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Response of NaCl and Brassinolide in *Stevia rebaudiana* under *in vitro* Condition

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The current work sought to investigate the impact of two chemicals (NaCl and Brassinolide) on growth parameters, chlorophyll pigments in stevia shoots cultured under *in vitro* condition. For this, nodal explants of stevia were cultured for four weeks on MS medium supplemented with 0.2 mg l^{-1} BAP, Kinetin 0.2 mg l^{-1} and various concentrations of NaCl (0, 60, 70, 90, 120, 150 mM) and BL (0, 1, 2, 3 4, 5µM) for screening under *in vitro* condition. Explant treated with NaCl showed reduction with increasing salinity levels in all the studied parameters while explants treated with Brassinolide showed best results in all the studied parameters. From the results, it is concluded that the stevia plant was less impacted by 60mM and 70mM NaCl concentrations when compared to other concentrations of NaCl, but BL increased/improved all the examined parameters when compared to control; 1µM and 2µM concentrations of BL responded the best.

Keywords: Brassinolide (BL); growth parameters; in vitro; photosynthetic pigments; stress; Stevia spp.

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1. INTRODUCTION

Stevia rebaudiana (Bertoni) is a tiny shrub of the Asteraceae family. Attempts to cultivate the plant in Paraguay immediately after Bertoni's discovery were unsuccessful because it was difficult to grow from cuttings, and the seeds [1]. It is used as a natural sweetener in food. Due to the high demand for stevia, a cost-effective technology is required for mass-producing its planting materials.

In *S. rebaudiana* cultivation, high soil salinity is a key agricultural constraint because it restricts plant growth and output [2] Because under high levels of salt (Na^+) in the soil, most crops have a difficult time for surviving and resuming their normal development processes [3,4]. Several investigations on the impact of salt on stevia have recently been conducted and found to be influenced by varying degrees of salinity, indicating that this plant is relatively sensitive to salt stress [5-9]. Salt-stressed plants have fewer shoots, smaller dry leaves, and stunted growth, as well as the breakdown of plant pigments such as chlorophyll and carotenoids, which disrupt the photosynthetic machinery and impede overall growth [10,11]. As a result, it is critical to develop a new strategy for increasing plant salinity resistance.

Brassinolide (BL) is a naturally occurring plant steroid that has pleiotropic effects [12]. In the past few decades, BL application has been known as the main strategy for enhancing crop tolerance to abiotic stress. Singh et al. [13] reported the feedback of brassinosteroids and their analogs under *in vitro* conditions. BL is necessary for plant growth and development, cell division, cell elongation, photosynthesis as well as conferring resistance to biotic and abiotic stressors [14-16].

The objective of this study was to evaluate the performance of stevia plants under salinity stress *in vitro*, and to assess the effectiveness of BL in improving this NaCl induced stress responses, using different concentrations of NaCl and Brassinolide.

2. MATERIALS AND METHODS

2.1 Study Area

The present investigation was undertaken in the Laboratory of Plant Tissue Culture and Stress Physiology, Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India. Healthy plants of *S. rebaudiana* were collected from local nurseries. For selection and screening of best concentration of NaCl and BL, nodal segment (1.5-2 cm long) was employed as an explant that was cultivated in half strength Murashige and Skoog [17].

2.2 Experimental Procedures

MS media was supplemented with hormones 0.2 mgL^{-1} BAP, 0.2 mgL^{-1} Kinetin. The cultures were incubated in the culture room at 25±2°C with a photoperiod 16 h light and 8 h dark having 25 µMol m^{-2} s^{-1} light intensity provided by cool fluorescent tube lights. The relative humidity of about 70-75% was maintained in the culture room throughout the period.

Treatment details were as follow:

Concentrations used for *in vitro* screening of NaCl treatment: 0 mM, 60 mM, 70 mM, 90 mM, 120 mM and 150 mM gL^{-1}.

Concentrations used for *in vitro* screening of BL treatment:0 µM, 1 µM, 2 µM, 3 µM, and 4 µM gL^{-1}.

All these tests were carried out in three replicates. Following a 4-week incubation period in the presence of NaCl and BL, the following parameters were evaluated:

2.3 Days Taken for Primordial Initiation after Culture

The days taken for primordial initiation were counted just days after inoculation (DAI) of explants for screening experiment of NaCl concentrations. The observation was carried out till all the explants got regenerated.

2.4 Length of Shoot (cm explant^{-1})

The shoot length of explants were measured from the base of the explants to the tip of the shoot and average lengths were calculated on per explant basis and expressed in centimeters (cm).

2.5 Number of Leaves Explant^{-1}

The total number of leaves per explant was counted and average was calculated.

2.6 Survival Percentage (%)

The survival percentage was calculated using the given formula:

$$\text{Survival Percentage} = \frac{\text{Number of explants survived}}{\text{Total number of explants}} \times 100$$

2.7 Fresh Weight of Shoot (mg g^{-1})

The fresh weight of shoots was measured and expressed in mg g^{-1} for normal and salinity grown plants. The plant was removed from the test tubes containing media. These were immediately transferred to the lab for measurement of fresh weight of shoot. Shoots were separated before fresh weight was assessed via a Sartorius BT-224S electronic balance.

2.8 Dry Weight of Shoot (mg g^{-1})

Freshly weighed shoots were placed in the envelopes after which these were placed in a Hot Air Oven for one hour at 100°C. The temperature was then decreased to 65°C until the constant weights of the samples could be achieved.

2.9 Photosynthetic Pigments (mg g^{-1} FW)

The chlorophyll and carotenoid levels was estimated by Lichanthaler [18] method.

2.10 Statistical Analysis

Data were analysed using analysis of variance (ANOVA) using SPSS 26.0 for windows software package and the means were separated by Duncan's multiple range tests (DMRT).

3. RESULTS AND DISCUSSION

In vitro screening of NaCl data revealed that increasing salinity stress significantly reduced the days of primordial initiation, shoot length, number of leaves, fresh and dry weight of shoot, chlorophyll a, b, carotenoid content and number of explant that survived in the entire treatment (60-150 mM) (Table 1; Fig. 1.)

In case of days of primordial initiation, the number of days taken for initiation of shoot primordia was found lower at high concentration (150 mM) and higher at lower concentration (60 mM) when compared to control (0 mM). In case of shoot length, lowest value was found in higher concentration of NaCl (150 mM) with around 62.06% reduction. While at lower salinity level (60 mM), reduction of shoot length was 13.79% was observed. In leaf number, the lowest value was found in higher concentration of NaCl (150 mM) (73.68% reduction). While at lower salinity level (60 mM) the reduction of leaves number was only 21.05%. This is in agreement with the previous research where shoot length, number of leaves decreased when salinity stress was applied to *in vitro* cultured stevia plant [9,19].

In case of shoot fresh weight, lowest value was found in higher concentration of NaCl (150 mM) with around 63.82% reduction. While at lower salinity level (60 mM), reduction of 29.78% was observed. In shoot dry weight, lowest value was found in higher concentration of NaCl (150 mM) with around 70% reduction. While at lower salinity level (60 mM) reduction of 20% was observed. Similar studies of reduction in shoot fresh weight of shoot was reported by Ors et al. [20] in tomato, Dolatabadian et al. [21] in soybean, and reduction in shoot dry weight by Dong et al. [22] in sugarbeet, Ibraham et al. [23] in Indian Pennywort.

In case of chlorophyll-a content, lowest value was found at NaCl (150 mM) with around 88.42% reduction. While at lower salinity level (60 mM) reduction of 57.89% was observed. In chlorophyll-b content, lowest value was found in higher concentration of NaCl (150 mM) with around 79.16% reduction. While at lower salinity level (60 mM) reduction of 23.61% was observed. In case of carotenoid content, lowest value was found in higher concentration of NaCl (150 mM) with around 72.54% reduction. While at lower salinity level (60 mM) reduction of 13.72% was observed. Similar findings were reported in stevia [5, 24, 25].

With regard to survival percentage, the lowest value was found in higher concentration of NaCl (150 mM) with about 71% reduction. While at lower salinity level (60 mM) reduction of 21% was noticed. Our findings are supported by Al-Taey et al. [26] in sweet orange, Lotfi et al. [27] in pistachio and Nabati et al. [28] in lentil.

In vitro screening of BL data revealed that BL significantly increased shoot length, number of leaves, shoot fresh and dry weight, chlorophyll a, b and carotenoid content in the entire treatment (60-150mM) (Table 2; Fig. 2.).

Table 1. Effect of different concentrations of sodium chloride (NaCl) on different parameters in *Stevia rebaudiana* Bertoni cultured under *in vitro* condition after 30 days of culturing

NaCl (mM)	DPI	Shoot Length (cm)	Number of leaves	Shoot Fresh Weight (mg g^{-1})	Shoot Dry Weight (mg g^{-1})	Chlorophyll-a (mg g^{-1} FW)	Chlorophyll-b (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	Survival %
0	2.00±0.15a	2.9±0.15a	19±1.53a	0.47±0.02a	0.10±0.01a	1.90±0.02a	0.72±0.02a	0.51±0.02a	100±1.53a
60	3.21±0.06b	2.5±0.06b	15±1.00b	0.33±0.01b	0.08±0.01b	0.80±0.02b	0.55±0.05b	0.44±0.03b	79±1.00b
70	3.42±0.06b	2.4±0.06b	12±0.58c	0.30±0.03c	0.07±0.00c	0.63±0.02c	0.42±0.04c	0.39±0.01c	68±0.58c
90	4.50±0.10c	2.3±0.10c	10±1.00d	0.25±0.01d	0.06±0.00d	0.42±0.02d	0.31±0.01d	0.35±0.01d	56±1.00d
120	5.30±0.10d	1.5±0.10d	8±1.00e	0.21±0.02e	0.05±0.00e	0.33±0.02e	0.21±0.02e	0.24±0.02e	50±1.02e
150	6.89±0.15e	1.1±0.15e	5±1.00f	0.17±0.02f	0.03±0.01f	0.22±0.03f	0.15±0.01f	0.14±0.02f	29±1.00f

where, NaCl=Sodium chloride, DPI=Days of primodia initiation. Data are in the form of mean± SEM, and means followed by the same letters within the columns are not significantly different at P≤0.05

Table 2. Effect of different concentrations Brassinolide (BL) on different parameters in *Stevia rebaudiana* Bertoni cultured under *in vitro* condition after 30 days of culturing

BL (µM)	Shoot length (cm)	Number of Leaves	Shoot Fresh Weight (mg g^{-1})	Shoot Dry Weight (mg g^{-1})	Chlorophyll-a (mg g^{-1})	Chlorophyll-b (mg g^{-1})	Carotenoid (mg g^{-1})
0	2.83±0.06f	19.33±1.50de	0.41±0.01f	0.10±0.01d	1.90±0.01a	0.72±0.02c	0.52±0.01c
1	4.50±0.10a	26.00±0.60b	0.59±0.01b	0.15±0.00b	1.98±0.00ab	0.85±0.03a	0.54±0.01a
2	4.20±0.00b	31.00±1.00a	0.61±0.01a	0.18±0.01a	1.99±0.01a	0.87±0.02a	0.55±0.01a
3	4.10±0.10cb	21.00±1.00c	0.51±0.02c	0.13±0.00c	1.97±0.01bc	0.77±0.01c	0.52±0.01c
4	3.90±0.10c	17.00±1.53e	0.47±0.01d	0.13±0.00c	1.95±0.01dc	0.76±0.02c	0.51±0.01bc
5	3.10±0.30d	9.00±1.00f	0.45±0.01e	0.11±0.00d	1.94±0.01d	0.73±0.02c	0.50±0.01c

where, BL=Brassinolide Data are in the form of mean± SEM, and means followed by the same letters within the columns are not significantly different at P≤0.05

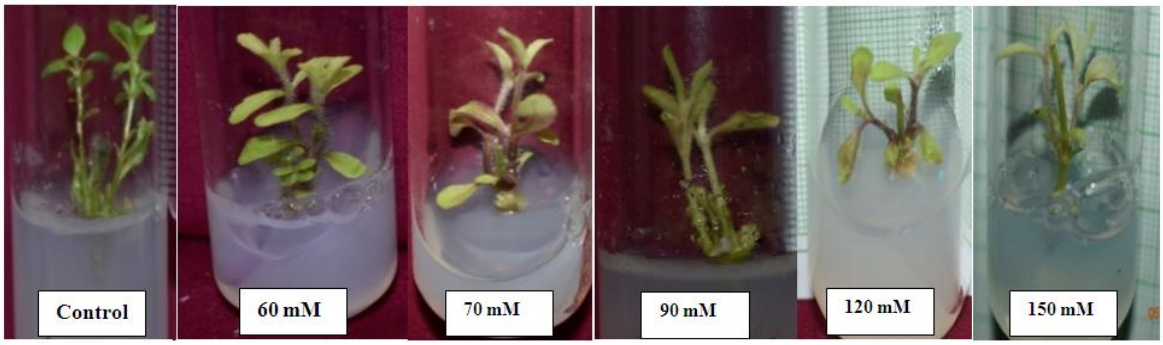

Fig. 1. Effect of different concentrations of sodium chloride (NaCl) on stevia

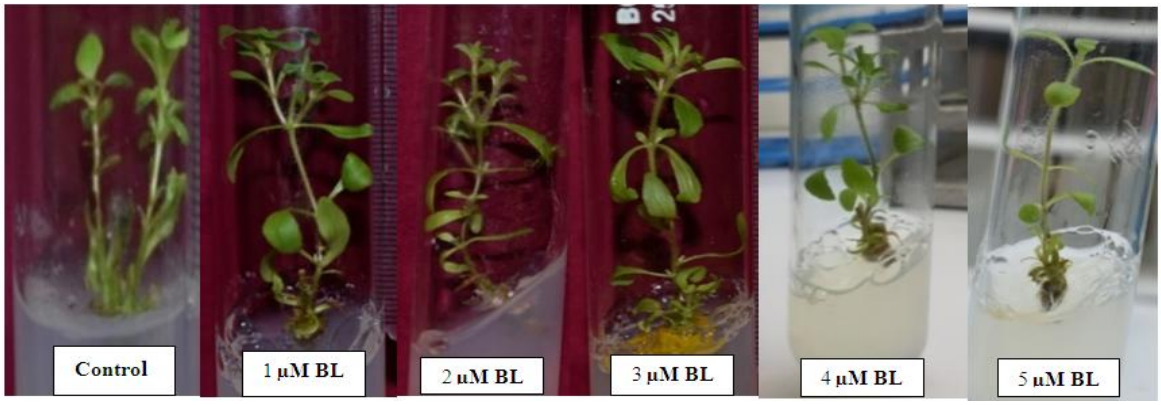

Fig. 2. Effect of different concentrations of Brassinolide (BL) on stevia

Data revealed that increasing BL increased the shoot length in the entire treatment (1µM to 5µM) as compared to control. The lowest value was found in higher concentration of BL (5 µM) with about 9.54% increment. While lower BL level (1 µM) could increased shoot length by 59.01%. In case of leaf number, lowest value was found in higher concentration of BL (5 µM) (9.54%) reduction. While lower BL level (2 µM) increased the value by 34.50%. These findings are in agreement with those of Chen et al. [29] in pear, Ghanbarali et al. [30]; Harshlata and Sai [31] in potato, Chavan et al. [32] in sugarcane; Pereira Netto et al. [33] in apple, in case of shoot length while Taha et al. [34] in bean; Chavan et al. [32] in sugarcane; Harshlata Tomar and Sai [31] in potato, in case of number of leaves.

The shoot fresh weight, at higher concentration of BL (5 µM) recorded about 9.75% increment. While lower BL level (2 µM) increased shoot fresh weight by 48.78%.The shoot dry weight, recorded lowest value in higher concentration of BL (5 µM) with about 50% increment. While lower BL level (2 µM) increased shoot dry weight by 10%. This is in agreement with the previous studies where foliar spraying of BL in chinese cucumber, peppermint increased the fresh and dry weight [35,36].

In case of chlorophyll-a content, lowest value was found in higher concentration of BL (5 µM) with about 2% increment compared to control. While lower BL level (2 µM) was increased chlorophyll-a by 4.7%. In case of chlorophyll-b content, lowest value was found in higher concentration of BL (5 µM) with about only 1.39% increase. While at lower BL level (2 µM), increase of 20.83% was noticed compared to control. Similar studies were observed by Zhang et al. [37] in soybean; Wang et al. [38] in capsicum; Sun et al. [39] in maize.

The lowest value for carotenoid content was found in higher concentration (5 µM BL) with about 5.76% increment. While lower BL level (2 µM) was increased the value by 3.84%. Similar findings was reported in watercress by Tang et al. [40].

4. CONCLUSIONS

Based on the findings, it can be concluded that *in vitro* salinity stress caused by NaCl

significantly lowered all of the examined parameters, with stevia being less affected by 60 and 70 mM NaCl in comparison to other concentrations of NaCl. Also, treatment with BL was found to significantly improve all the examined parameters, with 1 µM and 2 µM being the best of all the BL concentrations used. Hence our findings could be beneficial for future studies of BR response to stevia plants which have proven to have potential role in *in vitro* cultivation on stevia plants production. Additionally, the salinity screening at different concentrations could further be required for effect on biochemical and molecular mechanisms. Nevertheless, dose dependent salinity response in stevia plants needs further research, deciphering the mechanism involved along with economically important metabolites.

DISCLAIMER

The products used for this research are commonly and predominantly used in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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