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POLYETHYLENE GLYCOL-STIMULATED DROUGHT STRESS ENHANCED THE BIOSYNTHESIS OF STEVIOL GLYCOSIDES IN *STEVIA REBAUDIANA*

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Abstract

Rapid propagation of commercially important species can be achieved through plant tissue culture techniques. It is gaining importance in breeding methods due to its speedy process. Also, various stress conditions can be provided through this process to study the adoptive mechanisms of different plant species to stress. Mild drought stress increases the production of some valuable plant compounds have medicinal importance. *Stevia rabaudiana* is an important medicinal plant. It contains steviol glycoside, which is a natural sweetening agent. It has zero calories and is considered about 300 times sweeter than table sugar. This plant has also famous for its antioxidant activity. Mild stress conditions increase the production of plant secondary metabolites. PEG is a compound used to induce drought stress in culture media under *In vitro* conditions. For this purpose, five levels of PEG-6000, i.e., control, 1%, 2%, 3%, and 4% (w/v), were used in the culture medium. Cultured jars were kept for 45 days, and then different traits were evaluated, such as shoot length, fresh and dry weight of shoot, the total number of leaves per plant, chlorophyll content, phenol, flavonoids, SOD, POD, CAT, APX, MDA, proline, itsantioxidant activity, steviol glycosides, number of roots, and root length. The morphological traits were highly affected by PEG concentration. We found that drought stress improved enzymatic activity, and the level of antioxidant enzymes, including SOD, POD, CAT, and APX, increased to a certain extent, but further increases in the stimulated drought stress declined it. Proline content and MDA level also increased in the drought stressed plants. Similarly, it increased biochemical compounds such as phenols and flavonoids and improved antioxidant activity. Furthermore, it was found that the steviol glycoside contents were also increased by stimulated drought stress. These results suggested that the biosynthesis of steviol glycoside can be enhanced by using stimulated drought stress as an elicitor.

Key words: PEG; Drought stress; Stevia; Steviol glycoside, Biochemical compounds.

Introduction

Plant tissue and organ culture are advanced techniques in plant breeding, improvement, and biosynthetic pathways. It provides a convenient way for rapid multiplication of Stevia to avoid the above-mentioned problems referred to extensive production. This technique also help us to conserve rare medicinal plants species (Debnath *et al*., 2006). Plants are exposed to various types of stresses including biotic and abiotic under field conditions. These stresses adversely affect plant productivity (Aghighi, 2017). Drought is an important abiotic stress, for drought screening of different plant sp. *In vitro* culture techniques are used. Furthermore, it is reported that drought can modify the physiological and biochemical processes of plants and can increase the production of some valuable secondary compounds (Debnath *et al*., 2006). Plants subjected to different stresses and elicitors can increase the accumulation of secondary metabolites. These compounds are very important in the plant life cycle, especially under stress conditions (Yasin *et al*., 2021).

Various stresses such as low/high temperature, salinity, and drought badly influence plant growth and development (Akula & Ravishankar, 2011). To cope up with this type of condition, plants increase the production of various valuable compounds that upon accumulation adjust the plant mechanism and help with the survival of s plant. On the other hand, various types of elicitors are used to induce or enhance the biosynthesis of secondary metabolites. These elicitors might be the biotic or abiotic sources that can stimulate plant stress response (Thakur *et al*., 2019). Currently, the use of elicitors gaining attention to alter the biosynthetic pathway to enhance the production of different secondary metabolites that have medicinal importance. During invitro selection of plants for stress tolerance, different osmotic agents such as mannitol, sucrose, sorbitol, and Polyethylene glycol (PEG) were used. However, the most widely using osmotic agent that stimulates osmotic stress in the plant is PEG. Polyethylene glycol has a higher molecular weight due to which it cannot penetrate and hence reduce the water potential of the nutrient medium without entering into the plant body (Pérez-Clemente & Gómez-Cadenas, 2012).

S. rabaudiana Bertoni is a herbaceous perennial plant. Studies suggested that this plant is originated from

Paraguay and Brazil. Stevia is a very famous member of the Asteraceae family. For long times it had been used for medicinal purposes (Zeng *et al*., 2013). It is used as a medicinal herb by the Guarani tribes of Brazil and Paraguay about 1500 years ago. The most interesting character of *S. rabaudiana* is the presence of the natural sweetening agent known as stevioside. It is a zero-calorie sweetening agent synthesized in leaves. Stevioside is considered about 300 times sweeter than table sugar, while the leaves of *S. rabaudiana* are 30 times sweeter than normal sugar. More interestingly, the sweetening agent of Stevia is passing through the digestive system without affecting blood glucose levels. That's why it is considered safe for diabetic patients(Khalil *et al*., 2016). It has also antioxidant activity and some bioactive compounds, which have anti-hypersensitive, anti-cancerous, and anti-hyperglycemic properties. It is also used in the prevention of dental carries (Dey *et al*., 2013). Furthermore, it is a part of different foodstuff such as pickled vegetables, dried seafood, soy sauce, ice cream, candies and chewing gum. It is also incorporated in tooth paste and mouth wash (Anbazhagan *et al*., 2010).

There are some limitations associated with the propagation of Stevia via conventional method due to its seed capability and low germination rate Plants produce through seeds are not homogenous. Due to this the commercial properties are affected such as the ratio of secondary metabolites and the sweetening level (Tamura, 1984). An alternative method of plant propagation in Stevia is stem cutting. However, this technique is also restricted to specific conditions and plants produced through stem cuttings have a low acclimatization rate. Some researchers also suggested that plant propagated to this method have lower amount of steviosides (Khalil *et al*., 2016). The current study was designed to evaluate the effect of induced drought on micro-propagation of *S. rabaudiana* and its important secondary metabolites. In this study, we evaluated different parameters associated with plant growth, enzymatic activity and secondary metabolites. We found that growth growth related traits show decline.as a result of PEG stimulated drought stress. Further we observed that, enzymatic activity and proline content significantly improved to certain extent but then decreased suggested that PEG induced drought stress severely affect plant growth. On the other hands, plant secondary metabolites including steviol glycosides, phenols and flavonoids content improved by PEG stimulated drought stress.

Material and Methods

The experiment was conducted at plant tissue culture lab of Nuclear Institute for Food and Agriculture (NIFA), Peshawar-Pakistan.

Explant collection and sterilization: Healthy, young fast-growing leaves were collected from the field. The leaves were kept in distilled water to avoid moisture loss from leaves surface. The leaves were then surface sterilized with 70% ethanol for 2 minutes to remove surface contaminants and then treated with mercuric chloride 0.1% (w/v) for 90 seconds to disinfect explants. The leaf explants were then dried with filter paper to remove extra water from the leaf surface.

Establishment of culture: To study the effect of polyethylene glycol-induced drought on micro-propagation of *S. rabaudiana* under in-vitro condition, various concentrations of PEG i.e., 0, 1%, 2%, 3%, and 4% (w/v) was added to MS media. For this purpose, PEG was weighed with digital balance according to the treatment and then added to 200 ml distilled water. Beakers containing PEG and distilled water were kept on magnetic stirrer to dissolve properly. Different components of MS media were then added to the beaker along with 3% sugar, after which growth regulator (2, 4D 2mgL^{-1}) was supplemented and the volume was then adjusted to one liter. The pH of the media was adjusted to 5.7. Agar was added to gel the media @ 7.5 gL^{-1}. Prepared media was then autoclaved at 121 ^{0}C for 20 minutes. After autoclaving, the media explants were cultured the next day under a laminar flow hood in jars containing 30-40 ml media. Cultured explants were transferred to the growth chamber. Which were kept for 4 weeks in the light duration of 16/8 hours at temperature 25±2^{o}C. Callus from each treatment was then collected and cultured on shooting media augmented with BAP 2mgL^{-1}, while PEG was applied according to the treatments. The culture was transferred to the growth chamber and kept for 6 weeks. Each treatment was replicated three times in the experiment designed on a Completely Randomized Design.

Studied attribute: Different aspects of *S. rabaudiana* subjected to various PEG-induced droughts were studied. Shoot length was determined by selecting plants from each treatment and the length was determined with measuring tape, a number of leaves of plants from each treatment was counted and then its average was determined, while fresh weight shoots from each treatment were selected and weighed on a digital balance. However, for dry weight shoots were oven dried at 55^{o}C for 36 hrs. Chlorophyll content was determined followed by the method used by (Azzam, 2021).

Analytical methods: The dried shoots obtained from the shoot dry weight of each treatment were crushed with pestle and mortar to make a fine powder. For total phenolic content protocols of (Khalil *et al*., 2016)were used. According to the 0.03 ml sample extract was collected and folin cheocalteu @ 0.1 ml was mixed with 2.25 ml distilled water. The solution was centrifuged at 14000 rpm for 10 mins and kept in dark for 30 mins. Then it was passed through filter paper to remove large particles. Samples from each treatment were put in the cuvette and the absorbance was noted at 760 nm. In a similar way total flavonoid content was determined according (Khalil *et al*., 2016) 0.25 ml extract, 0.075 ml $AlCl_3$(5% w/v) and NaOH 0.5 ml was mixed with 1.25 ml distilled water. The solution was centrifuged at 14000 rpm for 10 mins and kept in dark for 30 mins. Then it was passed through filter paper to remove large particles. Samples from each treatment were put in the cuvette and the absorbance was noted at 510 nm. For DPPH free radicals scavenging 5 mg dry powder was collected from each treatment and dissolved in 20 ml HPLC grade water according to (Khalil *et al*., 2016) for each 1 ml solution 2 ml DPPH was used. It was then kept in dark for 30 minutes for maximum radical scavenging. After which absorbance was noted at UV spectrophotometer.

Enzymatic activity

Preparation of enzyme extract: For determination of antioxidant enzymes activity, leaf samples of 0.5 g was grounded to fine powder in liquid nitrogen, with phosphate buffer (0.05 M, pH 7.8), and centrifuged at 12,000 × *g* for 20 min at 4°C. The upper solution was used for the deduction of antioxidant enzymes and MDA content. The activities of antioxidant enzyme were expressed as U mg^{-1} protein fresh weight.

Antioxidant enzymes assay: Superoxide dismutase (SOD) activity was carried out according to (Jahan, 2022)An aliquot of 20 µL enzyme extract was mixed with 3 mL SOD reaction mixture containing phosphate buffer (1.5 mL 50 mM, pH 7.8) nitro blue tetrazolium (NBT) 0.3 mL (0.75 mM) 0.3 mL 130 mM methionine, 0.3 mL 0.02 mM riboflavin, 0.3 mL 0.1 mM EDTANa2 and 0.25 mL distilled water). The control covered with aluminum foil was kept in dark, while the enzyme solution incubated at 4000 lx fluorescent light for 30 min. Reaction started and appearance of dark blue color indicated end of reaction. The absorbance was measured using spectrophotometer at 560 nm wavelength.

The procedure of Jahan *et al*., (2022) was used to check the activity of peroxidase (POD). A 20-µL aliquot of the enzyme extract was added to 3 mL POD reaction mixture 0.1 mM phosphate buffer, (pH 6.0), 16 mM guaiacol and 19 µL 10% (w/v) H2O2. Activity was recorded at 470 nm absorbance at 30 s interval for 3 min.

Next, we measured catalase (CAT) activity. For determination of CAT activity, an aliquot of 50 µL enzyme extract was added to 2.5 mL reaction mixture 0.1 mM phosphate buffer having pH 7.0, 0.1 mM H2O2 and absorbance measured at 240 nm at 30 s interval for 3 min (Jahan, 2022).

The ascorbate peroxidase (APX) was find out following the protocol of (Jahan, 2022). in a reaction mixture containing phosphate buffer (50 mM, pH 7.0), 0.1 mM EDTA, 0.5 mM AsA, 1.0 mM H2O2 and 0.2 mL enzyme solution and absorbance measured at 290 nm.

Malondialdehyde (MDA) content was determined as described previously Tiwari *et al*., (2010). A 1-mL enzyme extract was added to reaction mixture having 0.65% (w/v) thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) and put in hot water for 15 min at 100°C, followed by immediate cooling to stop the reaction. That solution was then centrifuged for 10 min at 10,000 × *g* and absorbance read at 532, 600, and 450 nm.

Proline accumulation in the leaf was determined by the method recommended by Bates *et al*., (1973) (Bates, 1973). 0.5 g leaf sample was extracted in 10 mL of 3% sulfosalicylic acid and 1 mL supernatant was mixed with glacial acetic acid and acid-ninhydrin, upon incubation for 1 h at 100°C the reaction was stopped by putting it in ice bath. Then, 2 mL toluene was added, mixed and kept for 30 min. at room temp. Afterward, the mixture was divided into two phases. The optical density of the chromophore comprising toluene was determined at 520 nm with a UV-120 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Further, it was estimated as fresh weight of leaf sample proline (μMg^{-1}) (Jahan, 2022).

Stevioside quantification: Stevioside was measured by using the techniques reported by (Dey *et al*., 2013). 20 mg dry powder was dissolved in 10 ml ethanol for extract preparation. HPLC (Shimadzu system, LC-8A; Kyoto, Japan) having C-18 column (150×4.6 mm) was used for quantification of sativoside. 70% methanol and 30% water were used as mobile phase having 1.5 ml min-1 flow rate. In 200 µg ml^{-1} HPLC grade water was used for standard preparation of steviosides and then compared with results of samples, which was calculated in mg/g of dry weight (DW).

Statistical analysis

Statistix version 8.1 was used for data analysis and for mean comparison LSD test was used at α 1%. While for figures, Sigma Plot V14.0 was used and correlation heat maps were constructed trough graphpad prism.

Results

Morphological attributes: Various morphological and biochemical attributes of *S. rabaudiana* were studied during the experiment. The culture was kept for 45 days in the growth room and then data were recorded. Shoot length of *S. rabaudiana* was investigated which is significantly affected, subjected under different PEG concentrations. Shoot length was negatively affected by PEG concentrations shown in (Fig. 1A). When PEG concentration increased from zero to 4% (w/v), shoot length was decreased gradually. Similarly, Results regarding number of leaves was also negatively influenced by PEG concentrations. Highest number of leaves $plant^{-1}$ was recorded in the control treatment that is not receiving any PEG, decreased gradually with an increase in PEG concentration, in the same way a minimum number of leaves was observed in the treatment having maximum PEG concentration (4% w/v). It indicated that increase in concentration decreased the number of leaves $plant^{-1}$ (Fig. 1B).

One of the important parameters related to plant growth and development is chlorophyll content. Chlorophyll content in the leaves of *S. rabaudiana* subjected under PEG induced drought stress showed a gradual decrease with the increase in PEG concentration which is shown in (Fig. 2A-D). This decrease may be due to a decrease in water uptake as a result of drought stress caused by PEG in the culture media. In the same way, the Root length of *S. rabaudiana* was also significantly affected by various PEG concentrations (Fig. 6). A negative impact of PEG induced drought stress was found on root length. With increase in PEG concentration, root length decreased however, maximum root length was found in the control treatment. Water content of the tissue decreased as a result of drought. It leads to decrease leaf turgor pressure and ultimately cell elongation is affected. We also noticed that roots fresh and dry weight are also inversely affected by PEG concentrations (Fig. 6A-D). Further, the correlation analysis show the negative impact of the induced drought stress on morphological traits (Fig. 7). We notice that there is a negative correlation exist among morphological traits and PEG concentrations. With increase in PEG concentration growth related traits were significantly declined.

Enzymatic activity: Plants are sessile in nature as they cannot moved and experience different types of stress conditions. For this purpose, they evolved some strategies to adjust with these conditions. One of these tactics to cope with harsh environmental conditions is the activation of enzymatic system. This enables the plant to scavenge Reactive Oxygen Species (ROS) which are destructive for plant. In our experiment, we evaluated some enzymes that are helpful to balance the plant growth and stress tolerance. Such as SOD activities as influenced by PEG concentration, in our results with increase in PEG concentration SOD activity increase smoothly but at 3% there is a big increase noticed which indicated that the ROS amount increased and plant modify its growth conditions to avoid further damage. Similarly, POD and CAT activity smoothly increased with increase in PEG concentration. While in case of APX, it is also followed the same trend as SOD (Fig. 3A, B, C, D).

Furthermore, MDA concentration increases with increase in PEG concentration, which have negative impact on growth of Stevia. However, proline accumulation also increased, which have the ability to adjust growth and stress response of the plant (Fig. 4C, D).

Biochemical attributes: *S. rabaudiana* is a perennial shrub famous for its low calories sweetening agent, which is non-carcinogenic. Its extracts are used in a wide range of medicines all over the world. The secondary metabolites found in its leaves known as steviol glycosides (SVglys) are useful for the human body. Stevioside contents are the major constituents of Stevia. The biosynthesis of these SVglys in the stevia plant is constituted through a complex metabolic pathway, which is still not clear. Drought stress influence biochemical process in the plants. It is also significantly increased due to stress. Results of the HPLC analysis showed that PEG induced drought stress positively influenced stevioside content in the leaves of stevia (Fig. 5A). Similarly, rabaudioside is another type of SVglys that are found in stevia leaves. It is also noticed that the concentration of rabaudioside was also increased with the increase in PEG concentrations up to 3%, however it decreases with further increase in PEG induced drought stress (Fig. 5B).

Phenols are the important plant secondary metabolites, which have an important role in the plant defense mechanism. In our experiment, Plant Phenolic content was significantly improved by PEG concentrations. Results showed that phenolic content in *S. rabaudiana* increased with increase in concentration up to 3% (w/v) but then it starts to decreased (Fig. 4A). This increase may be due to the response of Stevia to adverse conditions induced on it. Similarly, flavonoid also significantly increased in *S. rabaudiana*. Results showed that increase in PEG concentration increased the synthesis of Flavonoid upto 3% (w/v) however, further increase in concentration caused decline (Fig. 4B). This may be due to the disturbance in plant normal physiological process. dulcoside is also a type of SVglys, which have natural sweet taste. Its biosynthesis also increased at a certain level of stress and then decline (Fig. 5) all of these secondary plant compounds have an important role in plant tolerance and ROS scavenging.

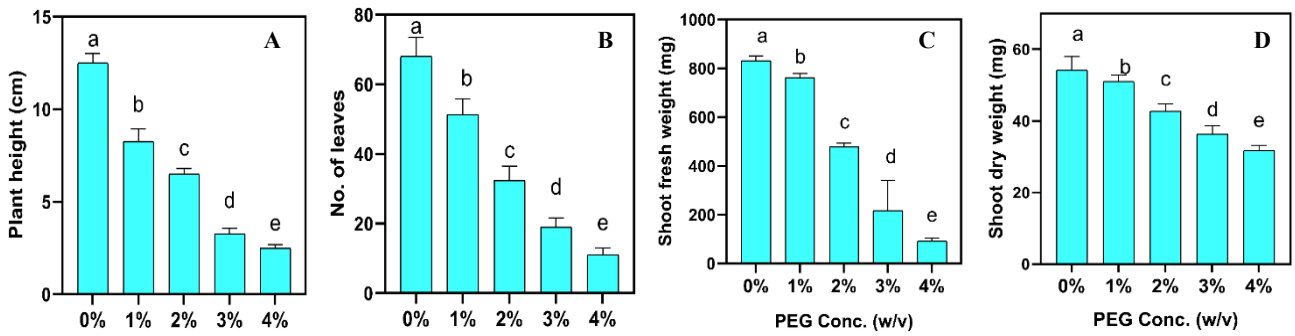

Fig. 1. PEG induced drought stress significantly influenced morphological traits of *S. rabaudiana* under *In vitro*. Figure 1. Show shoot length (cm), number of leaves, shoot fresh and dry weight (mg) (Fig. 1 A, B, C, D).

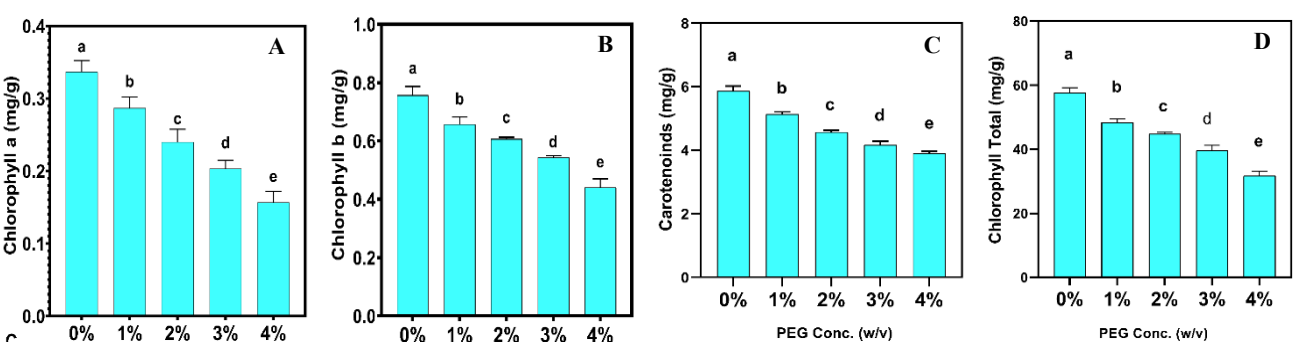

Fig. 2. PEG induced drought stress significantly influenced chlorophyll content (mg/g FW) of *S. rabaudiana* under invitro. Figure 2. Show chlorophyll a, b, total chlorophyll and carotenoids content (Fig. 2 A, B, C, D) subjected to various PEG concentrations.

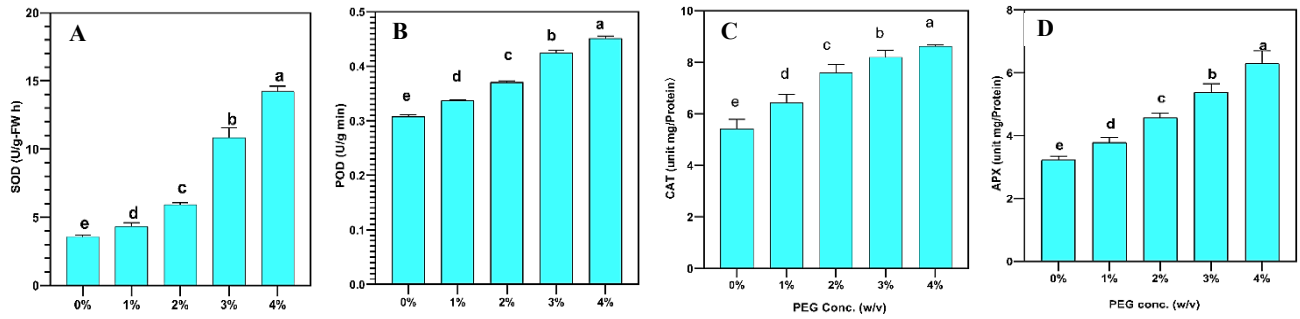

Fig. 3. PEG induced drought stress significantly influenced enzymatic activity of *S. rabaudiana* under invitro. Figure 3. Show SOD, POD, CAT APX, content (Fig. 3 A, B, C, D) subjected to various PEG concentrations.

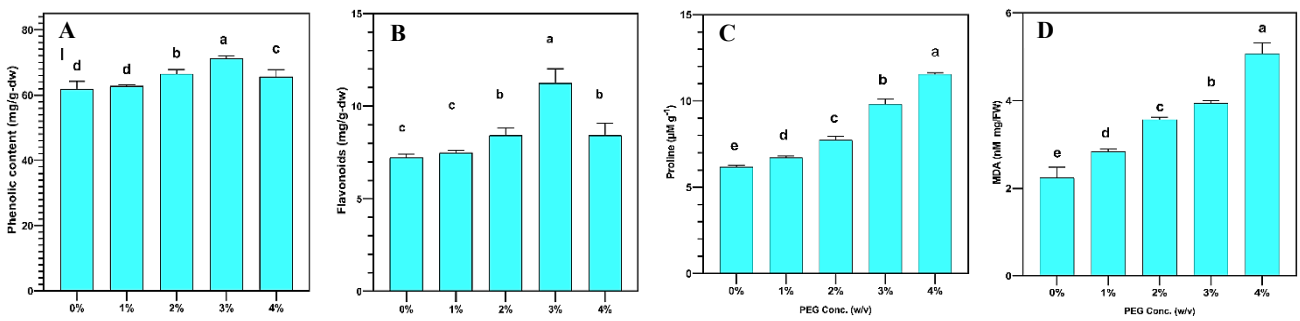

Fig. 4. PEG induced drought stress significantly influenced biochemical synthesis of *S. rabaudiana* under invitro. Figure 4. Show phenols, flavonoids, proline and MDA (Fig. 4A, B, C, D) of Stevia subjected to PEG concentrations.

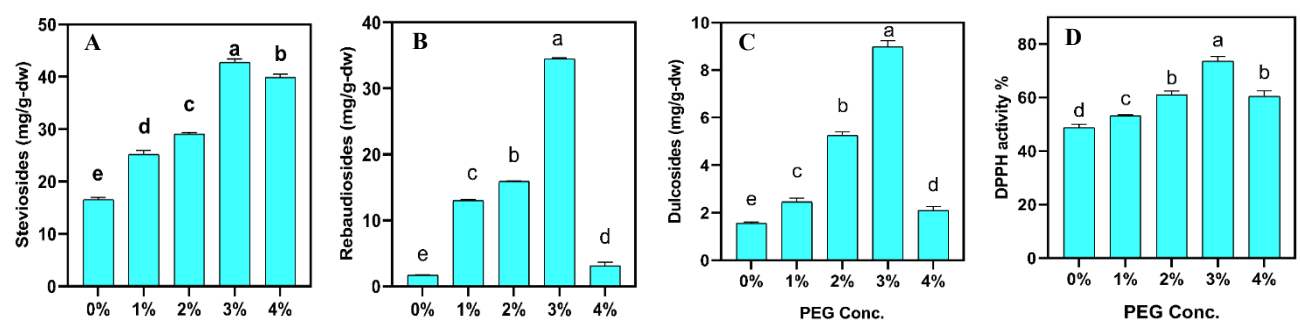

Fig. 5. Show stevioside, rabaudiosides, dulcosides, (A, B, C) and DPPH activity (D) subjected to various PEG concentrations.

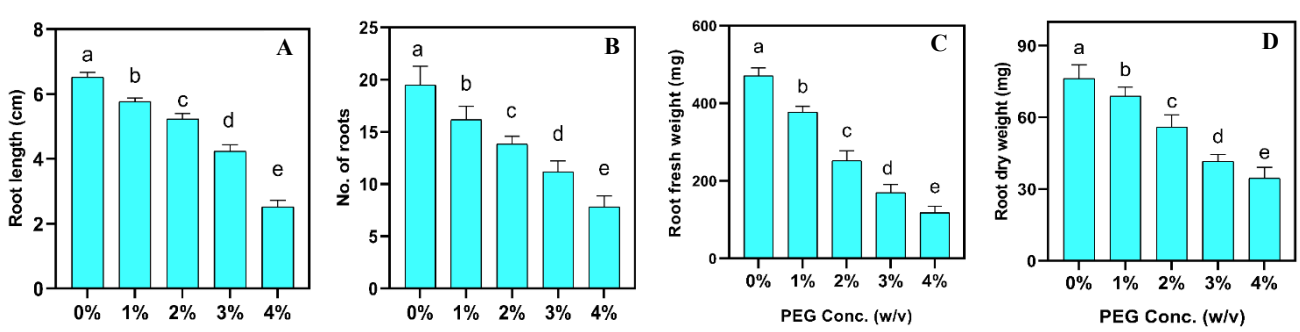

Fig. 6. show root length, number of roots, fresh and dry weight (mg) of the roots (A, B, C, D) subjected to various PEG concentrations.

Antioxidant activity plays a vital role in plant defense mechanism, especially during stress conditions. Increase in the total antioxidant activity was observed when PEG concentration increased from 0 to 3% however it declines when concentration increased above 3% (Fig. 5D). Enhancement in the antioxidant system is one of the finest tactics to lessen the consequence of abiotic stress like drought. Correlation analysis shows the magnitude of the stress response of *S. rabaudiana* to induced drought (Figs. 5-6). Considering enzymatic activity and biochemical compounds, we observed that Peg concentration can be positively correlate with biochemical compounds and enzymatic activity. There is an increase in the active biochemical compounds including phenols and flavonoids. The heat map also shows that the synthesis of steviol glycosides and other related compounds also improved.

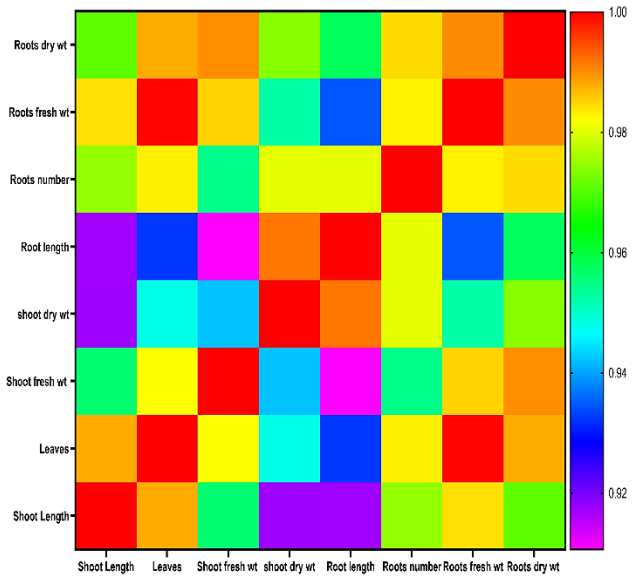

Fig. 7. Heatmap show the corelation analysis of morphological traits influenced by PEGinduced drought stress.

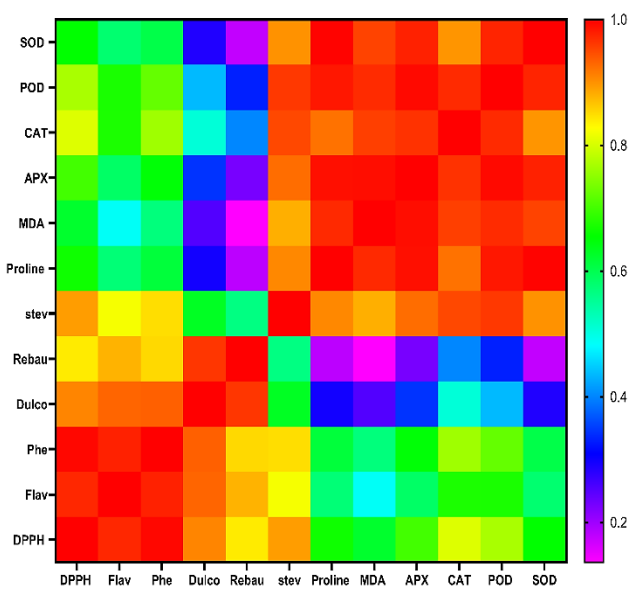

Fig. 8. Describe the correlation analysis of biochemical compounds and enzymatic activity associated with induced drought stress.

Discussion

Different types of elicitors are used to improve the biosynthesis of valuable plants compounds which have a vital role in medicines. These elicitors alter the biosynthetic pathways of these secondary metabolites. In order to improve the production of SVglys in stevia leaves, we used various PEG concentrations to induced drought stress under *In vitro* conditions. Our results show that mild drought stress not only enhances the concentration of SVglys in *S. rabaudiana* but also improves the antioxidant system of the plant. Our results are supported by some of the researchers who previously worked. We correlate the findings of some research to our results. Most of the morphological traits are inversely affected by PEG. This decrease may be due to a decrease in water influenced by PEG application in the media. It also affects nutrient uptake and reduced plant growth (Fig. 6). (Pandey & Chikara, 2014) reported that the shoot length of *S. rabaudiana* decreased under drought stress induced through PEG application in the media. (Ghaheri *et al*., 2015) also observed that shoot length is inversely correlated with PEG concentration. Drought stress affects nutrient uptake which decreased cell growth and ultimately the morphogenetic potential of the explant is affected (Gupta *et al*., 2016). Under stress conditions, one of the strategies adopted by the plant is to slow down the growth. This reduction in growth not only enables the plant to save energy and also reduced the risk of heritable damage (Hossain *et al*., 2007).

Our findings are similar to that reported by (Said*et al*., 2015) who concluded from their experiment that an increase in PEG concentration decreased average shoot length. Furthermore, they reported maximum shoot length was observed in the control treatment (Fig. 6). Said *et al*., 2015 found similar results that increased PEG concentration in the culture media decreased the number of leaves. A plant grown under drought stress have a low number of leaves. This decline in a number of leaves per plant is due to decreased rate of cell enlargement and occurrence of leaf senescence which results from reduced turgor pressure(Shao *et al*., 2008). Reduction in leaf size is the first response of plants facing drought stress (Fig. 7). Due to reduced water content in the plant tissues, turgor pressure of the leaf is also reduced which results in inhibition in cell elongation (Albiski *et al*., 2012).

PEG induced drought stress affects plant activities and as a result, growth indexes such as shoot length, number of leaves plant^{-1}, surface area of the leaf, fresh and dry weight are negatively affected (Toumi *et al*., 2007). Our findings are also in line with that observed by (Pandey & Chikara, 2014) they also reported a similar decrease in number of leaves per plant in *S. rabaudiana* subjected under different PEG concentrations. Shao *et al*., (Shao *et al*., 2008) reported that morphological and physiological process of the plants are strongly affected by drought stress. Rate of photosynthesis is strongly affected by low availability of water and reduce accumulation of carbohydrates. Subsequently, reduced growth and biomass accumulation occurs.

Our results are accordance to (Akte *et al*., 2016) who observed a significant decrease in shoot fresh weight subjected under various drought concentrations. Nejad (2011) stated that increasing drought concentration during growth, reduced root and shoot weight. This is due to imbalance among cations caused by their complex interaction with in xylem transport system. Furthermore, A significant decrease in shoot dry weight was found when drought stress induced through PEG concentration in the culture media (Akte *et al*., 2016). Chegah*et al*., 2013 also reported a decrease in shoot dry weight in plants grown under invitro drought stress. Due to drought stress blockage of pores occurred. It decreased the photosynthesis rate and ultimately lead to reduced growth (Nabati *et al*., 1994). Anjum *et al*., (2011) also reported similar results; drought stress caused a reduction in the number of pores. It affects the synthesis of fresh and dry weight. Our results are similar to those reported by (Pandey & Chikara, 2014) who found a similar decrease in shoot dry weight in *S. rabaudiana* treated with PEG to induce drought. Chlorophyll

content was also reduced due to drought stress. Which ultimately affects the synthesis of chlorophyll pigment. Due to drought stress, the photosynthesis rate decreased significantly. This reduction in photosynthesis is a kind of defense mechanism (Jones & Corlett, 1992) Lowering the photosynthesis, reduced in total chlorophyll content occurred. The content of photosynthetic pigments and their composition is strongly affected by water availability (Farooq *et al*., 2009) Oxidative stress in the plants generated due to water stress cause degradation of chlorophyll content and also reduce it synthesis (Kiani *et al*., 2008).

Our results are in conformation with that reported by (Akte *et al*., 2016) who observed decrease in chlorophyll content in plants subjected to drought stress conditions. Lattanzio *et al*., (2006) reported that, under diverse environmental conditions one of the responses of plants to cope that conditions, plants start to increase the production of phenolic contents. Due to stress conditions total phenolic content was significantly increased (Pennycooke *et al*., 2005). Our results are similar to those reported by (Hajihashemi & Ehsanpour, 2014) that a significant increase in phenolic content was observed when drought stress induced under in-vitro conditions. (Hajihashemi *et al*., 2012) reported that inducing drought stress through PEG increases the synthesis of Flavonoid in *S. rabaudiana* grown under invitro condition. Drought stress induced through various PEG concentrations significantly increased the amount of total Flavonoid content in *S. rabaudiana* (Hajihashemi & Ehsanpour, 2014). During stress situations free radicals produced in the plant body which is destructive for cell components. It can abolish molecules and split cell membranes (Sairam *et al*., 1998).

A significant increase in antioxidant activity of Stevia was detected when it has grown under drought stress conditions induced through PEG. It also found that enhancement in scavenging of oxidant compounds in plants developed under drought stress. Hajihashemi *et al*., 2012. Steviol glycosides are the major component of the Stevia, which is also significantly increased during drought. It may be due to alteration in the production pathway of stevioside, which is triggered by drought stress induced through PEG application. (Gupta *et al*., 2016) also reported that an increase in PEG concentration induced drought stress in the culture media. Which enhanced the production of total steviol glycosides in *S. rabaudiana*. Similarly, (Zeng *et al*., 2013) also found increase in steviol glycoside concentration of *S. rabaudiana* when subjected to mild stress. (Hajihashemi *et al*., 2012) also found similar results in *S. rabaudiana* treated with various PEG concentrations grown under in-vitro conditions. Our findings are consistent to those reported by (Albiski *et al*., 2012) who observed a similar decrease in the number of roots when exposed to drought stress. (Ghaheri *et al*., 2015) also reported that drought stress decreased number of roots in *S. rabaudiana* when grown under in-vitro conditions. Due to decreased cell elongation and division root length was affected (Taiz & Zeiger, 2006). PEG induced drought stress adversely affected various growth parameters such as root and shoot length. It is due to decrease water uptake. Which affects nutrient uptake resulting reduction in photosynthesis and biomass accumulation (Albiski *et al*., 2012). Pandey *et al*., (2014) also reported similar results when plants were subjected to induced drought stress.

Conclusions

Our findings demonstrated that mild drought stress influences plant growth, physiology, and biochemical processes. PEG induced drought stress negatively affects plant growth and morphological aspects. However, it enhances the production of SVglys and improves the ROS scavenging activity by improving the production of phenolic compounds. So, we may suggest that mild drought stress can be used to enhance the production of medicinally important secondary plant compounds.

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References

Aghighi, S.M., H. Omidi and S.J. Tabatabaei. 2017. Stevia (*Stevia rebaudiana* Bertoni) responses to NaCl stress: growth, photosynthetic pigments, diterpene glycosidesand ion content in root and shoot. *J. Saudi Soc. Agric. Sci*., 12(101). doi: https://doi.org/10.1016/j.jssas.2017.12.001.

Akte, J., S. Yasmin, M. Bhuiyan, F. Khatun, J. Roy and K. Goswami. 2016. *In vitro* screening of rice genotypes using polyethylene glycol under drought stress. *Prog. Agri.*, 27(2): 128-135.

Akula, R. and G.A. Ravishankar. 2011. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Sign. Behavior.,* 6(11): 1720-1731.

Albiski, F., S. Najla, R. Sanoubar, N. Alkabani and R. Murshed. 2012. *In vitro* screening of potato lines for drought tolerance. *Physiol. Mol. Biol. Plants*, 18(4): 315-321.

Anbazhagan, M., M. Kalpana, R. Rajendran, V. Natarajan and D. Dhanavel. 2010. *In vitro* production of *Stevia rebaudiana* Bertoni. *Emirates J. Food Agri.*, 216-222.

Anjum, S., L. Wang, M. Farooq, M. Hussain, L. Xue and C. Zou. 2011. Brassinolide application improves the drought tolerance in maize through modulation of enzymatic antioxidants and leaf gas exchange. *J. Agron. Crop Sci.*, 197(3): 177-185.

Azzam, C.R.A.T., S.K., R.M. Abdel-Aziz., K.M. Rabea., A.I.B.Abou-Sreea., M.M. Rady and E.F. Ali. 2021. Salinity effects on gene expression, morphological, and physio-biochemical responses of *Stevia rebaudiana* Bertoni *In vitro*. *Plants*, 10(820): 1-19.

Bates, L.S., R.P. Waldren and I.D. Teare. 1973. Rapid determination of free proline for water stress studies. *Plant Soil*, 39: 205-207.

Chegah, S., M. Chehrazi and M. Albaji. 2013. EffEcts of drought stress on growth and development frankenia plant (*Frankenia leavis*). *Bulg. J. Agri. Sci.*, 19(4): 659-666.

Debnath, M., C. Malik and P.S. Bisen. 2006. Micropropagation: a tool for the production of high quality plant-based medicines. *Curr. Pharm. Biotech.*, 7(1): 33-49.

Dey, A., S. Kundu, A. Bandyopadhyay and A. Bhattacharjee. 2013. Efficient micropropagation and chlorocholine chloride induced stevioside production of *Stevia rebaudiana* Bertoni. *Comp. Rend. Biol.,* 336(1): 17-28.

Farooq, M., A. Wahid, N. Kobayashi, D. Fujita and S. Basra. 2009. Plant drought stress: Effects, mechanisms and management *Sustainable agriculture* (pp. 153-188): Springer.

Ghaheri, M., D. Kahrizi and G. Bahrami. 2015. Effect of mannitol on some morphological characteristics of *In vitro Stevia rebaudiana* Bertoni. *Biharian Biol*., 11(2): 94-97.

Gupta, P., S. Sharma and S. Saxena. 2016. Effect of abiotic stress on growth parameters and steviol glycoside content in Stevia rebaudiana (Bertoni) raised *In vitro*. *J. Appl. Res. Med. Arom. Plants*, 3(4): 160-167.

Hajihashemi, S. and A.A. Ehsanpour. 2014. Antioxidant response of *Stevia rebaudiana* B. to polyethylene glycol and paclobutrazol treatments under *In vitro* culture. *Appl. Biochem. Biotech.*, 172(8): 4038-4052.

Hajihashemi, S., J.M. Geuns and A.A. Ehsanpour. 2012. Molecular and physiologicalanalysis of *Stevia rebaudiana* after treatment with polyethylene glycol, paclobu-trazol and gibberellic acid. *Stevia*, 6: 71-95.

Hossain, Z., A.K.A. Mandal, S.K. Datta and A.K. Biswas. 2007. Development of NaCl-tolerant line in *Chrysanthemum morifolium* Ramat. through shoot organogenesis of selected callus line. *J. Biotechnol.*, 129(4): 658-667.

Jahan, M.S.H., F.S. Alotaibi, N.M. Alabdallah, B.M. Alharbi, K.M.A. Ramadan, E.S.A. Bendary, D. Alshehri, D. Jabborova snd D.A. Al-Balawi. 2022. Exogenous putrescine increases heat tolerance in tomato seedlings by regulating chlorophyll metabolism and enhancing antioxidant defense efficiency. *Plants,* 11(1038):1-22.

Jones, H. and J. Corlett. 1992. Current topics in drought physiology. *J. Agri. Sci.,* 119(3): 291-296.

Khalil, S.A., N. Kamal, M. Sajid, N. Ahmad, R. Zamir, N. Ahmad and S. Ali. 2016. Synergism of polyamines and plant growth regulators enhanced morphogenesis, stevioside content, and production of commercially important natural antioxidants in *Stevia rebaudiana* Bert. *In vitro Cell. Develop. Biol.,* 52(2): 174-184.

Kiani, S.P., P. Maury, A. Sarrafi and P. Grieu. 2008. QTL analysis of chlorophyll fluorescence parameters in sunflower (*Helianthus annuus* L.) under well-watered and water-stressed conditions. *Plant Sci.,* 175(4): 565-573.

Lattanzio, V., V.M. Lattanzio and A. Cardinali. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochemistry: Adv. Res.*, 661(2): 23-67.

Nabati, D., R. Schmidt and D. Parrish. 1994. Alleviation of salinity stress in Kentucky bluegrass by plant growth regulators and iron. *Crop Sci.,* 34(1): 198-202.

Nejad, T.S. 2011. Effect of drought stress on shoot/root ratio. *World Academy of Science, Engineering and Technology.,* 5: 539-541.

Pandey, M. and S.K. Chikara. 2014. *In vitro* regeneration and effect of abiotic stress on physiology and biochemical content of *Stevia rebaudiana* 'Bertoni'. *J. Plant Sci Res.*, 1(3): 1-9.

Pennycooke, J.C., S. Cox and C. Stushnoff. 2005. Relationship of cold acclimation, total phenolic content and antioxidant capacity with chilling tolerance in petunia (Petunia× hybrida). *Environ. Exp. Bot.*, 53(2): 225-232.

Pérez-Clemente, R.M and A.Gómez-Cadenas. 2012. *In vitro* tissue culture, a tool for the study and breeding of plants subjected to abiotic stress conditions. *Recent advances in plant In vitro culture*: InTech. 91-108.

Said, E., R. Mahmoud., R. Al-Akshar and G. Safwat. 2015. Drought stress tolerance and enhancement of banana plantlets. *In vitro. Austin J. Biotechnol. Bioeng*, 2(2): 1040-1046.

Shao, H.B., L.Y. Chu., C.A. Jaleel and C.X. Zhao. 2008. Water-deficit stress-induced anatomical changes in higher plants. *Comp. Rend. Biol.*, 331(3): 215-225.

Taiz, L. and E. Zeiger. 2006. Plant Physiology, Sinauer Associates, Sunderland, MA. *Plant physiology. 4th ed. Sinauer Associates, Sunderland, MA.*

Tamura, Y., S. Nakamura., H. Fukui and M. Tabata. 1984. Clonal propagation of *Stevia rebaudiana* Bertoni by stem-tip culture. *Plant Cell Rep.*, 3(5): 183-185.

Thakur, M., S. Bhattacharya, P.K. Khosla and S. Puri. 2019. Improving production of plant secondary metabolites through biotic and abiotic elicitation. *J. Appl. Res. Med. Arom. Plants,* 12: 1-12.

Toumi, I., W.M. Sehli, S. Bourgou, N. Jallouli, A. Bensalem-Fnayou, A. Ghorbel and A. Mliki. 2007. Response of ungrafted and grafted grapevine cultivars and rootstocks (*Vitis* sp.) to water stress. *J. Int. des Sciences de la Vigne et du Vin,* 41(2): 85.

Yasin, M., A. Ahmad, T. Khaliq, M. Habib-ur-Rahman, S. Niaz, T. Gaiser and G. Hoogenboom. 2021. Climate change impact uncertainty assessment and adaptations for sustainable maize production using multi-crop and climate models. *Environ. Sci. Pollut. Res.*, 1-22.

Zeng, J., A. Chen, D. Li, B. Yi and W. Wu. 2013. Effects of salt stress on the growth, physiological responses, and glycoside contents of Stevia rebaudiana Bertoni. *J. Agri. Food Chem.*, 61(24): 5720-5726.

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