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EFFECT OF SALINITY ON GROWTH AND ANTIOXIDANT ENZYME ACTIVITIES OF *STEVIA REBAUDIANA* BERTONI

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Abstract

The current study is focused on the salt tolerance capacity of *Stevia rebaudiana* (Asteraceae) and to observe the effect of salt on growth and antioxidant enzyme activities. After surface sterilization, nodal segments from *In vitro* raised plants were cultured on Murashige and Skoog (1962; MS) basal medium containing various concentrations of sodium chloride (NaCl). After 30 days of salt stress, it was observed that various morphological and biochemical parameters of the plant were strongly affected. Shoot length was gradually decreased by increasing the NaCl concentration from 0 to 150 mM. Similar trend was found regarding the number of leaves and nodes. Total soluble protein contents and antioxidant enzyme activities were increased due to the activation of defense system of plant under stress. Total soluble protein content was 4.74, 5.8, 6.2 and 7.25 mg/g at 0, 50, 100 and 150 mM salt level. Peroxidase activity was changed from 0.30 U/mL of enzyme (control) to 2.37 U/mL of enzyme (150 mM) while CAT activity was increased from 2.2 U/mL of enzyme (control) to 4.0 U/mL of enzyme (150 mM). Similarly, there was a gradual increase in superoxide dismutase activity with increasing NaCl concentration from 0-150 mM compared to control. Changes in antioxidant enzymes' activities indicate their important role in scavenging reactive oxygen species produced during stress episode.

Key words: *In vitro*; Sodium chloride; *Stevia rebaudiana*; Plant tissue culture.

Introduction

Soil salinity has developed as one of the major limitations to crop production all over the world. Human impacts have increased the process of soil salinization in recent decades (Ali *et al*., 2022). When the concentration of NaCl exceeds 40 mM in the soil, it is known as saline (Acosta-Motos *et al.*, 2017). Saline soils have a high electrical conductivity (EC), a limited water potential, and a higher proportion of ionic salts, rendering plants and other life forms difficult to survive (Gull *et al.*, 2022). Salinity is reported to have impacted over 800 million hectares of agricultural land worldwide (Yasin *et al.*, 2018). According to a report, salinity degrades about 1–2% of fertile soils globally per year (FAO, 2021). Furthermore, data shows that the annual cost of land affected by salt has been calculated to be US$ 27.3 billion over the last few decades (Quadir *et al.*, 2014). Total area of Pakistan is 79.6 mha, of which about 6 million hectares are affected by salinity (Sajid & Aftab, 2014; Syed *et al.*, 2021).

Stevia rebaudiana, a member of *Asteraceae* family, is a native of South America, known for its sweat compounds Steviol glycosides (Petulik, *et al.*, 2021). It is used as natural sweetener due to the presence of diterpene glycoside (Ramesh *et al.*, 2006). Synthetic sweetener such as saccharin, when used in heavy quantities, was the possible risk of bladder cancer (Jaroslav *et al.*, 2006). These days, leaves of *Stevia* plants and steviosides are being utilized as sweetener in Asia, Europe and South America. Being economically important, *Stevia* is cultivated commercially beyond its traditional production zones. It is native plant of Brazil and Paraguay (Ramesh *et al.*, 2007). *Stevia* is rapidly becoming a common industrial crop all over the world. Now, it is widely grown in China, Japan, Mexico, Russia, Korea, the United States, India, and Canada (Khalil *et al.*, 2014; Pal *et al.*, 2015). The plant is grown for the sweetness of its leaves, which contain steviol glycoside sweeteners including stevioside and rebaudioside A. *Stevia* leaves contain diterpenoid steviol glycosides (SGs), which are sweet-in-taste and lower in calories (Lemus-Mondaca *et al.*, 2012; Ritu & Nandini, 2016).

Stevia rebaudaina is a moderately salt tolerant plant. In general, there is a negative correlation between salt stress and plant morphology (Debnath *et al.*, 2019). Salinity caused an oxidative stress due to the production of several reactive oxygen species like superoxide radical (O_2^-), hydroxyl radical (HO^-) or singlet oxygen (O_2^1), Hydrogen peroxide (H_2O_2). Over production of these reactive oxygen species inhibits the activity of several enzymes, membrane lipid peroxidation, DNA damage and ultimately severely reduces the yield of plants. During salt stress, some toxic ions like Na^+ and Cl^- become accumulated in leaves and damage tips and margins (Gupta & Huang, 2014). Photosynthesis is a vital phenomenon for all plants, but it is highly affected by the salinity. If biotic or abiotic stress causes a disturbance at any stage of photosynthesis, photosynthetic rate is usually decreased. Salt stress showed a negative impact on photosynthesis by decreasing CO_2 availability due to the limitations of stomatal conductance (Zahra *et al*., 2022). Due to salinity, toxic ions were accumulated and plants could not absorb required water which caused a decrease in water potential and turgor pressure in leaves and stem (Methenni *et al.*, 2018; Akhtar *et al.*, 2021). In response to up regulation of reactive oxygen species, antioxidant enzymes' activity is enhanced (Munir *et al.*, 2021). Superoxide dismutase is considered as a major scavenger of these ROS and convert them into H_2O_2. Peroxidase and catalase neutralize this H_2O_2 into water and oxygen and in this way improve the growth of plants under stress (Sajid & Aftab, 2009; Khilji *et al.*, 2022; Zahra *et al*., 2022). Looking at the importance of this plant, the present study aims at investigating the salt tolerance capacity of *Stevia* under *In vitro* conditions. Another objective of this study was to partially know the mechanism of salt tolerance by observing the activities of antioxidant enzymes and protein contents.

Material and Methods

Application of NaCl to *Stevia rebaudiana* Bertoni plants and growth conditions: The plants of *Stevia rebaudiana* Bertoni were procured from the Lahore Nursery located at Kalma Chock, Lahore, Pakistan. To check the effect of salinity on *Stevia* plant, Murashige and Skoog (1962; MS; Sigma) basal medium was prepared by adding macronutrient, micronutrient, vitamins, iron EDTA stock in required amount. Various concentrations of salt (NaCl) were measured in grams and added in MS media directly. Media containing four different concentrations of NaCl, *i.e*., 50, 100, 150 mM along with control (0 mM NaCl) were prepared. Then sugar and agar were added in appropriate amount and heated and finally culture tubes containing 10 ml media were autoclaved at 121°C for 15 minutes under 15 lbs./inch² pressure. Nodal segments (ca. 1cm) from *In vitro* raised plants were cultured on Murashige and Skoog (1962; MS) basal medium containing various concentrations of sodium chloride (NaCl). After inoculation, they were kept in 16-h photoperiod (fluorescent light of 40 $\mu molm^{-2}s^{-1}$) at 25 ± 2°C. The cultures were observed for 30 days and then the data were collected for various morphological parameters (shoot length, number of leaves and number of nodes).

Estimation of antioxidant enzyme activates of *S. rebaudiana* under salinity stress: Fresh leaves (1g) of *S. rebaudiana* were taken and ground into a fine powder form and centrifuged (Sorval RB-5 refrigerated super speed centrifuge) for 15 minutes at 4°C and 15000 rpm. Supernatant was obtained and poured into the tubes and stored at -20°C for analysis of catalase, SOD, POD and total soluble protein contents. Biuret method recommended by Racusen and Johnstone, (1961) was used for the estimation of total soluble protein contents. By using Guaiacol-H_2O_2 method of Luck (1974) with slight amendments peroxidase (POD; E.C 1.11.1.7) activity was determined. Catalase (CAT; E.C 1.11.1.6) activity was measured by using Beers & Sizer, (1952) method and protocol of Maral *et al.*, (1977) was used for the estimation of superoxide dismutase (SOD; E.C 1.15.1.1) using spectrophotometer (U4000 Germany). SOD activity was expressed as U/mg of protein.

Statistical Analysis

Statistical analyses were done by using SPSS version 21.0.0 (IBM). One way ANOVA was used to determine significant differences between mean values. To compare the means of treatments, Duncan's multiple range test was used at $p<0.05$.

Results

In this research work, we studied the effect of NaCl on morphological characteristics and antioxidant enzyme activities of *Stevia rebaudiana* under *In vitro* conditions. Data were collected after 30 days of inoculation of explants on MS media supplemented with various concentrations of NaCl.

Effect of salt on morphological parameters of *S. rebaudiana*: Different concentrations of salt (50, 100, 150 mM) along with control (0 mM) were used which showed a clearly different effect on morphology of plant. Plant length was decreased as the amount of salt was increased. Shoot length was decreased from 6.3 cm to 2.69, 1.78 and 0.6 cm at 50, 100 and 150 mM salt concentration, respectively. More than 50% reduction in plant length was observed when 50 mM salt was added in MS medium. In saline media, number of leaves and nodes were also gradually decreased with increasing concentration of salt compared to control medium. Number leaves was decreased from 12.2 (control) to 6.4 at 150 mM salt level. Minimum number leaves were also observed at 150 mM salt medium while highest number of nodes was observed at control treatment without salt in MS medium. A continuous decrease in number of nodes from 4.4 to 3.9, 2.9 and 1.6 was observed at 50, 100 and 150 mM salt treatment to stevia plants (Table 1; Figs. 1 & 2).

Changes in antioxidant enzymes' activities of *S. rebaudiana* under NaCl stress: Different concentrations of NaCl (50, 100, 150 mM) along with control (0 mM) were used which showed significant ($p<0.05$) differences in total protein content and antioxidant enzymes' activities. At maximum salt concentration (150 mM), maximum soluble proteins (7.25 ± 0.40 mg/g) were observed, however, minimum protein contents (4.74 ± 0.01 mg/g) were recorded in control treatment without salt. When amount of salt was increased gradually in media from 50 to 150 mM, protein contents were increased significantly ($p\leq0.05$). Soluble protein contents were 5.8 ± 0.05 mg/g, 6.2 ± 0.08 mg/g and 7.25 ± 0.40 mg/g at 50, 100 and 150 mM salt level (Table 2).

Peroxidase analysis of *S. rebaudiana* revealed that there was a significant ($p<0.05$) change after the addition of salt in MS medium. POD activity increased gradually with the increase of NaCl concentration. At maximum concentration of salt (150 mM) POD activity increased to its maximum level (2.37 ± 0.3 U/mL of enzyme). The decrease in the salt level from 100 mM to 50 mM resulted in the decrease of POD activity from 1.30 ± 0.02 U/mL of enzyme to 0.94 ± 0.11 U/mL of enzyme respectively. In control medium, a minimum POD activity (0.30 ± 0.01 U/mL of enzyme) was measured compared to 150 mM salt level.

Catalase (CAT) activity was determined after 30 days of salt treatment to plants and it was observed that it was increased by increasing the concentration of salt in MS medium. Catalase activity was 2.6± 0.2, 3.3± 0.1 and 4 ± 0.2 U/mL of enzyme at 50, 100 and 150 mM salt treatment, respectively compared to control treatment. Hence, there was significant ($p<0.05$) difference in control and salt treated plants.

There was a positive correlation between NaCl concentration and SOD activity. Maximum activity of SOD (6.49 ± 0.01 U/mg of protein) was observed in medium containing highest salt concentration (150 mM) compared to all other treatments. SOD activity also increased with the increase of the salt level. Minimum SOD activity (2.36 ± 0.15 U/mg of protein) was measured in control where no salt treatment was provided. At 50 and 100 mM salt level, SOD activity was 3.85 and 5.08 ± 0.01 U/mg of protein, respectively. In interactive term of medium and changes in various growth and biochemical parameters, a significant ($p<0.05$) difference was recorded.

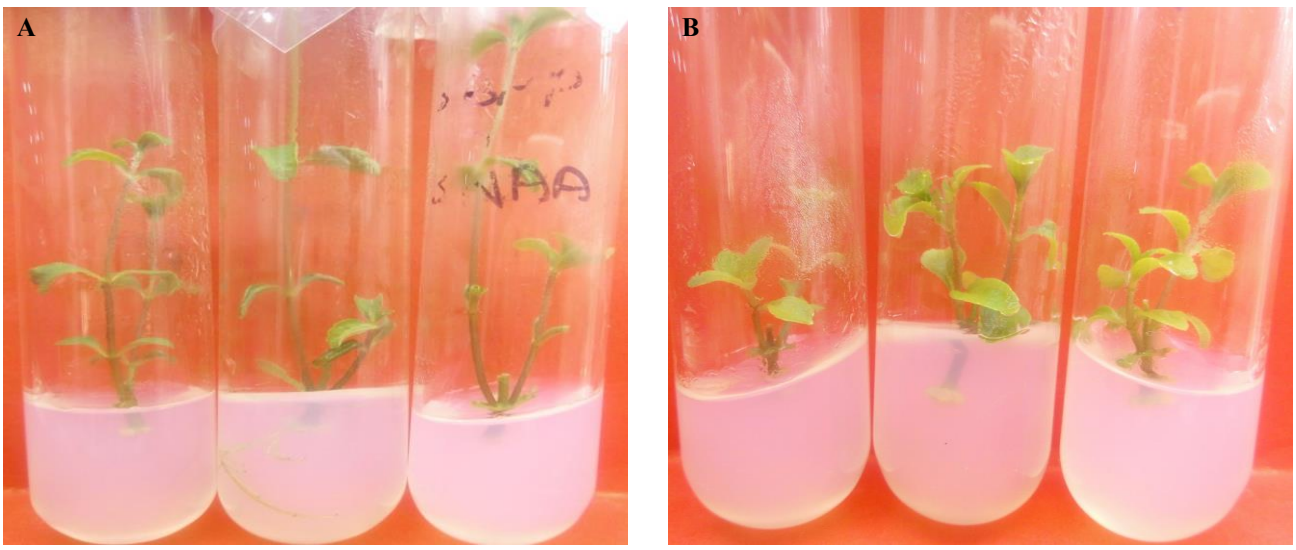

Fig. 1. *Stevia rebaudiana* growth after one month in control (A) and 50 mM (B) salt in MS medium.

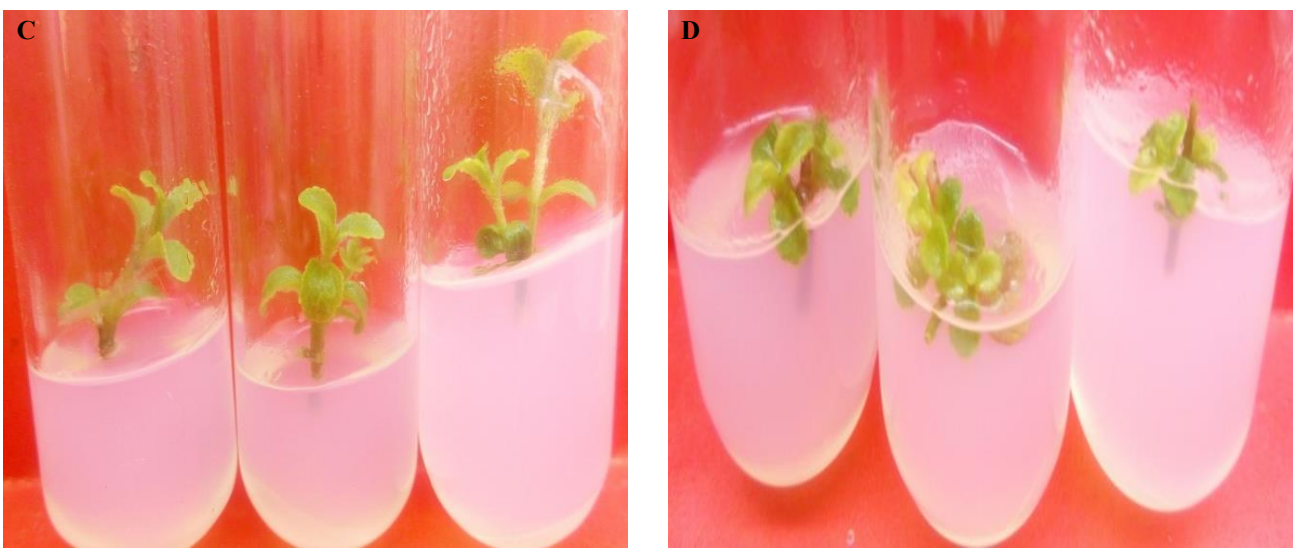

Fig. 2. *Stevia rebaudiana* growth after one month at 100 mM (C) and 150 mM (D) salt in MS medium.

Table 1. Morphological parameters of 30-day old *Stevia rebaudiana* under *In vitro* salt stress conditions.

Treatments of NaCl (mM)	Parameters		
	Shoot length (cm)	Number of leaves	Number of nodes
0	6.3 ± 1.66^{a}	12.2 ± 2.20^{a}	4.4 ± 1.17^{a}
50	2.69 ± 0.61^{b}	11.4 ± 0.96^{a}	3.9 ± 0.56^{a}
100	1.78 ± 0.41^{c}	9.2 ± 1.93^{b}	2.9 ± 0.73^{b}
150	0.6 ± 0.17^{d}	6.4 ± 2.63^{c}	1.6 ± 0.84^{c}

Means (± Standard deviation) of all parameters are mentioned in the table
According to DMRT (Duncan's multiple range test), different alphabetical letters are assigned to showing significant differences ($p \leq 0.05$) between the values

Table 2. Biochemical analysis of 30 days old *Stevia rebaudiana* under *In vitro* salt stress conditons.

Treatments NaCl (mM)	Parameters			
	Total protein content (mg/g of tissue)	POD (U/mL of enzyme)	SOD (U/mg of protein)	CAT (U/mL of enzyme)
00	4.74 ± 0.01^{d}	0.30 ± 0.20^{d}	2.36 ± 0.08^{d}	2.2 ± 0.05^{d}
50	5.8 ± 0.05^{c}	0.94 ± 0.01^{c}	3.85 ± 0.01^{c}	2.6 ± 0.08^{c}
100	6.2 ± 0.08^{b}	1.30 ± 0.01^{b}	5.08 ± 0.02^{b}	3.3 ± 0.11^{b}
150	7.25 ± 0.40^{a}	2.37 ± 0.10^{a}	6.49 ± 0.10^{a}	4.0 ± 0.08^{a}

Means (± Standard deviation) of all the parameters is mentioned in the table
According to DMRT (Duncan's multiple range test), different alphabetical letters are assigned to show significant differences ($p \leq 0.05$) between the values

Discussion

Salinity is a major problem for plants. It has a negative correlation with plants regarding various growth parameters such as reduction in shoot length, number of leaves and nodes. This study was carried out to observe the effect of salt stress on growth and changes in antioxidant enzymes' activities of *Stevia rebaudiana.* The experiment was established with four concentrations of salt (NaCl), *i.e*., control (0), 50, 100 and 150 mM. MS medium was fortified with NaCl to imposed salt stress condition during this investigation.

It was found that morphological characteristics such as plant height, number of leaves and nodes were decreased with increasing concentration of salt. Growth was maximum in control sample and minimum at 150 mM salt solution. Similar reduction in growth of *In vitro*-grown potato plants under salt stress (0-4%) was also reported by Gupta & Huang (2014). Reduction in growth due to insufficient water uptake, a common indicator of salt stress was reported by Munns (2002) and Rehman *et al*., (2022). Under salt stress, chloroplast cannot remain stable which causes a reduction in normal function of stomata and photosynthetic rate become low. These changes lead to reduced growth, *i.e*., shoot length, number of leaves and nodes of *Stevia* plants. By increasing NaCl concentration, reduction in plant height was also observed by Kavita & Alka, (2010). The plant height and leaves weight were decreased due to the increased amount of salt, which caused changes in enzyme activity that led to the suppression of growth (Gerami, 2020). NaCl stress usually influences the osmotic potential in the soil due to which plant can absorb limited water only. As a result, stomatal conductance is reduced in *Stevia* and growth is also affected, as demonstrated in other plants (Acosta-Motos *et al.*, 2015).

In our study, total soluble protein content were enhanced with the increase of salt concentration in MS medium. Total soluble protein content usually increase in plants under salinity stress because it might provide plants with a storage form of nitrogen which can be re-utilized. Sajid & Aftab (2022) reported that in salt stress conditions, soluble protein content become higher in some salt tolerant cultivars of potato. It was reported that salt tolerant plants had higher soluble protein content under salt stress conditions (Azzam *et al*., 2021). In contrast to this, Azevedo *et al*., (2009) studied total soluble protein under salt stress and demonstrated that salt sensitive plants had higher protein content than salt tolerant plants, *i.e*., spring maize and wheat. In contrast, Saed-Moocheshi *et al*., (2017) reported that total soluble protein was decreased under salt stress in *Rubus idaeus.* Amini & Ehsanpour (2005) measured the total soluble protein content of two cultivars of tomato and reported that increase and decrease of total soluble proteins depended on the genotype of a plant.

In our study, when stress was increased, activities of antioxidant enzymes were also increased which probably helped the plants to cope with reactive oxygen species (ROS). Under stress environment, a protective system known as the antioxidant enzyme system is triggered. Antioxidants are quenchers of ROS which helps the plants to compete the oxidative stress. When NaCl level was increased, amount of SOD was also increased to combat ROS and through this it played an important role in the survival of plants under stress. Under stress conditions, firstly plants increase the production of superoxide dismutase (SOD). Combined activity of SOD, CAT and POD is important in minimizing effects of ROS, as SOD is an enzyme which converts O_2^- into H_2O_2 which is further broken by CAT and POX (Wang *et al.*, 2022). SOD activity isalso increased under salt stress (Hasanuzzaman *et al.*, 2021). Talaat & Todorova (2022) investigated that under salinity stress, activities of POD enzyme were increased in cultivars of wheat. Salt tolerant plants have greater POD activity rather than salt sensitive plants alfalfa and potato under salt and drought stress condition (Sajid and Aftab 2012; Kumari *et al.*, 2015; Wang *et al.*, 2022).

Salinization causes an increase in Na^+ in the cytosol which impairs the photosynthetic system. Due to this, the absorbed light becomes higher than the demand of photosynthesis and finally produces ROS in plant organs (Asada, 2006). Salt stress causes the generation of ROS, such as O_2^-, O^-, OH^-, and H_2O_2 (Hasanuzzaman *et al.*, 2021). ROS are also produced in plants during normal functions and helps in signaling processes but in stress conditions they cause damage to DNA, proteins, chlorophyll pigments and enzymes. It is a complex phenomenon to tolerate NaCl stress which involves various processes such as ionic balance, protection of photosynthesis system, accumulation of osmolytes and ROS scavenging mechanism (Acosta- Motos *et al.*, 2015; De-Andrade *et al.*, 2021). Zeng *et al.*, (2013) and Cantabella *et al.*, (2017) characterized the *Stevia* as a slightly salt tolerant plant by altering the numbers and structure of chloroplasts to combat the NaCl stress. It is considered that like other herbs, *Stevia* has adopted different strategies to defeat the problems of osmotic stress and limited stomatal conductance.

Conclusion

Stevia rebaudiana is a crop of recent domestication in the world. Salinity is the basic problem spread around the world. It affects the physiological and biochemical features of plants. This study reveales that the plant growth is severely inhibited by salinity, however, increasing the activities of antioxidant enzymes might be helpful to reduce the damaging effect of salt stress. However, this necessitates further investigations to draw some conclusive picture regarding the role of antioxidant enzyme activities in enhancing growth of *In vitro* grown stevia plant under salt stress.

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