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Optimizing Growth through Systematic Evaluation of Salinity, Putrescine, and Sodium Nitroprusside Levels in *Stevia rebaudiana*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Stevia rebaudiana, a natural sweetener source, has gained significant attention in recent years due to its potential as a sugar substitute with minimal caloric impact. To maximize its cultivation and production efficiency, it is imperative to optimize its growth conditions. In the present study, we performed a pot experiment in the polyhouse of the Department of Horticulture at the Institute of Agricultural Sciences, Banaras Hindu University, Varanasi and investigated the effects of salinity stress (NaCl), exogenous application of putrescine (Put), a polyamine, and sodium nitroprusside (SNP), a donor of nitic oxide, as foliar spray on different morpho-physiological traits such as plant height, number of leaves per plant and SPAD or chlorophyll content in *Stevia* over different time intervals under *in vivo* conditions. Our findings revealed that mild salinity stress (15 and 30 mM NaCl) caused a lesser reduction in plant growth characteristics. However, excessive salinity (120

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mM NaCl) had adverse effects on plant health, particularly at 15 days. We observed that higher concentrations of Put (1 mM and 2 mM) positively influenced all the studied growth indices as compared to control and NaCl treated plants. This indicates the potential for Put to be used as a growth regulator in *Stevia* cultivation. Similarly, 150 µM and 200 µM SNP treatments produced best results in Stevia plants by significantly enhancing plant height, leaf number and SPAD at 3, 6, 9, 12 and 15 days. Therefore, SNP can be used as a potential growth promoting agent in *Stevia* plants. These findings have significant implications for sustainable *Stevia* cultivation, ensuring a stable supply of natural, low-calorie sweeteners for the food and beverage industry.

Keywords: Stevia; salinity; putrescine; sodium nitroprusside; morpho-physiological.

1. INTRODUCTION

Stevia rebaudiana, a perennial herb native to South America, commonly known as sweet leaf, honey leaf, candy leaf or simply *Stevia*, has emerged as a valuable and natural source of non-caloric sweeteners, primarily represented by secondary metabolites like steviol glycosides. Its sweetening compounds, especially stevioside and rebaudioside A, have gained significant attention as healthier alternatives to sugar due to their zero-calorie profile and potential health benefits. As the world grapples with escalating concerns about sugar consumption and its associated health risks, *Stevia's* importance in the global sweetener market continues to grow. Numerous researchers have revealed that *Stevia* has a number of health advantages in addition to its sweetening properties, including anti-diabetic, anti-obesity, anti-tumor, anti-hypertensive, anti-microbial, anti-caries, and antioxidant qualities [1,2].

Nevertheless, while *Stevia rebaudiana* holds significant promise, its commercial cultivation encounters numerous obstacles such as suboptimal growth, restricted yields, and vulnerability to various environmental stressors, notably soil salinity. This research paper focuses on investigating the crucial impact of three essential elements—sodium chloride (NaCl), putrescine (Put), and sodium nitroprusside (SNP)—in influencing the growth and physiological reactions of *Stevia rebaudiana*.

Salinity stress, often caused by high soil salinity or suboptimal irrigation practices, can substantially hinder plant growth and yield. Sodium chloride (NaCl), a common source of salinity stress, can significantly affect plant physiological processes, nutrient uptake, and osmotic regulation. In addition, elevated salinity levels display a profound effect on photosynthesis in plants, resulting in significant alterations in various aspects. These changes encompass modifications in the ultrastructure of organelles, shifts in the composition of pigments and metabolites, and adjustments in the activities of enzymes crucial to photosynthesis [3-5]. An investigation made by Cao et al.[6] showed that exposure to higher salinity levels resulted in a notable reduction in the total chlorophyll (Chl) content. This decline in chlorophyll content can potentially lead to the disruption of chloroplast structure. However, research on the impact of salt stress on *Stevia* remains limited and insufficient. Thus, a systematic evaluation of NaCl levels and their impact on *Stevia rebaudiana* growth is imperative to develop sustainable cultivation practices.

Polyamines, such as putrescine, have been recognized as vital regulators of plant growth, stress tolerance, and development [7]. Their application in agriculture has shown promising results in mitigating the harmful impact of abiotic stressors, including salinity [8,9]. The exogenous supplementation of putrescine (Put), a significant polyamine (PA), has been observed to exert detrimental effects under saline conditions, impacting growth and various biochemical parameters in different plant species such as *Luffa*[10], *Allium jesdianum*[11], and *Panax ginseng* [12]. However, the precise mechanisms by which Put influences *Stevia rebaudiana* growth under salt stress conditions remain underexplored. Systematic investigations into the optimal levels of putrescine for enhancing *Stevia* growth and salinity tolerance are essential for harnessing the full potential of this growth-promoting agent.

Another crucial player in plant growth and stress response is nitric oxide (NO). Sodium nitroprusside (SNP), a nitric oxide donor, has been shown to modulate various physiological processes, including seed germination, root development, and abiotic stress responses in plants [13,14]. SNP, or Sodium Nitroprusside offers a valuable solution in alleviating the negative effects of salinity in plants. It accomplishes this by orchestrating the modulation of various physiological factors, such as chlorophyll content, stomatal behaviour, cell water status, and electrolyte leakage. Furthermore, SNP also exerts control over key biochemical markers like membrane lipid peroxidation, proline levels, the synthesis of phenolic compounds, and the activation of antioxidants within plants. This comprehensive approach contributes to the plant's ability to thrive in saline conditions [15]. However, research on the influence of SNP on the growth of *Stevia* plants is notably scarce in the existing scientific literature. The exploration of the potential impact of SNP on the enhancement of *Stevia rebaudiana* growth and its ability to withstand high salt levels presents a captivating and promising avenue for scientific investigation.

Therefore, this research paper aims to fill existing knowledge gaps in cultivation of *Stevia rebaudiana* by determining the optimal concentrations of NaCl, Put and SNP supplementation, with a focus on assessing their impacts on the growth-related characteristics of *Stevia* plants grown under *in vivo* environmental conditions. Overall, this research paper not only advances our understanding of *Stevia rebaudiana's* growth requirements but also offers innovative approaches to address agricultural challenges. Its findings are relevant not only for researchers in the field of plant biology but also for those involved in sustainable agriculture and the development of natural sweeteners, making it a valuable contribution to the scientific community.

2. MATERIALS AND METHODS

2.1 Plant Materials and Growth Conditions

In this study, 25-day-old *Stevia* plants were purchased from a nearby nursery in Varanasi and transplanted into pots with a 25-cm diameter and a 25-cm height that contained 1.5 grams of organic manure and 5 grams of garden soil in the polyhouse of the Department of Horticulture at the Institute of Agricultural Sciences, Banaras Hindu University, Varanasi.

2.2 Experimental Design and Treatments

The experiment was designed as a completely randomized design (CRD) with threeindependent factors and four treatments along with one control for each factor: NaCl (control,15 mM, 30 mM, 60 mM and 120 mM); putrescine (Put) (control, 0.05 mM, 0.1 mM, 1 mM and 2 mM); sodium nitroprusside (SNP) (control, 40 µM, 100 µM, 150 µM and 200 µM). Each treatment was replicated three times. The induction of salinity stress was carried out after one month of transplanting. Different levels of salinity were applied to the soil around the root zone of the first set of *Stevia* plants by dissolving various concentrations of NaCl of high purity in distilled water. At the same time, varying concentrations of Put (Sigma-Aldrich, ≥ 97% purity) and SNP (Sigma-Aldrich, ≥ 99% purity) were applied in the form of foliar spray to the second and third sets of *Stevia* plants at regular intervals until the end of the stress. The control plants received distilled water without any treatment. Morpho-physiological observations including plant height, number of leaves per plant and chlorophyll content were recorded at every three days interval during the experiment.

2.3 Plant Height

Plant height (cm) was measured from the base of the stem to the tip of the tallest leaf with the help of a ruler.

2.4 Number of Leaves per Plant

The leaf count for each plant was recorded on five separate duration: at 3 days, 6 days, 9 days, 12 days and 15 days after exposure to different concentrations of NaCl, Put and SNP, respectively.

2.5 Leaf Chlorophyll Content (SPAD)

The chlorophyll content in three randomly selected fully matured leaves from each pot was assessed using a SPAD Chlorophyll meter, specifically the SPAD-502 Plus by Konica Minolta, Inc. Subsequently, the average SPAD value for each treatment was computed based on these readings.

2.6 Statistical Analysis

Data were analysed through a one-way analysis of variance (ANOVA) to evaluate the main effects of salinity, PUT, and SNP treatments respectively. Post hoc Duncan multiple range tests (DMRT) were used to identify significant differences between treatment means ($p < 0.05$). Statistical analyses were conducted utilizing IBM's SPSS version 26.

3. RESULTS AND DISCUSSION

The plant height of *Stevia rebaudiana* was evaluated at 3 days, 6 days, 9 days, 12 days, and 15 days after exposure to different concentrations of NaCl, Put, and SNP (Table 1). The control plants exhibited a gradual increase in plant height over time, with values ranging from 42.17 cm at 3 days to 48.27 cm at 15 days. Contrastingly, increasing NaCl concentrations resulted in a significant reduction in plant height at $p < 0.05$. The NaCl treatment (120 mM) had the most pronounced inhibitory effect, with plant heights as low as 31.33 cm at 15 days. These results find alignment with the previous studies performed in different crops such as rice, coriander, alfalfa, tomato, chickpea, spearmint etc., [16-21]. This salinity-induced reduction in plant height may be attributed to the osmotic stress and ion toxicity caused by elevated NaCl concentrations, leading to impaired water and nutrient uptake. However, *Stevia* plants treated with Put showed a pronounced growth-promoting effect. Foliar spraying with Put at concentrations of 0.05 mM to 2 mM generally led to an increase in plant height compared to the control. The highest concentration (2 mM) of Put had the most substantial positive impact, with plant height reaching 51.23 cm at 15 days. Similar results were also documented by Mohammadi et al*.,* 2018 in *Thymus vulgaris*. Similar to Put, the SNP treatments showed a positive effect on plant height. At 3 days, there were no significant differences among the treatments. However, as the duration of exposure increased, plants treated with higher concentrations of SNP (100 µM, 150 µM, and 200 µM) exhibited significantly taller heights compared to the control. At 15 days, the highest concentration of SNP (200 µM) resulted in the tallest plants (52.30 cm), whereas the control plants had a height of 48.67 cm. Arab et al*.*[22] also observed an elevation in chlorophyll b content following the application of SNP, while Gohari et al*.*[23] showed that 200 µM SNP caused a notable enhancement in both chlorophyll a and b as compared to the control and NaCl treatments in *Ocimum basilicum* under salt stress. Our results suggest that SNP can enhance the growth of *Stevia rebaudiana*, particularly at higher concentrations. Further, the dose-dependent response observed in both Put and SNP treatments indicates that an optimal concentration of these substances can be beneficial for enhancing plant growth. These findings have practical implications for the cultivation of *Stevia rebaudiana* in saline-prone regions. The use of Put or SNP treatments may be a promising method to mitigate the damaging effects of salinity stress and improve crop yields.

In Table 2, the impact of various concentrations of NaCl, put (Putrescine), and SNP (Sodium Nitroprusside) on the number of leaves per plant of *Stevia rebaudiana* at various time intervals (3, 6, 9, 12, and 15 days) is presented. In the present study, we observed statistically significant differences between control and NaCl-treated plants ($P < 0.05$) at all concentrations and time points. The decline in leaf number was more pronounced with higher NaCl concentrations. For example, 120 mM NaCl treatment had the most pronounced effect, with only 37 leaves per plant at 15 days. The consistent decline in leaf number over time suggests that the impact of salt stress accumulates and becomes more severe with prolonged exposure. This reduction in leaf number can be attributed to the osmotic stress caused by high salt concentrations, which restricts water uptake and hampers the overall plant growth. Additionally, high salt concentrations can disrupt the ion balance within the plant, leading to reduced leaf formation. These results agree with earlier research on the negative impact of salt stress on plant growth [24-26]. The foliar spray of Put at concentrations ranging from 0.05 mM to 2 mM resulted in a slight increase in the number of leaves compared to the control group, with the highest number (119 leaves) observed at 15 days with 2 mM Put. When Put levels are increased in plants, it can stimulate cell division and differentiation, promoting the formation of new leaves [27]. However, the precise mechanisms by which putrescine influences leaf development can be complex and context-dependent, as it interacts with other factors and signaling pathways within the plant. Similarly, the application of SNP at concentrations from 40 µM to 200 µM resulted in variable effects on the number of leaves. The 200 µM SNP treatment showed the highest number of leaves (132.33) at 15 days, while other concentrations had intermediate effects. Similar findings have been reported in crops like safflower [28], wheat [29]. Our results indicate that SNP may serve as a growth-promoting agent for *Stevia rebaudiana* leaves, especially at higher concentrations. In addition, SNP is known to act as a signaling molecule in plants and can influence various physiological processes [30].

SPAD values are indicative of chlorophyll content and, thus, can be used as an indicator for plant health and photosynthetic activity. Table 3 provides valuable insights into the impact of various concentrations of NaCl (sodium chloride), Put (putrescine), and SNP (sodium nitroprusside) on the SPAD values of *Stevia rebaudiana* at different time intervals (3 days, 6 days, 9 days, 12 days, and 15 days). The application of NaCl to *Stevia rebaudiana* exhibited a dose-dependent effect on SPAD values over time. A significant decrease in SPAD values was observed as the NaCl concentration increased. At 15 mM NaCl, the SPAD values decreased by approximately 7.44% after 15 days compared to the control. However, at higher concentrations (60 mM and 120 mM), a more pronounced reduction in SPAD values was noted, with reductions of approximately 37.63% and 42.22%, respectively, after 15 days. Kazemi et al. [31] also reported a significant reduction in SPAD values in rice cultivars under salinity. This suggests that excessive salt stress negatively affects the chlorophyll content in *Stevia rebaudiana* leaves, resulting in reduced SPAD values. Put application exhibited contrasting results compared to NaCl treatment. SPAD values increased with increasing Put concentrations. At 2 mM Put, a significant increase of approximately 10.16% in SPAD values was observed after 15 days compared to the control. Put promotes chlorophyll synthesis by enhancing the activity of enzymes such as glutamate-1-semialdehyde aminotransferase (GSA-AT) and glutamyl-tRNA reductase (GluTR), which are essential in the chlorophyll biosynthetic pathway [32]. In a similar way, SNP treatment displayed a concentration-dependent response in SPAD values. At 200 µM SNP, a remarkable increase of approximately 28.12% in SPAD values was observed after 15 days compared to the control. This indicates that SNP application can enhance chlorophyll content in *Stevia rebaudiana* leaves. These findings are in agreement with Maslennikova et al.,[33], who observed that the pre-treatment with SNP could improve plant growth by enhancing chlorophyll content in wheat seedlings subjected to salinity stress. Jabeen et al.[15] reported that SNP application improved chlorophyll biosynthesis leading to enhanced productivity in soybean. However, lower concentrations (40 µM and 100 µM) also demonstrated positive effects but to a lesser extent, with increases of approximately 8.49% and 9.35%, respectively, after 15 days. This is due to the fact that SNP can influence chlorophyll content indirectly by participating in signal transduction pathways that regulate photosynthesis and chloroplast development. Overall, the positive impact of Put and SNP on chlorophyll content or SPAD values contributes to improved photosynthetic capacity and, consequently, enhanced plant growth and productivity [34].

Table 1. Effect of different concentrations of NaCl, Put and SNP on plant height of *Stevia rebaudiana*

Factor	Treatment	Plant Height (cm)				
		3 days	**6 days**	**9 days**	**12 days**	**15 days**
NaCl	Control	42.17c	42.57c	44.23c	45.43d	48.27c
	15 mM	41.33c	39.87b	39.63b	37.80c	35.97b
	30 mM	39.63b	38.93b	37.63b	36.60c	35.30b
	60 mM	38.37ab	35.23a	34.80a	34.50b	31.47a
	120 mM	37.10a	34.43a	33.30a	33.10a	31.33a
Put	Control	44.17a	44.40a	46.40a	47.50a	50.53abc
	0.05 mM	45.33ab	47.13b	47.80ab	47.90ab	49.30ab
	0.1 mM	46.53bc	47.43bc	48.43b	48.47ab	48.80a
	1 mM	48.07cd	48.97c	48.93b	49.30bc	50.67bc
	2 mM	48.63d	48.13bc	48.97b	50.40c	51.23c
SNP	Control	40.07a	43.10a	44.07a	46.33a	48.67a
	40 µM	41.57b	43.33a	45.00a	47.17ab	49.53ab
	100 µM	44.33c	45.17b	45.50a	48.37b	50.03ab
	150 µM	44.63c	47.97c	48.47b	50.53c	51.03bc
	200 µM	46.63d	48.30c	48.57b	51.17c	52.30c
SEM ±	**NaCl**	**0.54**	**0.84**	**1.06**	**1.16**	**1.67**
	Put	**0.49**	**0.46**	**0.32**	**0.33**	**0.32**
	SNP	**0.64**	**0.63**	**0.56**	**0.53**	**0.41**
SD	**NaCl**	**2.08**	**3.26**	**4.11**	**4.49**	**6.48**
	Put	**1.91**	**1.76**	**1.24**	**1.26**	**1.23**
	SNP	**2.49**	**2.43**	**2.18**	**2.06**	**1.58**

Mean values from three replicates are displayed. Within each column, means followed by distinct letters are considered statistically significant (P < 0.05) as determined by the DMRT post-hoc analysis. SEM (standard error mean) and SD (standard deviation) are also presented.

Table 2. Effect of different concentrations of NaCl, Put and SNP on number of leaves of *Stevia rebaudiana*

Factor	Treatment	Number of leaves per plant				
		3 days	6 days	9 days	12 days	15 days
NaCl	Control	108.67d	119.33c	134.33e	140.00e	155.33e
	15 mM	72.33c	71.33b	68.67d	66.33d	61.33d
	30 mM	70.00c	68.67b	61.67c	60.00c	48.67c
	60 mM	66.00b	64.33a	59.00b	50.33b	42.00b
	120 mM	62.67a	61.67a	53.67a	46.00a	37.00a
Put	Control	99.67a	102.67a	105.00a	108.00a	113.33b
	0.05 mM	103.00b	104.00a	106.00ab	109.00a	111.00ab
	0.1 mM	106.33c	107.67b	108.00b	110.67a	110.67a
	1 mM	109.00d	109.00b	110.33c	113.67b	117.00c
	2 mM	111.00d	113.33c	111.33c	117.00c	119.00c
SNP	Control	113.67a	115.00a	120.00a	123.33a	126.33ab
	40 µM	114.00a	119.67b	123.00b	125.67ab	128.00bc
	100 µM	116.33ab	123.67c	124.33bc	126.00ab	125.33a
	150 µM	117.33c	126.00cd	126.33cd	128.00bc	130.00cd
	200 µM	119.00c	127.67d	127.67d	129.67c	132.33d
SEM ±	**NaCl**	**4.47**	**5.73**	**7.98**	**9.22**	**11.76**
	Put	**1.12**	**1.08**	**0.70**	**0.94**	**0.93**
	SNP	**0.64**	**1.26**	**0.80**	**0.67**	**0.74**
SD	**NaCl**	**17.32**	**22.19**	**30.90**	**35.71**	**45.56**
	Put	**4.35**	**4.19**	**2.72**	**3.64**	**3.61**
	SNP	**2.46**	**4.87**	**3.08**	**2.59**	**2.85**

Mean values from three replicates are displayed. Within each column, means followed by distinct letters are considered statistically significant (P < 0.05) as determined by the DMRT post-hoc analysis. SEM (standard error mean) and SD (standard deviation) are also presented.

Table 3. Effect of different concentrations of NaCl, Put and SNP on SPAD values of *Stevia rebaudiana*

Factor	Treatment	SPAD				
		3 days	6 days	9 days	12 days	15 days
NaCl	Control	52.87c	53.63c	55.97d	57.63d	59.07d
	15 mM	49.00ab	48.73b	47.03c	44.07c	41.63c
	30 mM	47.10a	46.47ab	43.77b	41.37b	37.83b
	60 mM	50.13bc	45.40a	41.13a	38.47a	36.57b
	120 mM	49.33ab	45.23a	39.40a	36.77a	34.03a
Put	Control	43.30a	44.27a	48.43a	48.73a	50.40a
	0.05 mM	45.63b	45.70a	49.90ab	50.60ab	52.83b
	0.1 mM	46.80bc	47.70b	51.70bc	52.73b	54.57b
	1 mM	46.97bc	48.00b	52.73c	57.27c	54.67b
	2 mM	47.93c	48.10b	53.60c	57.73c	58.57c
SNP	Control	42.60a	43.33a	46.07a	48.63a	51.63a
	40 µM	42.80a	45.03ab	46.30a	49.10a	52.33ab
	100 µM	43.33a	45.97bc	49.30b	49.40a	54.30bc
	150 µM	45.77b	47.30c	48.57b	51.43b	54.50bc
	200 µM	46.13b	50.07d	52.33c	54.53c	55.03c
SEM ±	**NaCl**	**0.60**	**0.91**	**1.59**	**2.00**	**2.41**
	Put	**0.47**	**0.45**	**0.55**	**1.01**	**0.76**
	SNP	**0.47**	**0.64**	**0.65**	**0.63**	**0.45**
SD	**NaCl**	**2.33**	**3.53**	**6.16**	**7.76**	**9.32**
	Put	**1.81**	**1.73**	**2.15**	**3.90**	**2.93**
	SNP	**1.81**	**2.49**	**2.52**	**2.42**	**1.75**

Mean values from three replicates are displayed. Within each column, means followed by distinct letters are considered statistically significant (P < 0.05) as determined by the DMRT post-hoc analysis. SEM (standard error mean) and SD (standard deviation) are also presented

4. CONCLUSION

Salinity stress has a detrimental effect on morpho-physiological characteristics including plant height, leaf number and chlorophyll content in *Stevia rebaudiana*. However, Put and SNP foliar treatments boosted plant growth by enhancing these attributes over different time intervals. Our study revealed that lower salinity levels of 15 and 30 mM had a significantly less detrimental effect on the morpho-physiological traits of *Stevia* when compared to higher concentrations, highlighting the importance of carefully managing salinity in its cultivation for optimal growth and yield. Based on the findings, it is inferred that Put and SNP may be utilized as mitigating agents against salinity-induced growth inhibition in *Stevia*. Additionally, it is concluded from the current study that higher concentrations of Put (1mM and 2mM) and SNP (150 µM and 200 µM) treatments provided best results by increasing plant height, number of leaves and SPAD values. Moreover, these results hold significant implications for the cultivation and commercial production of *Stevia rebaudiana*, offering a foundation upon which future research and agricultural practices can build to maximize yield and quality in a sustainable manner.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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