Influence of Abiotic Salt Stress and Salicylic Acid on callus metabolic study of Bacopa monnieri during In Vitro Culture

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Abstract
This study aimed to examine the morphological, physiological, biochemical and secondary metabolite responses of an important medicinal plant, Bacopa monnieri, under salt stress conditions and salicylic acid elicitation in In vitro micropropogation. This study investigated the effect of the association of sodium chloride (NaCl) and salicylic acid (SA) on the growth of callus and the accumulation of bacoside in Bacopa monnieri explants by growing them on Murashige and Skoog (MS) medium containing various concentrations of these salts. Significant differences in the callus proliferation, biomass production, growth rate and metabolite production were noted between different treatment conditions with the callus. Concentrations of salt (50 and 100 mM) stimulated callus proliferation and adaptive physiological responses. Conversely, 150 mM salt concentrations reduced callus growth, browning and necrosis of tissue. Salicylic acid was used as an elicitor which promoted stress resistance, healthy green callus and improved tissue vitality. The moderate stress of salt and salicylic acid (SA) produced a significant increase in both fresh and dry weights and growth index of the cultures. Biochemical analyses suggest that optimization of stress and elicitation conditions were advantageous with respect to production of bacoside. The treatment comprising 100 mM NaCl and 1.0 mM salicylic acid produced a maximum bacoside content which was verified by HPLC as compared to the control and treatments. To conclude, it has been established that controlled salt stress along with salicylic acid elicitation affected the growth responses of Bacopa monnieri callus and enhanced the amount of accumulation of bacoside in the cultures. The results offer a new strategy to improve the production of valuable bioactive compounds in plant tissue culture and for abiotic stress-induced elicitation strategies.

Keywords: Bacopa monnieri, Sodium chloride (NaCl), Salicylic acid (SA), Plant tissue culture, Abiotic stress, In Vitro Culture, Bacoside

Introduction

Brahmi, or Bacopa monnieri, is a small creeping perennial herb, of the family Plantaginaceae. Its succulent nature helps it to retain water effectively such that it can withstand wetland habitats such as river banks, ponds and irrigated fields. [1]

Bacopa monnierihas become a matter of much concern in modern pharmacology and plant biotechnology in few decades. A scientific research has discovered a set of bioactive compounds called bacosides that are the main cause of its medicinal effects. They are triterpenoidsaponins and have been documented to significantly enhance cognitive functioning, protect the neuronal cells and relieve oxidative stress. Research indicates that Bacopa improves memory, learning capacity and information processing particularly on a long-term basis. [2]

In the agriculture study in found about 33% irrigated agricultural land affected by salinity. It is estimated that by 2050, may 50% of irrigated agricultural areas will be salted. [10]

In addition to its cognitive benefits, Bacopa monnierihas excellent antioxidant properties and these can compensate the impact of free radicals and result in the destruction of cells. It also has anti-inflammatory, anti-anxiety and anti-depressant effects, believed to take place via its effect on neurotransmitters such as serotonin and dopamine. In addition to its medicinal uses, Bacopa monnierihas emerged as a key topic of study in plant biotechnology, especially in the field of secondary metabolite biosynthesis. The environmental factors such as water availability, temperature, light intensity and soil salinity are significant factors influencing the growth of plants and bacosides accumulation.

In the micropropagation, Bacopa monnierican be quickly multiplied under controlled laboratory conditions. This technique enables us to have massive amounts of genetically pure and disease free plants and this ensures quality and quantity uniformity.

The salt stress and salicylic acid elicitation have become a useful tool of enhancing the production of bacoside in plants. Though salt stress can cause the activation of defense mechanisms and the production of secondary metabolites, the salicylic acid is a signaling molecule that enhances stress tolerance and metabolic activity. Combined, the techniques can boost the biomass and phytochemical yield to a significant extent provided the conditions are optimized appropriately. [4], [5]

Material and Method

Plant Materialand Sterilization

Bacopa monnieri plant was taken from Nature Nursery, Indore, after collecting healthy and disease free plant material, well washed with running tap water 10 Minutes then cut the nodal explant and treated with 30% sodium

Hypochlorite (Sunchem) for 10 minutes and 1 minutes in 1% HgCl(Sunchem, 5210A) solution respectively then washed with sterilized distilled water.

Culture media:
Take Murashige and Skoogmedium (Himedia, PT021-5L) 4.4g/liter. Sucrose (Sunchem) (3% w/v) BAP (Sunchem, 1431) 1.0 mg/Land IBA (Sunchem, 5523) 1.0 mg/L. Agar (Himedia) 0.8% w/v was used. Different concentrations of NaCl(Sunchem) and salicylic acid (Sunchem) for stress and elicitation were added to the medium before autoclaving for salt stress experiments. [6]

Preparation of working solutions:
Take stock solution 5, 10 and 15 ml for 100 ml working solution of 50, 100 and 150 mM respectively. The same formula was applied to prepare 0.5 mM, 1mM, and 1.5 mM concentrates of salicylic acid to take stock solution 0.5ML, 1 ml & 1.5 ml and was diluted to 100 ml to a final volume.

Establishment of plant tissue culture (PTC):
Explants of Bacopa monnieri were subjected to plant tissue culture (PTC). All explants were washed under running water and surface sterilized under sterile conditions with 0.1% solutions of HgCl2 and Tween-20. The explants were sterilized and inoculated into MS medium with the appropriate plant growth regulators for shoot induction and shoot multiplication. Cultures were grown at 25±2 °C and a 16 h photoperiod. [8]

Stress treatment methods:
The supplementation of medium with various concentrations of sodium chloride (NaCl) 50 mM, 100 mM and 150 mM and also the combination of salicylic acid (SA) was implemented as salt stress treatment for those shoots that were cultured. The cultures were then grown for 4–6 weeks under controlled conditions to assess the impact of salinity on plant growth, physiology and accumulation of bacosides in the plants. [9], [10]

Elicitation treatment method:
Shoots were grown in media containing varying concentrations of salicylic acid (0.5 mM, 1.0 mM, 1.5mM) and in combination with salt and were treated with salicylic acid. Under stressful conditions, it promoted callus proliferation, sustained green color and vigour of tissues. [11]

Incubation:
The explants of Bacopa monnieri were inoculated in freshly prepared Murashige and Skoog (MS) culture vessels and sealed with sealing film and labeled with treatment combinations for easy identification and monitoring during the experimental period. Cultures were grown in a controlled growth room with 16 h light and 8 h dark, a light intensity of ~40–50 µmol m^{-2} s^{-1} and a temperature of 25 ± 2°C. Usually swelling and bud break first occurred within 1 week after inoculation, and shoot initiation and multiplication occurred. [12]

Callus Induction Percentage:
Count total no of explant produced callus in total inoculated explant and then calculated by using the following formula:

$$\text{Callus Induction \%} = \frac{\text{Number of explants producing callus}}{\text{Total number of explants inoculated}} \times 100$$

Growth Index (GI):
For the growth index count initialweight of explant is 0.5g then calculated final wieght. It is calculated by the formula –

$$\text{GI} = \frac{\text{Final Weight} - \text{Initial Weight}}{\text{Initial Weight}}$$

Bacoside Extraction:
Make methanolic extraction as per slight modification of M. Deepak extraction method. Collected callus was shade dried at room temperature to dry constant weight and then 1g dried callus powder was extracted with methanol by ultrasonication for 30–45 minutes, and then concentrated at reduced temperature with Rotary Evaporator. The extract was then concentrated and re-dissolved in a known amount of HPLC grade methanol and filtered through a 0.45 µm membrane filter before performing chromatography.[13]

Assessment of Bacoside Based on HPLC:
High Performance Liquid Chromatography (HPLC) method, as described by Poornima Raj with a few modifications. Bacosides were separated and quantified by a reverse-phase HPLC system which was coupled with C18 column and UV detector. The mobile phase comprised of acetonitrile and water in 70:30 ratio with an optimized flow rate of 1.0 mL/min. The detection of bacosides was performed at 205 nm. [11]

Statistical Analysis:
The statistical treatment of the experimental data was done by the Analysis of Variance (ANOVA) method which was described by Fisher and the computations were done by using IBM SPSS Statistics software. [15]

Result &Observation

Callus Induction Percentage:

Table 1: Effect of Salt Stress and Salicylic Acid on Callus Induction Percentage in Bacopa monnieri.

Treatment Code	NaCl + SA Treatment	Callus Induction (%)	Mean ± SE
Control	0 mM NaCl + 0 mM SA	78	78 ± 1.2
S1	50 mM NaCl	84	84 ± 1.5
S2	100 mM NaCl	88	88 ± 1.7
S3	150 mM NaCl	52	52 ± 1.4
SA1	0.5 mM SA	86	86 ± 1.6
S1+SA1	50 mM NaCl + 0.5 mM SA	91	91 ± 1.8
S2+SA2	100 mM NaCl + 1.0 mM SA	95	95 ± 2.0
S3+SA3	150 mM NaCl + 1.5 mM SA	65	65 ± 1.5

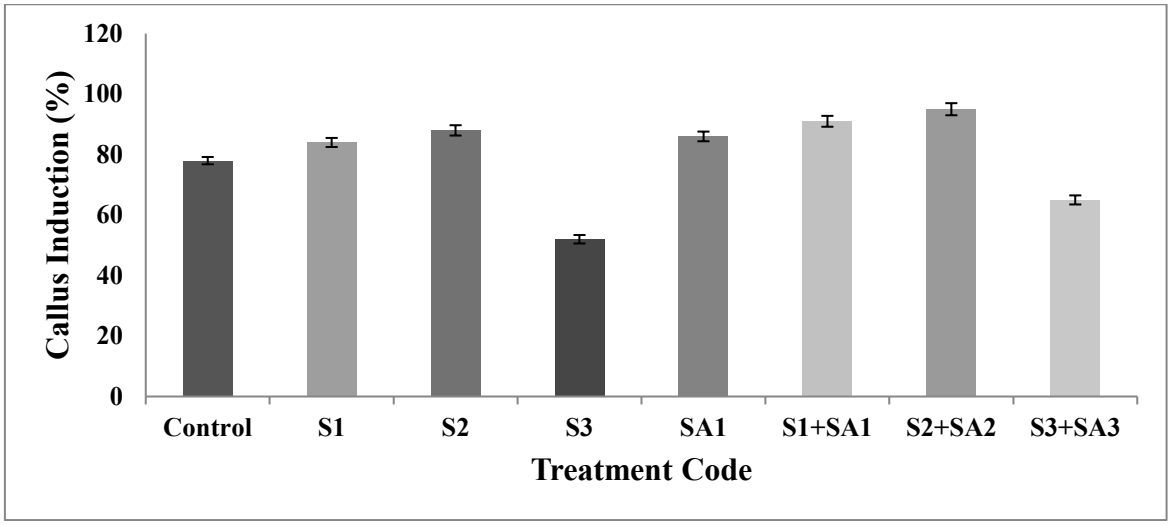


Figure 1: Effect of Salt Stress and Salicylic Acid on Callus Induction Percentage in Bacopa monnieri.

Fresh Weight of Callus:

Table 2: Effect of Salt Stress and Salicylic Acid on Fresh Weight of Callus in Bacopa monnieri

Treatment Code	Treatment Details	Fresh Weight of Callus (g)	Mean ± SE
Control	0 mM NaCl + 0 mM SA	1.82	1.82 ± 0.05
S1	50 mM NaCl	2.15	2.15 ± 0.06
S2	100 mM NaCl	2.48	2.48 ± 0.07
S3	150 mM NaCl	1.20	1.20 ± 0.04
SA1	0.5 mM SA	2.30	2.30 ± 0.06
S1+SA1	50 mM NaCl + 0.5 mM SA	2.75	2.75 ± 0.08
S2+SA2	100 mM NaCl + 1.0 mM SA	3.10	3.10 ± 0.09
S3+SA3	150 mM NaCl + 1.5 mM SA	1.65	1.65 ± 0.05

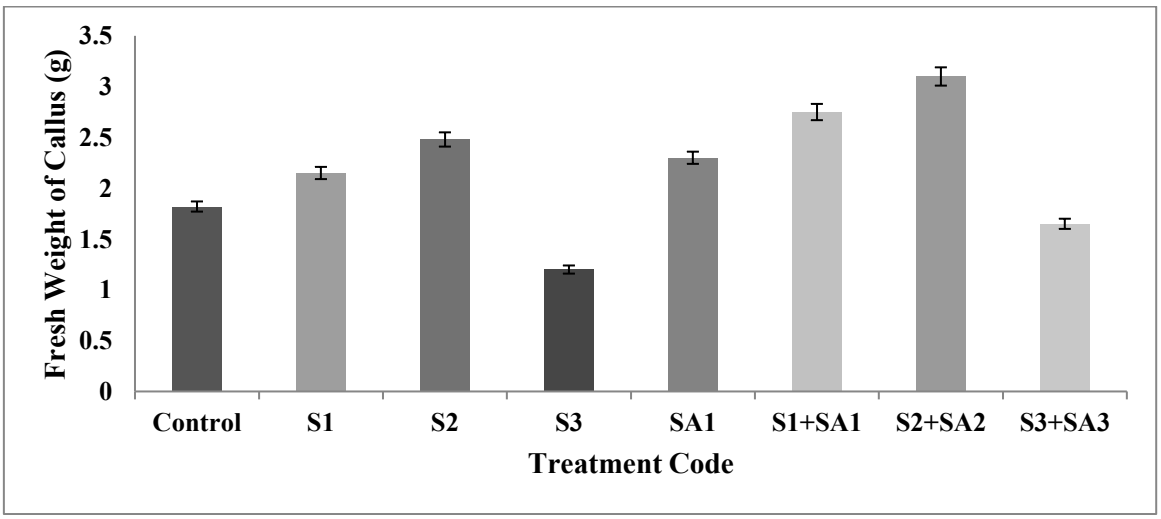
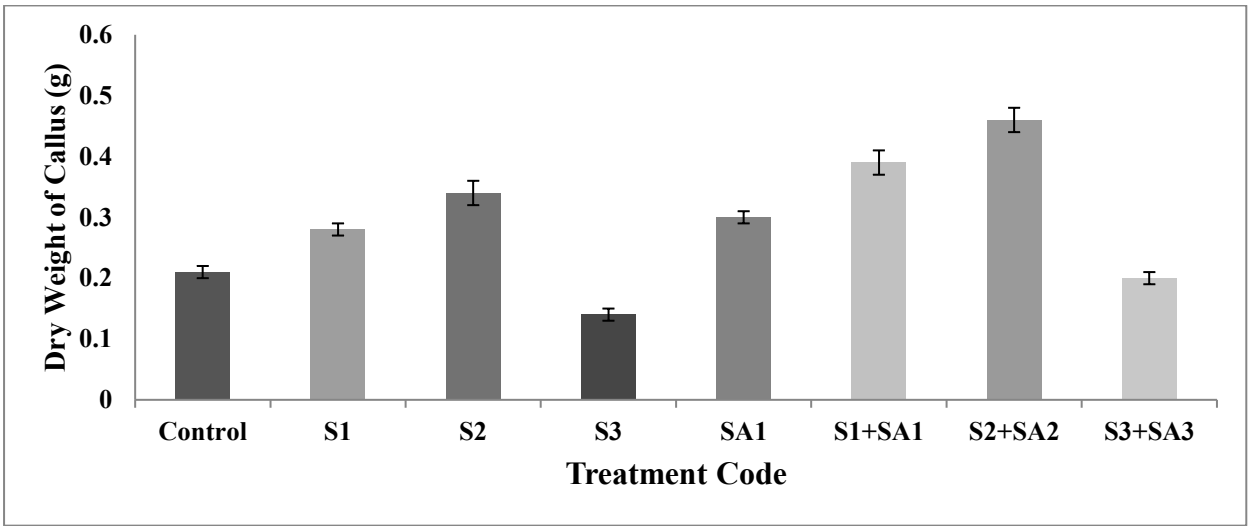


Figure 2: Effect of Salt Stress and Salicylic Acid on Fresh Weight of Callus in Bacopa monnieri

Dry Weight of Callus:

Table 3: Effect of Salt Stress and Salicylic Acid on Dry Weight of Callus in Bacopa monnieri

Treatment Code	Treatment Details	Dry Weight of Callus (g)	Mean ± SE
Control	0 mM NaCl + 0 mM SA	0.21	0.21 ± 0.01
S1	50 mM NaCl	0.28	0.28 ± 0.01
S2	100 mM NaCl	0.34	0.34 ± 0.02
S3	150 mM NaCl	0.14	0.14 ± 0.01
SA1	0.5 mM SA	0.30	0.30 ± 0.01
S1+SA1	50 mM NaCl + 0.5 mM SA	0.39	0.39 ± 0.02
S2+SA2	100 mM NaCl + 1.0 mM SA	0.46	0.46 ± 0.02
S3+SA3	150 mM NaCl + 1.5 mM SA	0.20	0.20 ± 0.01



(Figure 3:Effect of Salt Stress and Salicylic Acid on Dry Weight of Callus in Bacopa monnieri)

Growth Index of Callus:

Table 4: Effect of Salt Stress and Salicylic Acid on Growth Index of Callus in Bacopa monnieri

Treatment Code	Treatment Details	Initial Weight (g)	Final Fresh Weight (g)	Growth Index (GI)
Control	0 mM NaCl + 0 mM SA	0.50	1.82	2.64
S1	50 mM NaCl	0.50	2.15	3.30
S2	100 mM NaCl	0.50	2.48	3.96
S3	150 mM NaCl	0.50	1.20	1.40
SA1	0.5 mM SA	0.50	2.30	3.60
S1+SA1	50 mM NaCl + 0.5 mM SA	0.50	2.75	4.50
S2+SA2	100 mM NaCl + 1.0 mM SA	0.50	3.10	5.20
S3+SA3	150 mM NaCl + 1.5 mM SA	0.50	1.65	2.30

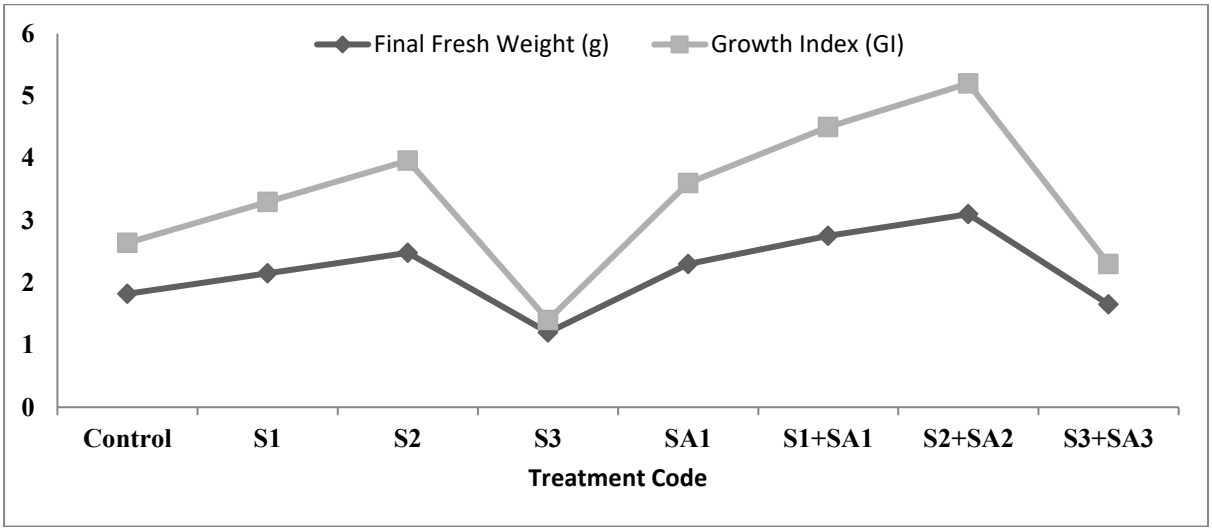


Figure 4: Effect of Salt Stress and Salicylic Acid on Growth Index of Callus in Bacopa monnieri

Bacoside Content:

Table 5: Effect of Salt Stress and Salicylic Acid on Bacoside Content in Bacopa monnieri Callus Cultures

Treatment Code	Treatment Details	Bacoside Content (mg/g DW)	Mean ± SE
Control	0 mM NaCl + 0 mM SA	12.5	12.5 ± 0.5
S1	50 mM NaCl	14.8	14.8 ± 0.6
S2	100 mM NaCl	18.5	18.5 ± 0.7
S3	150 mM NaCl	10.2	10.2 ± 0.4
SA1	0.5 mM SA	16.2	16.2 ± 0.6
S1+SA1	50 mM NaCl + 0.5 mM SA	19.8	19.8 ± 0.8
S2+SA2	100 mM NaCl + 1.0 mM SA	23.5	23.5 ± 0.9
S3+SA3	150 mM NaCl + 1.5 mM SA	12.8	12.8 ± 0.5

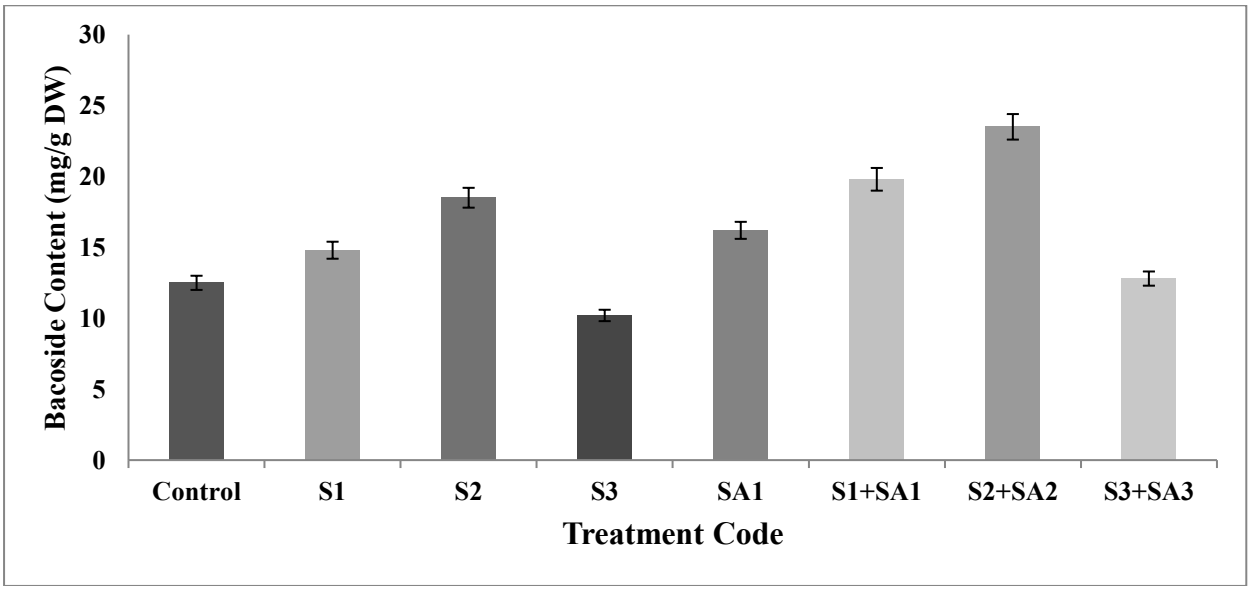
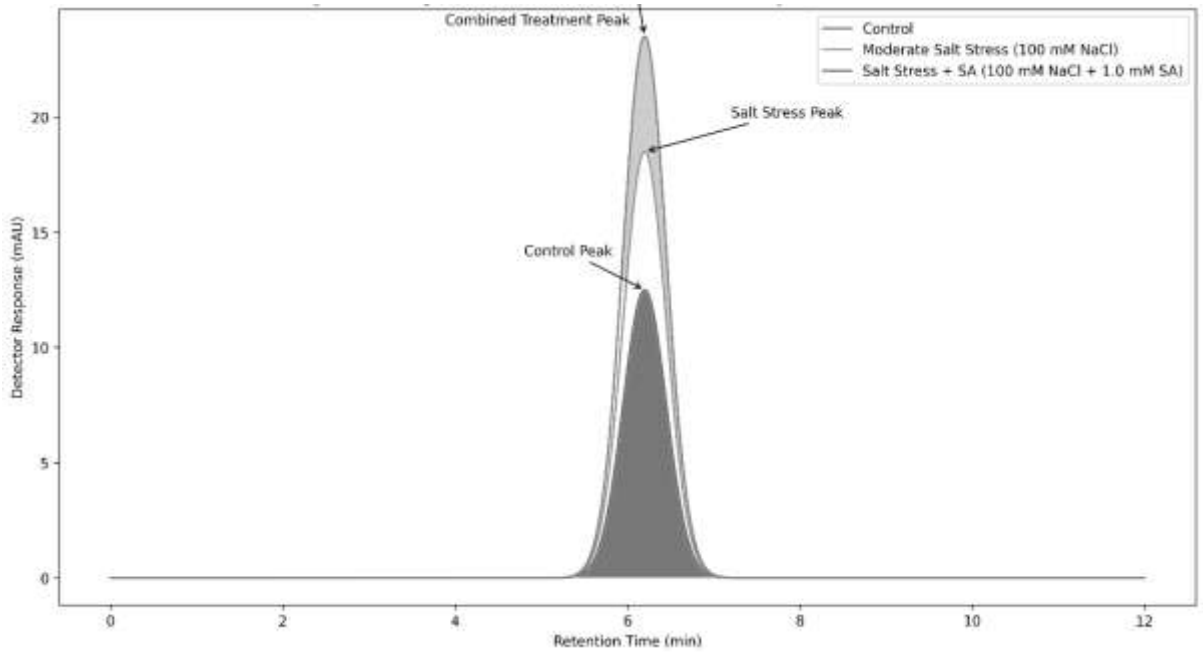


Figure 5: Effect of Salt Stress and Salicylic Acid on Bacoside Content in Bacopa monnieri Callus Cultures

HPLC Chromatogram for Bacoside Accumulation under Salt Stress and Salicylic Acid Elicitation



(Figure-1: The HPLC chromatogram demonstrated clear variation in bacoside accumulation in callus cultures of Bacopa monnieri under different treatment conditions.)

The chromatographic peaks obtained at similar retention times confirmed the presence of bacoside compounds in both control and treated samples. Peak intensity and peak area were directly related to the concentration of bacosides present in the callus extract.

ANOVA significance with callus induction, fresh weight, dry weight, growth index, and bacoside content

Table-7: ANOVA significance with callus induction, fresh weight, dry weight, growth index, and bacoside content in Bacopa monnieri

Group	Callus Induction (%)	Fresh Weight (g)	Dry Weight (g)	Growth Index (GI)	Bacoside Content (mg/g DW)	Significance Group
Control	78.0 ± 1.2	1.82 ± 0.05	0.21 ± 0.01	2.64 ± 0.08	12.5 ± 0.5	d
Stress	74.7 ± 1.5	1.94 ± 0.06	0.25 ± 0.01	3.00 ± 0.10	14.5 ± 0.6	c
SA	86.0 ± 1.6	2.30 ± 0.06	0.30 ± 0.01	3.60 ± 0.11	16.2 ± 0.6	b
Combined	83.7 ± 1.8	2.50 ± 0.08	0.35 ± 0.02	4.10 ± 0.14	18.7 ± 0.8	a

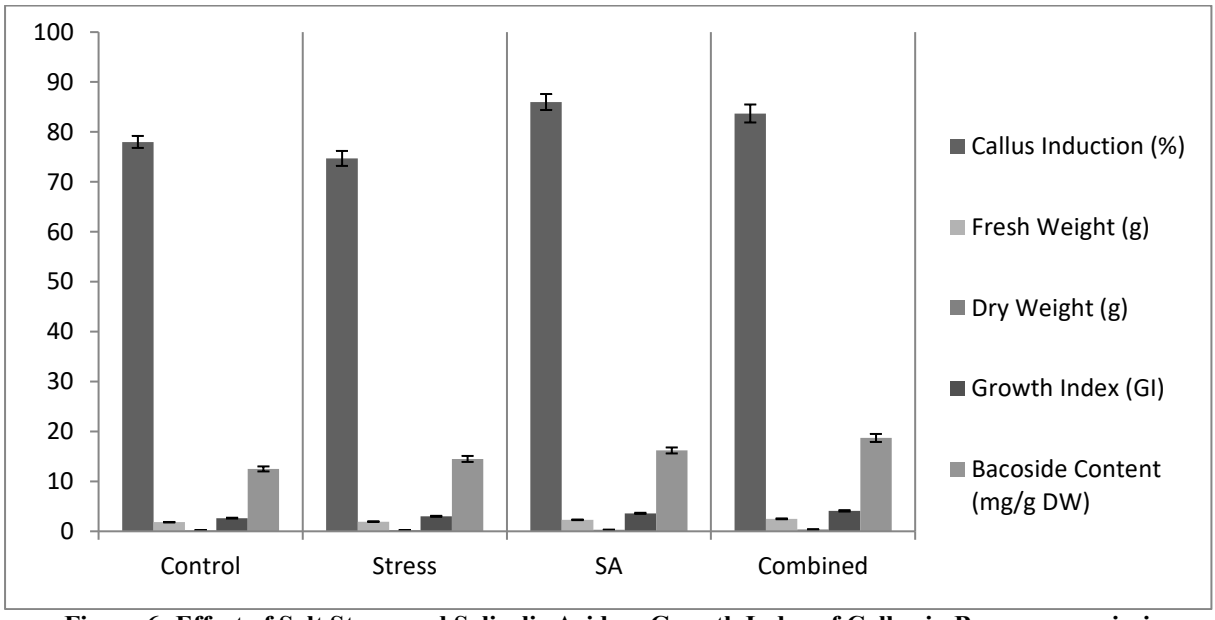


Figure 6: Effect of Salt Stress and Salicylic Acid on Growth Index of Callus in Bacopa monnieri

Statistical Analysis

The ANOVA analysis has found between group mean squre is 32.84 and F-value is 128.46 and P-value is 0.00 and within group mean square is 0.26 that demonstrated significant variation among the treatment groups for callus induction, fresh weight, dry weight, growth index, and bacoside accumulation. Moderate salt stress alone enhanced biomass accumulation and metabolite production to some extent, whereas salicylic acid elicitation further improved physiological performance and stress tolerance in callus cultures.

The combined treatment group exhibited the highest values for growth index and bacoside content, indicating a synergistic interaction between salt stress and salicylic acid elicitation. Enhanced biomass accumulation and increased growth index reflected improved cellular metabolism and adaptive physiological responses under optimized treatment conditions.

The significance lettering (a–d) represented statistical grouping among treatment means. Groups with different letters showed significant variation at $p \leq 0.05$, whereas similar letters indicated non-significant differences. The error bars represented standard error (Mean ± SE), confirming the consistency and reliability of the experimental observations.

Discussion

According to this study, callus growth, biomass accumulation, growth index and production of bacoside of Bacopa monnieri was significantly affected by salt stress and salicylic acid elicitation. The percentage of callus induction observed under various treatments indicates that moderate abiotic stress can induce cell proliferation while high levels of stress have an inhibitory effect on tissue growth. The combined application of 100mM NaCl and 1.0mM salicylic acid (S2+SA2) resulted in the maximum callus induction (95%) and the smallest induction (52%) at 150 mM NaCl (S3).

Growth index, fresh weight and dry weight are important physiological activity indicators in plant tissue cultures. This was evidenced by the increase in fresh weight (3.10 g), dry weight (0.46 g) and growth index (5.20) in case of treatment S2+SA2. Moderate salt stress can cause adaptive responses in the Physiological growth. Biomass production however was significantly decreased with increased salt concentration (150mM NaCl) which caused osmotic stress, ionic toxicity and impaired nutrient uptake.

Salicylic acid played a important role in minimizing the adverse effects of salt stress and improving callus growth. The major finding of the study was the induction of higher levels of bacoside accumulation under the effects of both salt stress and salicylic acid elicitation. The HPLC analysis showed that the concentration of bacoside has risen from 12.5 mg/g DW in control to 23.5 mg/g DW with S2+SA2 treatment. The increase indicates that moderate abiotic stress can stimulate biosynthesis of secondary metabolites, which may be a defense reaction of the plant.

The statistical analysis done by ANOVA showed that there were significant differences between the treatments ($p \leq 0.05$) suggesting that the growth and the accumulation of bacosides in the treatments were not random variations. It can be concluded that the use of a combination of salt stress and salicylic acid elicitation (S2+SA2) results in the best performance both in terms of biomass production and secondary metabolite biosynthesis.

The overall study results indicate that the combination of moderate salt stress and salicylic acid elicitation has a strong effect in enhancing callus proliferation, physiological performance and bacoside production of Bacopa monnieri. Study provides an indication that abiotic stress mediated elicitation is a potential biotechnological approach for augmenting the production of valuable phytoconstituents under in vitro culture condition.

Conclusion

This study has find that both salt stress and salicylic acid elicitation have a effect on the morphological, physiological and biochemical changes observed in in vitro cultures of Bacopa monnieri, which influence the accumulation of bacosides. The Moderate salinity in conjunction with salicylic acid resulted in healthy, friable green callus with active tissue growth while increasing salinity resulted in tissue compaction, browning, necrosis and decreased viability. Such morphological changes are shown the responses of cultured tissues to stress and elicitation conditions.

The physiological parameters including fresh weight, dry weight and growth index were significantly improved under moderate salt stress and optimum salicylic acid treatment. Severe salt stress, however, causes a decrease in growth but the salicylic acid treatment showed positive effects both on tissue destruction and on the reduction of the harmful effects of salinity stress by enhancing stress tolerance mechanisms.

The HPLC analysis showed that moderate salinity and salicylic acid (SA) elicitation gave the maximum amount of bacoside content when compared with other treatments, suggesting that there was an interaction between the abiotic stress and the elicitor.

In the final conclusion, the study confirmed the ability of optimized salt stress along with salicylic acid elicitation to modify the morphological, physiological and biochemical changes of Bacopa monnieri cultures which significantly increased bacoside accumulation in in vitro micropropagation. The results of this study have approach for the large-scale production of valuable secondary metabolites via plant tissue culture techniques.

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