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In vitro Regeneration and Effect of Abiotic Stress on Physiology and Biochemical Content of *Stevia Rebaudiana* 'Bertoni'

Research Article

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Abstract

The present work aim to regenerate *S. rebaudiana* 'Bertoni' under *in vitro* condition and also investigating the effect of salinity and drought stress on growth, physiological response and biochemical content. Cultures were established on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and Kinetin (Kn) individually and/or in combination. The highest frequency of bud break (90%) as well as bud elongation (95%) was obtained on MS medium supplemented with 0.5 mg/l BAP and highest mean number of shoot (30) and shoot length (12.2cm) with 1.0 mg/l BAP + 0.5 mg/l Kn along with rooting at 2.0 mg/l IBA. Moreover, when plants were subjected to NaCl and mannitol, significant decrease in shoot number, shoot length, root number and root length were observed with the increasing concentration of NaCl and mannitol (25, 50, 75 and 100mM) while significant increase was found in all parameters with increasing number of days (10, 20 and 30) as compared to control. In addition, shoot/root ratio, leaf no., leaf dry weight, leaf fresh weight, stem dry weight, root dry weight and shoot dry weight were continuously decreased ($p < 0.05$) as NaCl and mannitol concentration increased. The rate of proline accumulation was continuously increased and protein content was continuously decreased at different concentration of NaCl and mannitol. Moreover, significant reduction in chorophyll content was also observed with increasing concentrations of NaCl and mannitol. The results showed that salinity and drought stress significantly reduces the growth and yield components of *S. rebaudiana* 'Bertoni' by affecting endogenous growth hormones.

Keywords: *In vitro* regeneration; Salinity stress; Drought stress; *S. Rebaudiana*; Biochemical content

Abbreviations: BAP: 6-Benzylaminopurine; NAA: α-Naphthaleneacetic acid; IAA: Indole-3 acetic acid; 2 4-D: 2,4-Di-chlorophenoxyacetic acid; Kn: Kinetin; FBB: Frequency of bud break; FBE: Frequency of bud elongation; MNS: Mean no. of shoot; MSL: Mean shoot length; MNR: Mean no. of root; MRL: Mean root length

Introduction

In view of current situation of food insecurity, particularly in developing countries, a number of factors cause a decrease in crop productivity. Of them, availability of agricultural land, fresh water resources, ever-increasing abiotic stresses, and low economic activity in agricultural sector are the most important factors. However, it is generally believed that abiotic stresses are considered to be the main source of yield reduction. Both water and salt stress affected more than 10% of arable land, which results in rapid increase in desertification and salinization world-wide. As a consequence, average yields of major crops reduced by more than 50% [1]. Due to this reason, there is an increasing demand for new plant cultivars that have a potential for higher yield under such abiotic adversaries.

Stevia [Stevia rebuaidana (Bertoni); family: Asteraceae] is a

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perennial shrub widely planted in many countries such as China, Japan, Korea, India and certain countries of South America [2]. It is a natural sweetener plant known as sweet wee, sweet leaf, sweet herb on honey leaf, which is estimated to be 300 times sweeter than sugarcane [3]. Stevioside and rebaudioside are the active principal constituents of diterpene glycosides with varying sugar molecules present in leaves of stevia plant are responsible for high sweetness. Stevioside is abundantly available in leaves (13-20% in dry leaves) with 250-350 times sweetness. Rebaudioside (1-3% in dry leaves) is 350-450 times sweeter while that of fresh healthy leaves are only 30 times sweeter than table sugar. This is used as a substitute for saccharose, and in the treatment of diabetes mellitus, obesity, and hypertension and in caries prevention [4].

The seed of Stevia show very low germination percentage [5] and propagation by seeds does not allow the production of homogenous population resulting in variability in sweetener level and composition. Vegetative propagation by stem cuttings is also limited by the low number of individuals that can be obtained simultaneously from single plant. Micropropagation can provide genetically uniform plants in large numbers. However, with climate change and land clearing or irrigation, a large quantity of salt and alkali land have occurred in these areas, which possessed excellent light conditions for the growth and glycoside accumulation of *S. rebaudiana*. Previous studies on plants under salt stress had revealed alterations in the biomass, antioxidant enzyme activities and osmolytes, mineral contents, and secondary metabolites [6]. However, salt effects on the survival and productivity of *S. rebaudiana* have rarely been reported [7]. Thus, investigating these responses is difficult under field conditions, but plant tissue culture techniques are performed under aseptic and controlled environmental conditions. The advantages of plant tissue culture allow various opportunities for researcher to study the unique and complex responses of plants against environmental stresses.

Materials and Methods

Plant Material and *In vitro* Regeneration

A plant of family *Asteraceae* was selected as experimental material, obtained in the form of *Stevia rebaudiana* Bertoni plant growing in the Botanical Garden of Anand Agricultural University, Anand (Gujarat) INDIA. Axillary bud from the actively growing new shoots were excised and used as explant. The explants were rinsed in running tap water for 20 min, cleaned in 10% liquid soap solution for 5 min and then rinsed with double distilled water 2-3 times. The explants were surface sterilized with 0.1% mercuric chloride solution and for 2-3 min in 70% ethanol. Finally, the explants were washed in sterilized double distilled water and cultured on MS medium (Murashige and Skoog 1962) with vitamins, 3% sucrose, 0.8% agar supplemented with different concentrations (0.5-2.0 mg/l) of 6-benzylaminopurine (BAP) and Kinetin (Kn) for establishment and multiplication of culture. The pH of the nutrient medium was adjusted to 5.8 and 0.8% agar (Himedia, Mumbai) was added prior to autoclaving. The regenerated shoots were cultured on half-strength MS medium fortified with auxins viz. indole acetic acid (IAA) at different concentrations (0.5-2.0 mg/l) to study their effects on rooting. All the cultures were incubated at 27±2 ˚C under 2,000-2,500 lux for 16 h/day.

Plantlets with well developed roots were washed thoroughly with distilled water and cultured in a mixture of sand and peat (1:1) in pots and transferred to green house. The potted plants were irrigated and initially covered with plastic bags, which was gradually eliminated within four weeks for completing their acclimatization.

In vitro salinity and drought stress studies

In vitro salinity and drought stress conditions was developed as described by Debnath et al. [8] with modification in the concentration of growth regulators, NaCl and mannitol. For this, cluster of multiple shoots were separated and re-inoculated on MS medium supplemented with 1 mg/l BAP + 0.5mg/l Kn and different concentration of NaCl (0, 25, 50, 75 and 100 mM) and mannitol (0, 25, 50, 75 and 100 mM) for salinity and drought stress, respectively. These growing explants were retrieved from the modified MS medium at 10, 20 and 30 days interval for evaluation of plant development along with biochemical quantification of proline, protein and chlorophyll.

Biochemical constituent's analysis

Extraction and estimation of Proline Assessments of proline content were performed twice during the experimental period, at 10, 20 and 30 days after the onset of the experiment. Proline was extracted from sample of 0.5g fresh leaf material samples in 3% (w/v) aqueous sulphosalycylic acid and estimated using the ninhydrin reagent according to the method of Bates et al. [9]. The absorbance of fraction with toluene aspired from liquid phase was read at a wave length of 520 nm.

Extraction and estimation of Protein Protein extraction was performed as described by Hurkman and Tanaka [10]. 1 g fresh tissue was grinded in a chilled mortar and pestle with 2.5 mL of Tris (pH 8.8) buffered phenol and 2.5 mL of extraction media (0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2- mercaptoethanol, 0.9 M sucrose). The homogenate was centrifuged for 10 min at 5000 g for 4 ˚C. The aqueous phase at the bottom was mixed with 2.5 mL of extraction media and phenol by vortexing. The phenol phase at the top of the extract was separated twice by this method and pooled together. Proteins was precipitated from the phenol extract by adding 5 volumes of 0.1 M ammonium acetate in 100% methanol (stored at -20 ˚C) and centrifuging at 20,000 g, 4˚C for 20 min. The precipitate protein was incubated overnight at – 20 ˚C.

Protein quantification was performed as described by Lowry et al. [11]. To 1 ml of above solution, 5 ml of freshly prepared alkaline CuSO4 reagent [1 ml of Alkaline Na2CO3 reagent to 50 ml of Copper Sulphate reagent] was added mixed properly and after 10 min Folin's reagent (0.5 ml) was added. The solution was mixed instantaneously and allowed to develop the colour. Absorbance was recorded at 750 nm.

Extraction and estimation of chlorophyll Following extraction of liquid-nitrogen frozen leaf with 80% acetone, the concentration of chlorophyll was quantified by spectrophotometer method of Porra et al. [12].

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Statistical analysis and growth evaluation

The morphogenetic responses of the explants was assessed and recorded regularly up to a maximum of 15 passages. For each treatment, 12 replicates were maintained and each experiment was repeated at least three times. The results were noted in the form of Mean ± Standard Error (SE). The growth parameters was calculated as frequency of bud break (FBB), frequency of bud elongation (FBE), mean number of shoot (MNS), mean shoot length (MSL), mean number of root (MNL) and mean root length (MRL). The data recorded were analyzed statistically using analysis of variance technique including one-way and two-way ANOVA.

Results and Discussions

In vitro Regeneration of *Stevia rebaudiana* 'Bertoni'

Initiation or establishment of culture In vitro propagation studies show that plant hormones play an important role in regeneration of culture. For establishment of culture, different cytokinin (BAP and Kn) was used. The axillary bud explants were cultured on MS media supplemented with different concentration (0.5, 1.0, 1.5 and 2.0 mg/l) of BAP and Kn. BAP at 0.5 mg/l stimulated proliferation of the bud meristems to form bud clusters and maximum frequency of bud break (FBB) reached 90% and bud elongation reached 95% as compared to higher concentrations of BAP where slow growth was observed. Similar result was obtained by Abd-Alhady [13]. The stimulating effect of BAP on bud break has been reported earlier by Debnath [14]. In the initiation stage, Kn also showed maximum frequencies of bud break (85%) and bud elongation (92%) at 0.5 mg/l Kn (Figure 1a; Figure 2a) but lower than BAP while at concentration greater than 0.5 mg/l treatment resulted decline in all parameters.

Multiplication and elongation of Shoots The proliferation and multiplication of the shoots were also influenced by hormones when added singly or in combination. The mean number of shoot (MNS) per explant and mean shoot length (MSL) varied under various cytokinin concentrations and combinations (Figure 1b; Figure 2b, c). The effect of different concentration of BAP used was found to be significant for increasing the number of shoots (mean number of shoot, MNS) after 3-4 weeks of inoculation. Maximum number of shoots (14±0.94) and shoot length (10.2±0.83) was observed at 0.5mg/l concentration while at concentration higher than 0.5 mg/l treatment, the shoot number declined. It was clear that among different concentration of Kn used, only at 0.5 mg/l Kn concentration, the highest number of shoots (12±0.65) and shoot length (10.4±0.14) after 3-4 weeks was observed while at higher concentration than 0.5 mg/l Kn, the shoots number declined. Between the two cytokinins tested, BAP was more effective than Kn for shoot multiplication. When cultures were subjected to different combinations and concentrations of BAP and Kn, significant changes were observed. Our data revealed that *Stevia rebaudiana*, Bertoni cultures treated with 1.0 mg/l BAP in combination with 0.5 mg/l Kn found significant increase in shoot number (30±1.83) and shoot length (12.2±1.83) while concentration lower or higher declined MNS and MSL (Figure 1b). Abd-Alhady

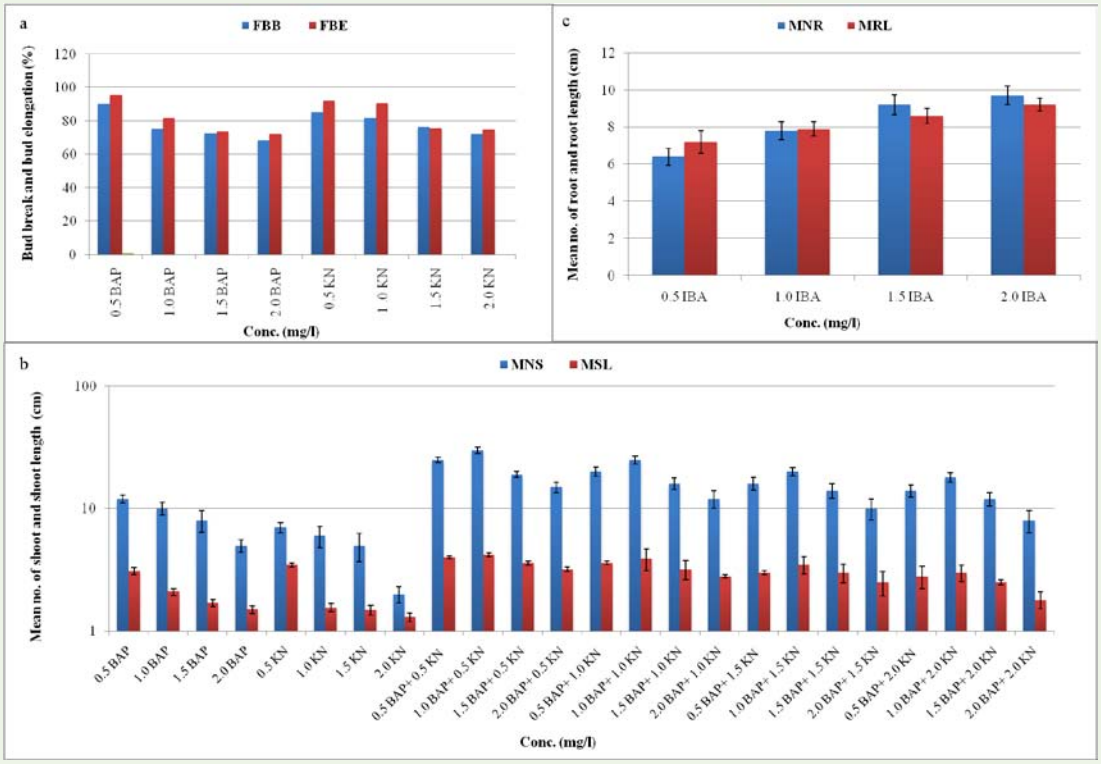

Figure 1: *In vitro* regeneration of *Stevia rebaudiana* Bertoni under aseptic medium a) Initiation or establishment of culture at different concentration (0.5, 1.0, 1.5 and 2.0mg/l) of BAP and Kn b) Multiplication of explants at different concentration and combination of BAP and Kn c) Rooting of plantlets at different concentration of IBA (0.5, 1.0, 1.5 and 2.0mg/l).

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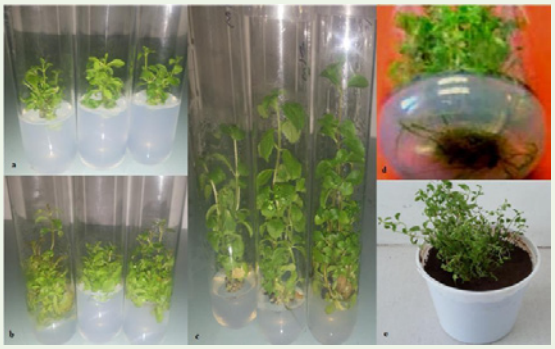

Figure 2: *In vitro* regeneration of *S. rebaudiana*, 'Bertoni' a) Initiation and/ or establishment of culture in MS supplemented with 0.5 mg/ l BAP b, c) multiplication and elongation of culture in MS supplemented with 1 mg/l BAP + 0.5 mg/l Kn d) rooting of plantlet on ½ strength MS medium supplemented with 2.0 mg/l IBA and e) Hardening of plantlet in pot containing sand and peat mixture (1: 1).

[13] reported highest shoot multiplication in medium supplemented with 1.0 mg/l BAP + 0.5 mg/l Kn (36.9 shoots per explant) and lowest shoot multiplication at 0.5 mg/l + 0.5 mg/l Kn in *Stevia rebaudiana*, Bertoni. Supplemented Kn to the medium enhanced the elongation of shoots.

Root Induction and acclimatization of plant After two cycles of sub-culturing, elongated shoots of 2-3 cm in length were excised and cultured on MS medium having different concentration of IBA. The experiments were conducted twice, with 3 replications (with 3 shoots per tubes). Initiation of rooting took place after 1-2 week of inoculation. Single and multiple roots were formed from the base and the nodal portions within 2-3 weeks. The highest number of root (MNR; 9.7±0.45) and root length (MRL; 9.2±0.17) was observed on ½ strength MS medium supplemented with 2.0 mg/l IBA while lowest number of root (6.4±0.45) and root length (7.2±0.60) occurred in ½ strength MS medium supplemented with 0.5 mg/l IBA (Figure 1c; Figure 2d). Similar results were observed by Debnath [14]. Abd-Alhady et al. [15] also stated that IBA gave better response for rooting of *Periploca angustifolia* than NAA.

Plantlets with well-developed root systems were transferred to pots containing sand and peat mixture (1:1) and covered with translucent plastic bags to ensure high humidity around the plants. The use of this procedure during the acclimatization phase ensured that most of the plantlets transplanted to ex vitro conditions continued to grow vigorously. After two months when the plastic bags were completely removed, 70% of the plantlets survived in the greenhouse and showed normal growth of the plant.

The result of the experiment and other earlier research reports clearly support the possibility of propagating *S. rebaudiana,* Bertoni, by adopting *in vitro* techniques. Thus, *In vitro* propagation can become an important alternative to conventional propagation and breeding procedures for wide range of plant species.

In vitro salinity and drought stress studies

Fundamentally, plants require energy (light), water, carbon and mineral nutrients for growth. Abiotic stress is defined as environmental conditions that reduce growth and yield below optimum levels. Plant responses to abiotic stresses are dynamic and complex [16]; they are both elastic (reversible) and plastic (irreversible).

Effect of NaCl and mannitol on plant growth Plant growth inhibition is a phenomenon often occurring in glycophyte or salt-sensitive plant species under salinity and drought conditions. In the present study, salt and drought stress induced significant differences on plant growth during the experimental period. The significant decrease in the shoot number and shoot length was observed with the increasing concentration of NaCl (25, 50, 75 and 100mM) and significant increase in shoot number and shoot length with increasing number of days (10, 20 and 30) (Supplementary file 1A; Figure 3a,b; Figure 4). The highest number of shoot (~25) and shoot length (10.7cm) was observed at 25mM after 30 days and lowest number of shoot (~7) and shoot length (6.5cm) at 100mM after 10 days of treatment as compared with control. The plants grown under control (no salinity application) exhibited the maximum values for shoot number (~28) and shoot length (12.6cm) as compared to remaining treatments after 30 days of culture, which indicated that salinity is responsible for reduction in shoot number and shoot length. Shahid et al. [17] observed the reduction in shoot length with increasing concentration of NaCl. Ashraf et al. [18] also reported that salt stress affects the plant growth and development by influencing fresh and dry weight of roots, shoots along with shoot length. This might be explained as the inadequate photosynthesis caused by stomatal closure and the reduction of carbon assimilation rate under salt stress [6].

Drought is a multidimensional stress affecting plants at various levels of their organization [19]. The response to drought at the whole plant and crop levels is complex because it reflects the integration of stress effects and responses at all underlying levels of organization over time and space. In the present investigation, drought stress exhibited significant differences on shoot number and shoot length during the experimental conditions. After 20 stress days, decreases in shoot number and shoot length was observed in plants grown with 25, 50 75 and 100mM mannitol (Figure 3a, b; Figure 5). In case of shoot number, significant ($p < 0.05$) differences were not observed with the increasing number of days (Supplementary file 1A). The means values of shoot number and shoot length were 16, 14.3, 10.3, 7.3, 3.7 and 10.5, 10.3, 9.2, 7.4, 7.1 cm for treatments of 0, 25, 50, 75, and 100mM mannitol, respectively.

Salinity and drought stress also exhibited significant differences on root number. With the increasing concentration of NaCl and mannitol significant decrease in root number were observed after 10, 20 and 30 days. Interestingly, at 100mM Mannitol, decrease in root number 4, 3 and 2 were observed with increasing number of days 10, 20 and 30 days, respectively (Supplementary file 1A). Moreover, salinity stress significantly increases the root number after 10, 20 and 30 days while drought stress significantly decreases the root number during the experimental condition. Similar result was observed by Abdel-Hussein [20] who evaluated MM106 and Omara apple rootstocks for salt tolerance *in vitro*. The reduction in rooting percentage might be due to the inhibitory effects of salt on the metabolic activities which associated with cell division, differentiation and elongation [21].

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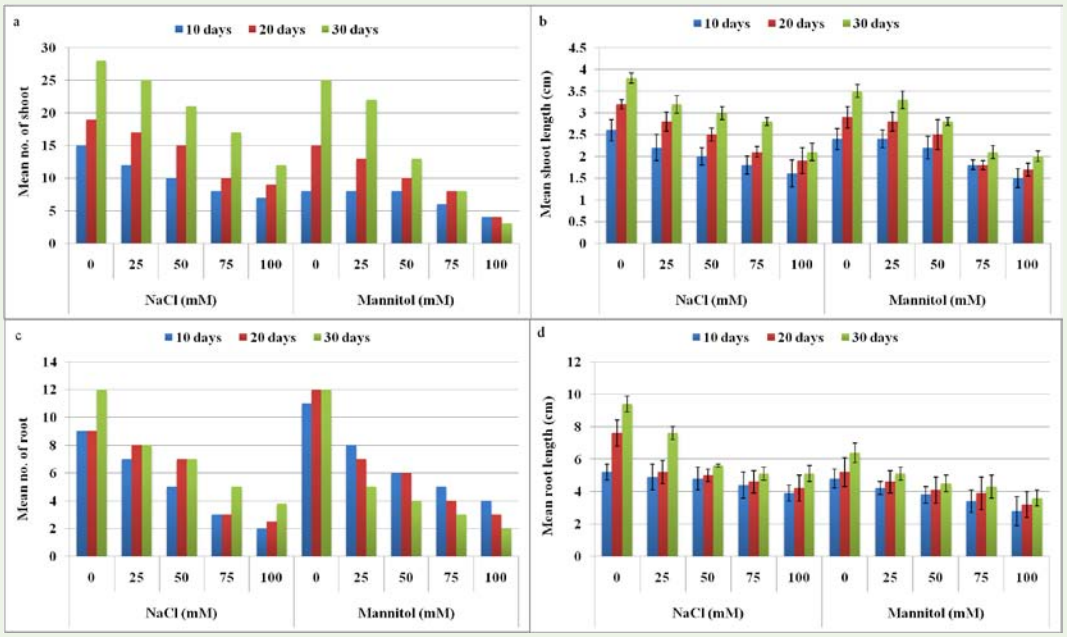

Figure 3: *In vitro* effect of different concentrations (0, 25, 50, 75 and 100mM) of NaCl and mannitol stress on *Stevia rebaudiana* 'Bertoni' a) Mean number of shoot after 10, 20 and 30 days of culture b) Mean shoot length after 10, 20 and 30 days of culture c) Mean number of root after 10, 20 and 30 days of culture d) Mean root length after 10, 20 and 30 days of culture.

Figure 4: Effect of salinity stress on plant growth and development at different concentrations 25 (a), 50 (b), 75 (c) and 100mM (d) of NaCl.

Figure 5: Effect of drought stress on plant growth and development at different concentrations 25 (a), 50 (b), 75 (c) and 100mM (d) of mannitol.

By following the root length of the plants under salinity and drought stress during the treatment periods, Fig 3 it appears there is a general trend for the decrease in the root length of the plant using the concentration 25, 50, 75 and 100mM while significant increase in the root length after 10, 20 and 30 days. The means values of root length were 7.4, 5.9, 5.1, 4.7 and 4.4 cm and 5.5, 4.6, 4.1, 3.9 and 3.2 cm for treatments of 0, 25, 50, 75, and 100mM NaCl and mannitol, respectively. Ashraf et al. [18] report that plants grown under control (no salinity application) exhibited the maximum values (10.11 cm) for root length as compared to remaining treatments, which indicated that salinity is responsible for reduction in root length. Thiam et al. [22] reported that root length was adversely affected with a significant reduction of NaCl concentration (50 and 100 mM) and reached 6.95cm and 6.33 cm, respectively. Their findings suggest that high NaCl concentration significantly affect the root growth.

Moreover, salinity and drought stress resulting significant decrease in the leaf FW, leaf DW, stem DW, shoot DW and root DW after 25, 50, 75 and 100mM treatment. The significant reduction of leaf FW 36% and 54%, leaf DW 79% and 55%, stem DW 66% and 60%, shoot DW 38% and 55%, root DW 72% and 51% were observed at 100mM NaCl and mannitol treatment, respectively. Furthermore, the significant percentage difference in between the salinity and drought stress conditions were 25%, 21% and 6% of leaf DW, root DW and stem DW whereas no significant difference were observed in shoot DW and leaf FW (Supplementary file 1B; Table 1, 2).

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Zeng et al. [23] reported that plants treated with 90 and 120 mM NaCl showed significantly lower plant height, leaf number, branch length, stem dry weight, leaf fresh/dry weight, and shoot dry weight than control. They also found that high salt treatment (120 mM NaCl) drastically decreased the total dry weight of *S. rebaudiana* by 40% in comparison to control. In addition, shoot/ root ratios of salt-treated plants were inhibited markedly by 30-50% compared with that of the control. The results obtained in the present experiment verified this conclusion that as the salinity increases, the biomass content of *S. rebaudiana* significantly reduces than control. Srivastav and Srivastav [24] also presented the similar findings that severe drought stress conditions reduce the plant biomass of *S. rebaudiana*. These results were similar to the findings of Pirzad et al. [25] and Mohammadian et al. [26].

Leaf is the main plant organ responsible for light absorption and transformation and plays an important role in providing energy and nutrients for the growth and development of plants. The growth inhibition caused by salt and drought stress mainly originated from the damage to leaf function. Results presented in Table 1 and Table 2 showed that higher levels of salinity and drought decrease leaf number throughout the experiment. In the present study, the number of fully expanded leaves decreased dramatically with increasing salt level, which could be seen as the combined effect of increasing the senescence rate of older leaves and decreasing the production of new leaves. Zeng et al. [23] reported leaf chlorosis, wilting, and necrosis were in plants treated with 90 and 120 mM NaCl for 28 days, respectively. It was found that the general trend of the treatment reflects a gradual decrease in the number of plant leaves with the increase of NaCl and mannitol concentration, compared with the plants of the control experiment.

Leaf number significantly reduced as increasing concentration of NaCl and mannitol. It was found that the general trend of the treatment reflects a gradual decrease in the number of plant leaves with the increase of salt concentration, compared with the plants of the control experiment. The maximum number of leaves 44.8 and 43.5 were found at 25mM and minimum number of leaves 22.5 and 26 at 100mM NaCl and manitol stress treatments, respectively. According to Silva et al. [27] leaves were more sensitive than plant height after 7 days of treatment. After 36 days, significant reduction in leaves number at all NaCl levels was observed. Shao et al. [28] reported that reduction in number of leaves under water stress is may be due to decline in cell enlargement and more leaf senescence resulting from reduced turgor pressure. On the other hand, significant reduction in shoot/root ratio was also observed as NaCl and manitol concentration increase (Table 1). Shoot growth was probably more susceptible to salt than root, which generally led to a lower shoot/root ratio in salt treatments. Zeng et al. [23] found significant reduction in shoot/root ratios in all NaCl treatments than in control. Moreover, Nejad [29] studied on effect of drought stress on shoot/root ratio with four levels of water stress as the first factor and plant growth periods as the second factor with four replications. Their findings suggest that increasing drought stress with each period of growth, shoot and root weight decreases per plant. Previous studies suggested that this sensitivity could be due to an imbalance among cations as a result of the complex interaction in the xylem transport system.

Effect of salinity and drought stress on protein content Figure 6b

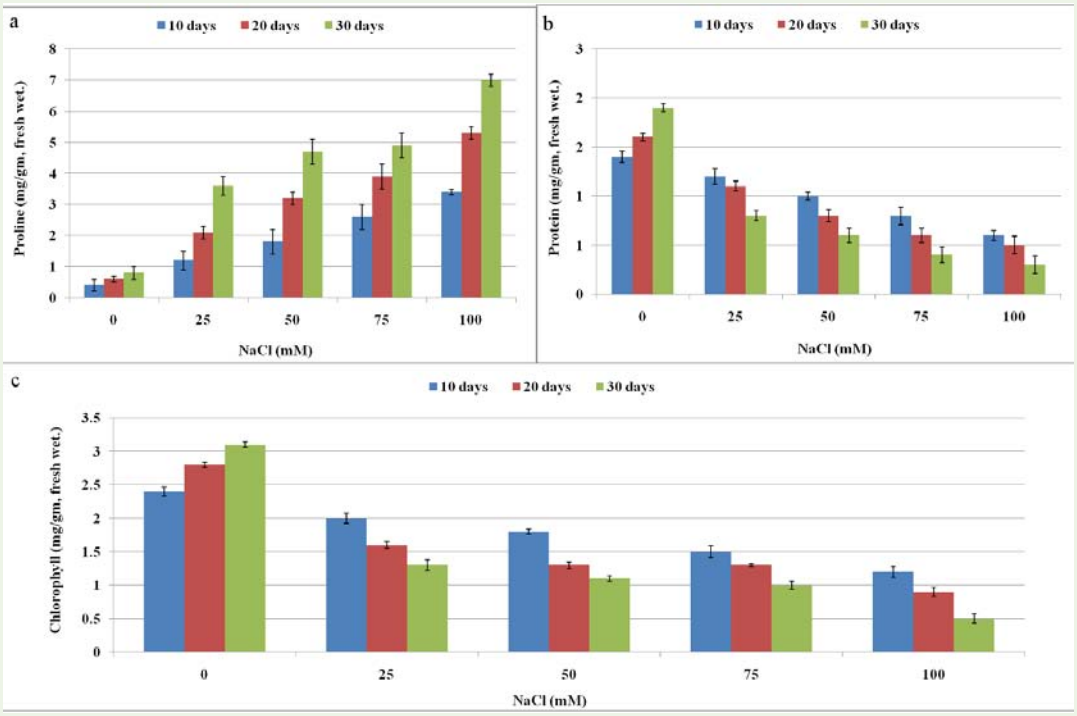

Figure 6: Effect of different concentrations (0, 25, 50, 75 and 100mM) of NaCl on biochemical content of Stevia rebaudiana 'Bertoni' a) Effect of salinity stress on proline content of plant b) Effect of salinity stress on protein content and c) Effect of salinity stress on chlorophyll content.

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and Figure 7b results indicate a reverse effect of sodium chloride and mannitol using various concentrations on total protein of *S. rebaudiana* plant after 10, 20 and 30 days. It appears from the data that there was a general decrease in protein content that corresponded with the increase in NaCl and mannitol concentrations as compared with the control plant. Statistical analysis did show a significant difference during the three measurement periods (Supplementary file 2A, B).

Chen et al. [30] found that exposing *Vigna unguiculata* (L.) plants, at the age of 14 days, to salt treatment using 75mM of NaCl, reduced soluble protein content in the plant. The results of the previous study was confirmed by Cheruth et al. [31], with their study on *Cathranthus roseus* (L.) and Khosravinejad et al. [32] with their study on barley

Table 1: Effect of different concentration of NaCl on growth parameters of *S. rebaudiana 'Bertoni'*.

NaCl (mM)	Leaf Number	Shoot/root ratio	g plant^{-1}				
			Leaf DW	Stem DW	Root DW	Shoot DW	Leaf FW
0	55.9	10.9	0.87	0.37	0.15	1.27	4.48
25	44.8	9.7	0.85	0.31	0.13	1.02	4.01
50	36.2	7.8	0.73	0.29	0.13	0.80	3.30
75	24.1	6.2	0.71	0.26	0.12	0.58	2.71
100	22.5	4.8	0.69	0.24	0.11	0.48	1.63

Table 2: Effect of different concentration of mannitol on growth parameters of *S. rebaudiana 'Bertoni'*.

Mannitol (mM)	Leaf Number	Shoot/root ratio	g plant^{-1}				
			Leaf DW	Stem DW	Root DW	Shoot DW	Leaf FW
0	47.0	9.5	0.83	0.30	0.18	1.33	4.95
25	43.5	7.3	0.78	0.27	0.17	1.25	4.65
50	37.0	5.4	0.57	0.25	0.15	1.21	4.10
75	31.5	4.8	0.53	0.23	0.13	0.83	3.40
100	26.0	4.1	0.45	0.18	0.09	0.73	2.65

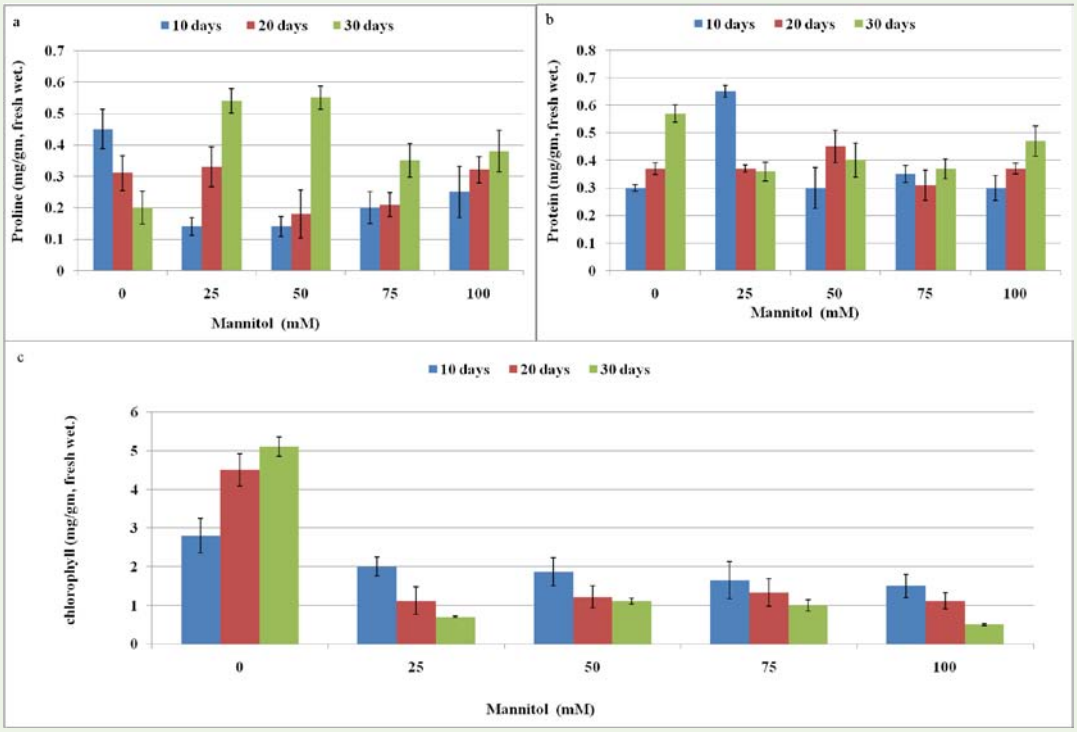

Figure 7: Effect of different concentrations (0, 25, 50, 75 and 100mM) of mannitol on biochemical content of *Stevia rebaudiana* 'Bertoni' a) Effect of drought stress on proline content of plant b) Effect of drought stress on protein content and c) Effect of drought stress on chlorophyll content.

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Hordeum vulgare L. On the contrary, Sibole et al. [33] reported that the treatment of clover plant (*Medicago citrna* L.) for 30 days with concentrations of 0, 1, 50, 100, 200 mM of NaCl increased soluble protein content in the seedlings, compared with control plants. Another study by Kapoor and Srivastava [34] on *Vigna mungo* (L.) supports the previous results.

In contrast to effect of drought stress on protein concentration, there were no significant differences observed with the increasing concentration of mannitol as compared to control plants. However, the amount of protein (mg/gm) increases after 10, 20 and 30 days of culture in control plant. Several researches indicated that soluble protein decreases with increasing drought stress time [35,36]. The water stress injury causes damage to protein synthesizing mechanism. The possible reason for decreased protein content under water stress may be due to increased activity of protease and also it may be due to proteolysis or decreased synthesis or both. Leaf proteins undergo accelerated hydrolysis as water stress develops.

Effect of salinity and drought stress on proline content We analyzed the proline content of plant grown at 0, 25, 50, 75 and 100mM NaCl and mannitol. Results showed that amount of proline increased with the increasing concentration of NaCl and mannitol (Figure 6a; Figure 7a). Statistically significant differences in proline accumulation were determined between the control and treatment periods of NaCl while in drought stress condition no significant differences were observed (Supplementary file 2A, B). Proline accumulation in salt stressed plants is a primary defense response to maintain the osmotic pressure in a cell, which is reported in salt tolerant and salt sensitive cultivars of many crops [37-39]. Zhang et al. [23] reported that proline content enhanced significantly with increasing concentration of NaCl and was 17-, 31-, and 42-fold higher in 60, 90, and 120 mM NaCl than in the control, respectively. Several studies have indicated that proline content increases during drought stress [40,41].

Effect of salinity and drought stress on chlorophyll content Decrease in chlorophyll content was determined as a result of increasing NaCl and mannitol concentration. The highest content of chlorophyll was found at 25mM after 10 days of culture while lowest content was found at 100mM after 30 days in both salinity and drought treatment conditions. The significant decreasing percentage of chlorophyll was 50 % at 100mM NaCl treatment after 10 days of culture that further reduced (16 %) after 30 days of stress treatment. Moreover, significant reduction (54 %) was observed at 25mM after 100 days of mannitol that decrease (10 %) further after 30 days of culture (Figure 6C; Figure 7C). Statistical analysis shows that significant reduction in chlorophyll content was found at each concentration level (25, 50, 75 and 100mM) but data obtained after 10, 20 and 30 days of culture was found no significant in both salinity and drought stress plants (Supplementary file 2A, B). The decrease in chlorophyll content may be due to an increase of chlorophyll degradation or to a decrease of chlorophyll biosynthesis. Change in chlorophyll contents due to salinity is the most evident biochemical response [42]. Amira et al. [43] also found the similar results on propagation of plant *Cathranthus roseus* under salinity and drought conditions where they reported that reduction in chlorophyll associated with reduced photosynthetic activity and transpiration rate in plants at all salinity levels is considered a defense mechanism against damaging reactive oxygen species by diminishing light absorbing capacity that reduces the flow of electrons through the photosysteme. Moreover, disruption of the thylakoid and stromal membranes may result in leaf chlorosis and necrosis, Qureshi et al. [44] which indicated that salt concentration above 90 mM NaCl could impair the chloroplast structure of *S. rebaudiana*. In contrast to drought stress condition, decrease in the chlorophyll was observed which is reported first time in *S. rebaudiana*. No such study was previously reported in *S. rebaudiana*.

Summarizing, salinity and drought stress significantly affect the growth and development as well as biochemical content of *S. rebaudiana*. As the concentration of NaCl and mannitol increased physiomorphologic and biochemical components i.e. proline, protein and chlorophyll contents negatively affects the growth and development of plants but 50 % of plant was survived at the concentration (50mM) of NaCl and mannitol. These results indicate the mutant development at such concentration. Further research is in progress to decipher the expression profile of four major genes involved in steviol glycoside pathway under salinity and drought conditions and compare with HPLC results for stevioside and rebaudioside i.e. responsible for sweetening property, content with control plant.

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