proline dehydrogenase (PDH; **D**) in *S. rebaudiana* callus. Values are expressed as mean ± SEM of three independent experiments. Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's test at $p < 0.05$

content in *Stevia* plant (Hajihashemi and Ehsanpour 2013, 2014; Hajihashemi et al. 2013). Therefore, it became imperative to study the effect of GA or PBZ treatments and their interaction with PEG on calli of *Stevia* without SVglys biosynthesis capacity.

The results demonstrate that unorganized cell masses of *S. rebaudiana* callus accumulated high amount of $H_2O_2$ in response to PEG which supports our previous finding in *Stevia* plant (Hajihashemi and Ehsanpour 2013). PBZ and GA treatments reduced $H_2O_2$ accumulation in PEG-treated calli which confirms results of PBZ and PEG interaction in *Stevia* plant (Hajihashemi and Ehsanpour 2014). Water stress is associated with increased oxidative stress due to accumulation of ROS, particularly $H_2O_2$ in chloroplasts, mitochondria, and peroxisomes. In order to overcome the oxidative stress, plants employ strategy of antioxidant enzyme activities induction (Foyer and Noctor 2003). PEG provoked a severe drought stress in *Stevia* plant that could partly be restored by antioxidant capacity associated with active enzymatic and non-enzymatic defense systems (Hajihashemi and Ehsanpour 2014). Non-enzymatic antioxidants of ST and Reb A showed very potent radical scavenging activity towards both ROS and reactive nitrogen species (Hajihashemi and Geuns 2013). Hence, antioxidant mechanism in PEG-treated calli (in absence of SVglys) maybe different from *Stevia* plant. The total antioxidant activity of calli (FRAP) significantly increased in PBZ, GA and PEG treatments and their interactions. On contrary to callus results, PBZ treatment did not affect FRAP in *Stevia* plant (Hajihashemi and Ehsanpour 2013). It shows a variation between antioxidants capacity of *Stevia* calli and plants.

Results revealed a positive correlation between $H_2O_2$ and SOD activity in the PEG-treated calli which was not found out in *Stevia* plants (Hajihashemi and Ehsanpour 2014). $H_2O_2$, which resulted from the action of SOD, is toxic to cells. Therefore, it is important to scavenge $H_2O_2$ rapidly by the antioxidative defense system to water and oxygen (Guo et al. 2006). It should be mentioned that SOD activity in PEG, PEG + PBZ, PEG + GA and PEG + PBZ + GA treatments was almost the same while $H_2O_2$ contents in PEG + PBZ, PEG + GA and PEG + PBZ + GA treatments is significantly less than PEG treatment. It suggests that all of these treatments produce the same amounts of $H_2O_2$ whereas PBZ and GA treatments activated some ROS scavengers to reduced oxidative stress. The overexpression of SOD, if accompanied by enhanced $H_2O_2$ scavenging mechanisms like CAT enzyme, has been considered as an important anti-drought mechanism to cope with oxidative stress during water deficit conditions (McKersie et al. 1999). Interestingly, CAT and APX played an important role in $H_2O_2$ detoxification in calli which was followed by less accumulation of $H_2O_2$ in PEG, PEG + PBZ, PEG + GA and PEG + PBZ + GA treatments, comparing to PEG treatment. Also, the results of present study showed that there is no correlation between FRAP with antioxidant enzymes of SOD, APX and CAT. Tadhani et al. (2007) found a linear

correlation between FRAP with flavonoids and phenols, which confirms our previous study on PEG treatment in *Stevia* plant (Hajihashemi and Ehsanpour 2014). However, PBZ-treatment showed higher FRAP in callus than PEG treatment, which can be due to an increase in some other antioxidants (e.g. ascorbate pool and α-tocopherol; Hajihashemi 2017), besides phenols and flavonoids. Interestingly, in GA-treated *Stevia* plants, the increased FRAP was not due to flavonoids and phenols because they have not shown any significant changes in response to GA treatment. Therefore, it can be concluded that different enzymatic and non-enzymatic antioxidants are responsible for total antioxidant capacity in *Stevia*, and further studies on antioxidants is suggested.

A common adverse effect of drought stress is ROS production which reacts with proteins, resulting in protein denaturation (Torres-Franklin et al. 2008). Improvement in protein accumulation in response to drought stress not only helped in maintaining tissue water status but also protected from drought induced ROS (Anjum et al. 2012). According to the results of present study, PEG treatment alone increased proteins of calli, while PBZ and/or GA treatments significantly increased proteins accumulation in PEG-treated plants. Therefore, higher protein accumulation in PBZ and GA in combination of PEG is a promising approach to increase drought resistance in calli. According to obtained results in present study, no correlation was detected between protein contents and activity of APX, SOD and CAT enzymes, which confirms our previous studies on *Stevia* plants (Hajihashemi and Ehsanpour 2013, 2014). More study on proteins is a promising approach to better understand the responsible mechanism that can be used in biotechnology to produce drought resistance cells.

The osmotic adjustment under drought stress can be achieved by accumulation of amino acids, proline, proteins, and other solutes in the cytoplasm improving water uptake from drying soil (Anjum et al. 2012). The total amino acids and proline contents significantly increased in PEG, PBZ and GA-treated calli, with the highest accumulation in PBZ treatment alone or in its interaction with GA or PEG. It is reported that free amino acid accumulation in chickpea is due to protein hydrolysis (Khalil et al. 2014). However, in the present study it cannot be correlated to proteins degradation because proteins content increased under different treatments. Thus, it can be suggested that they serve as an organic nitrogen pool to be used for protein synthesis. Proline accumulation in response to water-deficit preserves the structure of complex proteins, maintains membrane integrity, reduces injury to proteins and cells, oxidation of lipid membranes or photo-inhibition, and increases drought-tolerance (Anjum et al. 2012). P5CS catalyzes proline biosynthesis, while PDH mediates proline oxidation (Raymond and Smirnoff 2002). In our experiment, even though the P5CS and PDH activity were not affected by any of PEG, PBZ and GA treatments, the proline content significantly increased in response to PEG, PBZ and GA treatments. In contrast, there is a report of increasing PDH and P5CS activity in response to water deficit in mulberry, which was followed by increased proline content (Chaitanya et al. 2009). The result of proline accumulation confirms our previous observations in *S. rebaudiana* plants under PEG and PBZ treatments (Hajihashemi and Ehsanpour 2013, 2014) which shows the ability of *Stevia* plants and calli to maintain drought tolerance by proline accumulation. Also, it can be suggested that PBZ treatment is more effective than GA treatment in reducing PEG-induced turgor pressure by higher accumulation of proline and amino acids. The results showed no clear correlation between proline and amino acids with antioxidant system because both GA and PBZ increased FRAP at the same level, while the amino acids and proline contents in PBZ-treated calli were significantly higher than in GA-treated calli. The results of present study combined by an in vitro regeneration of *S. rebaudiana* enable us to produce a large scale of drought tolerance plants for cultivation in field.

In conclusion, PEG-induced drought stress resulted in oxidative stress in callus of *Stevia* by ROS accumulation, supporting the results of PEG treatment in *Stevia* plant. The PBZ and GA treatments increased proteins and the activity of antioxidant enzymes against PEG treatment to remove toxic oxygen radicals. Moreover, the PBZ and GA treatments increased total amino acids and proline in PEG-treated calli to reduce drought-induced turgor pressure, more effectively in PBZ treatment. These results provided information that could be used for producing drought resistance crops from calli using somaclonal variation techniques. To this aim, genome wide analyses are suggested to achieve a comprehensive outlook of the traits involved in antioxidants mechanisms.

**Funding** No fund or grant was received for this project and corresponding author paid the experiments cost.

## References

Aebi H (1984) Catalase in vitro. Method Enzymol 105:121–126

Anjum SA, Farooq M, Xie XY, Liu XJ, Ljaz MF (2012) Antioxidant defense system and proline accumulation enables hot pepper to perform better under drought. Sci Hortic 140:66–73

Asada K (1992) Ascorbate peroxidase—a hydrogen peroxide-scavenging enzyme in plants. Physiol Plant 85(2):235–241

Bates L, Waldren R, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39(1):205–207

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44(1):276–287

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1–2):248–254

Brandle J, Telmer P (2007) Steviol glycoside biosynthesis. Phytochemistry 68(14):1855–1863

Chaitanya KV, Rasineni GK, Reddy AR (2009) Biochemical responses to drought stress in mulberry (*Morus alba* L.): evaluation of proline, glycine betaine and abscisic acid accumulation in five cultivars. Acta Physiol Plant 31(3):437–443

Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant 119(3):355–364

Geuns JMC (2003) Stevioside. Phytochemistry 64:913–921

Guo R, Sun D, Tan Z, Rong D, Li C (2006) Two recessive genes controlling thermophotoperiod-sensitive male sterility in wheat. Theor Appl Genet 112(7):1271–1276

Gupta S, Prakash J (2009) Studies on Indian green leafy vegetables for their antioxidant activity. Plant Food Hum Nutr 64(1):39–45

Gupta P, Sharma S, Saxena S (2015) Biomass yield and steviol glycoside production in callus and suspension culture of *Stevia rebaudiana* treated with proline and polyethylene glycol. Appl Biochem Biotechnol 176:863–874

Hajihashemi S (2017) Physiological, biochemical, antioxidant and growth characterizations of gibberellin and paclobutrazol-treated sweet leaf (*Stevia rebaudiana* B.) herb. J Plant Biochem. https://doi.org/10.1007/s13562-017-0428-4

Hajihashemi S, Ehsanpour AA (2013) Influence of exogenously applied paclobutrazol on some physiological traits and growth of *Stevia rebaudiana* Bertoni under in vitro drought stress. Biologiae 68:414–420

Hajihashemi S, Ehsanpour AA (2014) Antioxidant response of *Stevia rebaudiana* B. to polyethylene glycol and paclobutrazol treatments under in vitro culture. Appl Biochem Biotechnol 172(8):4038–4052

Hajihashemi S, Geuns JM (2013) Free radical scavenging activity of steviol glycosides, steviol glucuronide, hydroxytyrosol, metformin, aspirin and leaf extract of *Stevia rebaudiana*. Free Radic Antioxid 3:S34–S41

Hajihashemi S, Geuns J (2016) Gene transcription and steviol glycoside accumulation in *Stevia rebaudiana* under polyethylene glycol-induced drought stress in greenhouse cultivation. FEBS Open Bio 6(9):937–944

Hajihashemi S, Geuns JM, Ehsanpour AA (2013) Gene transcription of steviol glycoside biosynthesis in *Stevia rebaudiana* Bertoni under polyethylene glycol, paclobutrazol and gibberellic acid treatments in vitro. Acta Physiol Plant 35(6):2009–2014

Khalil SE, Hussein MM, Khalil AM (2014) Interaction effects of different soil moisture levels, arbuscular mycorrhizal fungi and three phosphate levels on: II-mineral ions, protein, aminoacids contents of garden cress (*Lepidium sativum* L.) plant. Int J Adv Res 2(12):263–278

McKersie BD, Bowley SR, Jones KS (1999) Winter survival of transgenic alfalfa overexpressing superoxide dismutase. Plant Physiol 119(3):839–848

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473–497

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Biol 49(1):249–279

Raymond MJ, Smirnoff N (2002) Proline metabolism and transport in maize seedlings at low water potential. Ann Bot 89(7):813–823

Rena AB, Splittstoesser WE (1975) Proline dehydrogenase and pyrroline-5-carboxylate reductase from *Pumpkin cotyledons*. Phytochemistry 14(3):657–661

Stines AP, Naylor DJ, Høj PB, Van Heeswijck R (1999) Proline accumulation in developing grapevine fruit occurs independently of changes in the levels of Δ1-pyrroline-5-carboxylate synthetase mRNA or protein. Plant Physiol 120(3):923

Swanson SM, Mahady GB, Beecher CW (1992) Stevioside biosynthesis by callus, root, shoot and rooted-shoot cultures in vitro. Plant Cell Tissue Organ Cult 28(2):151–157

Szôllôsi R, Varga IS (2002) Total antioxidant power in some species of Labiatae (adaptation of FRAP method). Acta Biol Szegediensis 46(3–4):125–127

Tadhani MB, Patel VH, Subhash R (2007) In vitro antioxidant activities of *Stevia rebaudiana* leaves and callus. J Food Compos Anal 20:323–329

Torres-Franklin ML, Contour-Ansel D, Zuily-Fodil Y, Pham-Thi A-T (2008) Molecular cloning of glutathione reductase cDNAs and analysis of GR gene expression in cowpea and common bean leaves during recovery from moderate drought stress. J Plant Physiol 165(5):514–521

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Sci 151(1):59–66

Yemm E, Cocking E, Ricketts R (1955) The determination of amino-acids with ninhydrin. Analyst 80(948):209–214