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ASSESMENT OF VARIOUS CONCENTRATION OF SALICYLIC ACID IN TISSUE CULTURE *IN VITRO* SYSTEMS FOR THEIR EFFECT ON MODULATING ABIOTIC STRESS TOLERANCE MECHANISMS IN PEPPER (*CAPSICUM ANNUUM* L.) PLANTS

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Abstract

Abiotic stress, particularly in climate-sensitive crops like Capsicum annuum L., severely impacts crop productivity. Understanding the mechanisms through which plants can mitigate these stresses is key to developing more resilient crop varieties for the future. Salicylic acid is a plant hormone known for its role in regulating plant growth and enhancing resistance to abiotic and biotic stresses. Thus, this study focuses on elucidating the dose-dependent effects of exogenous SA application on physiological, biochemical, and morphogenetic responses of pepper explants cultivated in vitro. The findings revealed a dose-dependent effect of SA, where lower concentrations significantly enhanced seed germination rates, shoot initiation and elongation while promoting robust root development. It also increased the total phenolic and chlorophyll content, suggesting an activation of defense mechanisms and improved photosynthetic efficiency.

Key words*: salicylic acid, in vitro; resilient, vegetable, crops.*

INTRODUCTION

The productivity of agricultural crops is significantly affected by abiotic stress, particularly in species that are highly sensitive to environmental fluctuations, such as *Capsicum annuum* L. (Zhang et al., 2024). Stress factors like drought, salinity, and extreme temperatures can trigger adverse physiological, biochemical, and molecular changes that impede plant growth and development (Dos Santos et al., 2022). These conditions often lead to reduced biomass accumulation, impaired photosynthetic capacity, and diminished crop yields. With the increasing impact of climate change, finding effective strategies to improve plant resilience has become a major priority in agricultural and biotechnological research (Garland and Curry, 2022).

Salicylic acid (SA), a naturally occurring plant hormone, plays a crucial role in the regulation of plant responses to both biotic and abiotic stress (Liu et al., 2022). Its involvement extends to modulating defence mechanisms, enhancing the activity of antioxidant enzymes, and stimulating osmolyte production. SA participates in key metabolic pathways and signaling cascades that influence plant adaptation to adverse conditions by improving stress tolerance, photosynthetic efficiency, and secondary metabolite synthesis (Salam et al., 2023).

Previous research has highlighted the beneficial effects of exogenous SA application in counteracting stress-induced damage that occurs in the presence of salt (Quamruzzaman et al., 2001; Li and Liu, 2023), UV-B (Khare et al., 2023), ozone (Liu et al., 2021), drought (Naz et al., 2021), heat (Shaukat et al., 2022), cold (Soualiou et al., 2022; Shaukat et al., 2022) and metal stress (Bilal et al., 2023). Studies indicate that SA enhances the activity of antioxidant defense systems, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which mitigate oxidative damage caused by stress (Fujita and Hasanuzzaman, 2022; Zulfiqar and Ashraf, 2021). Additionally, SA influences stomatal regulation, thereby optimizing water-use

efficiency under drought conditions. Moreover, its role in stimulating the production of secondary metabolites such as flavonoids and phenolic compounds is well-documented, with these compounds playing a protective role in reducing oxidative stress. However, the extent of SA's effectiveness depends on its concentration, with both stimulatory and inhibitory effects observed depending on the applied dose (Robertson and Li, 2021; Hasan et al., 2022).

In vitro tissue culture techniques offer a controlled environment to investigate the physiological and biochemical responses of plants to SA under stress conditions. These systems minimize external environmental variability, allowing precise analysis of SA's impact on plant growth and adaptation mechanisms. Additionally, *in vitro* cultures facilitate the study of morphogenetic responses, such as shoot and root development, callus formation, and somatic embryogenesis, which are indicative of stress resilience (Custódio et al., 2022; Wijerathna-Yapa and Hiti-Bandaralage, 2023).

This study aims to elucidate the effects of varying SA concentrations on *Capsicum annuum* L. explants cultivated under in vitro conditions. By assessing parameters such as germination rates, shoot initiation, root development, chlorophyll content, and total phenolic accumulation, we seek to identify the optimal SA concentration that enhances stress tolerance mechanisms. The outcomes of this research will deepen our understanding of SA-mediated stress adaptation and may provide valuable insights for improving the resilience of pepper plants in commercial agriculture and crop improvement programs.

The findings revealed a dose-dependent effect of SA, where lower concentrations (0.1 mM and 0.5 mM) significantly enhanced seed germination rates, shoot initiation, and elongation while promoting robust root development. These concentrations also increased the total phenolic and chlorophyll content, suggesting an activation of defense mechanisms and improved photosynthetic efficiency. Conversely, higher concentrations (1 mM SA) delayed germination and inhibited root and shoot development, indicating potential phytotoxic effects at elevated levels.

The study underscores the role of SA in modulating stress-responsive pathways, particularly through its interaction with endogenous hormonal signaling and defense-related processes. By optimizing SA concentrations, this research provides a foundation for improving pepper plant resilience to abiotic stresses in controlled environments, with potential implications for field applications and sustainable agriculture.

MATERIALS AND METHODS

For this study, seeds of pepper, 'Ionel' variety were selected as plant material. The experiments were conducted at the Vegetable Research and Development Station in Bacau, within the in vitro Tissue Culture Laboratory. The primary objective was to evaluate the impact of exogenous salicylic acid (SA) application on pepper plants' responses to abiotic stress. To ensure aseptic conditions for tissue culture experiments, the seeds underwent a surface sterilization process by immersion in a 0.1% mercuric chloride ($HgCl_2$) solution for 10 minutes, followed by thorough rinsing with sterile distilled water to eliminate any remaining sterilizing agents.

Murashige and Skoog (MS) medium (1962) were used as the basal growth medium. It was supplemented with 30 g/L sucrose as a carbon source to support plant growth. To solidify the medium, agar was added at a concentration of 8.0 g/L. Three different concentrations of SA (1 mM, 0.5 mM, and 0.1 mM) were incorporated into the medium to assess their effects on morphogenetic reaction and plant growth. The pH of the medium was adjusted to 5.8 before autoclaving.

Sterilized seeds were germinated on MS medium under optimal conditions, and germination indices were recorded to assess SA's effects. One-week-old seedlings provided apical and hypocotyl explants for tissue culture. Four treatment groups were established: control (V0, no SA), 1 mM SA (V1), 0.5 mM SA (V2), and 0.1 mM SA (V3). Each treatment variant was replicated three times to ensure result accuracy.

The tissue cultures were maintained under controlled environmental conditions, with an incubation temperature of 26±1°C. A 16-hour

photoperiod with a light intensity of 5000 lx was provided to simulate optimal conditions for photosynthesis. Subculturing was performed every 30 days to prevent overcrowding and maintain continuous growth. Daily observations were conducted to monitor morphological and physiological changes in response to the different SA concentrations.

To facilitate acclimatization, rooted plantlets were transferred to hydroponic conditions within bottles, while newly formed shoots were transplanted into fresh media for the microclonal propagation phase. Hydroponic solutions contained a Previcur 0.15%, and in order to maintain a relative humidity of 100%, the containers were covered with transparent bags. The newly formed plants were kept in an acclimatization room under a 16-hour light/8-hour dark photoperiod, with temperatures maintained between 20°C and 23°C.

After acclimatization, plants were transferred to a potting mixture consisting of sterilized sand and vermiculite (1:1) in plastic containers. They underwent a hardening phase inside a mist chamber with 80% relative humidity for two weeks before final transplantation to the greenhouse.

Chlorophyll content was assessed following the methodology described by Lichtenthaler (1987). Fresh plant samples (0.1 g) were homogenized and extracted using 80% acetone. The extract was centrifuged for 10 minutes at 3.530 rpm using a Hettich Universal Centrifuge 320 | 320 R. The supernatant was collected, and absorbance was measured at 470 nm, 647 nm, and 663 nm using a UV-VIS spectrophotometer.

The concentrations of chlorophyll a, chlorophyll b, and carotenoids were calculated using the following formulas:

- Chlorophyll a (Cla) concentration (mg/L) = 12.25 × Abs 663 nm - 2.79 × Abs 647 nm
- Chlorophyll b (Clb) concentration (mg/L) = 21.50 × Abs 647 nm - 5.10 × Abs 663 nm
- Carotenoids (Car) concentration (mg/L) = (1000 × Abs 470 nm - 1.82 × Cla - 85.02 × Clb) / 198

Tissue culture data were analyzed using ANOVA to assess the significance of SA's effects. Mean values and standard deviations were calculated for each treatment variant to facilitate comparative analysis and interpretation of results.

RESULTS AND DISCUSSIONS

Effects of salicylic acid on germination and seedling development

The application of salicylic acid (SA) at different concentrations significantly influenced the germination dynamics and early seedling development of *Capsicum annuum* L. Our results indicate that seeds cultivated on variant with 1 mM SA exhibited a pronounced delay in germination, approximately three days longer than the control group. This suggests that elevated SA concentrations interfere with dormancy release and germination initiation, potentially by modulating hormonal balance or altering cellular metabolic processes.

Following germination, seedlings exposed to 1 mM SA displayed severe root developmental anomalies, including stunted growth, calluses and, in some cases, complete inhibition of root formation. The irregular root architecture observed in these seedlings suggests that high SA concentrations may disrupt root elongation and differentiation, ultimately impairing the plant's ability to efficiently uptake water and nutrients (Figure 1).

Figure 1. Plantlets with abnormal development exhibited on variant with 1.0 mM SA

Conversely, at a lower concentration of 0.1 mM SA, germination rates remained comparable to those of the control group, indicating that this concentration does not negatively impact seed dormancy release. Moreover, the plants emerged on variant with 0.1 mM SA exhibited enhanced shoot growth and overall vigor,

highlighting the dose-dependent nature of SA's effects on early plant development (Figure 2).

Figure 2. Pepper plants regenerated on variant with 0.1 mM SA

Morphogenetic responses of explants to SA treatment

The morphogenetic response of *C. annuum* explants varied depending on both the type of explant used and the concentration of SA in the culture medium. Apical explants exhibited the highest morphogenetic potential, with rapid shoot initiation at the base of the explants. The addition of 0.1 mM SA further enhanced shoot multiplication and elongation, underscoring the positive role of this concentration in stimulating shoot formation.

In contrast, hypocotyl explants displayed a different developmental pattern, primarily forming roots rather than shoots (Figure 3).

Figure 3. Morphogenetic reaction of pepper hypocotyls under the influence of SA

However, on lower concentrations of SA, shoot formation was observed at one side of the hypocotyl segments, which subsequently developed into fully differentiated plantlets. These observations suggest that the developmental fate of hypocotyl explants is influenced by local hormonal gradients and the microenvironment created by SA supplementation.

Quantitative analysis revealed that shoot formation varied significantly across different SA concentrations. In media supplemented with 0.1 mM SA, 96.94% of explants successfully generated shoots, compared to 74.84% in the control group. The highest SA concentration (1 mM) led to a notable decline in shoot initiation, with only 67.74% of explants forming shoots. These results indicate that while moderate SA concentrations can enhance shoot proliferation, excessive levels may exert an inhibitory effect on shoot induction and development (Table 1).

Table 1. Effects of different concentrations of SA in MS medium for multiple shoot induction at pepper plants after 60 days of culture - means ± SE

Variant	% of explant showing response	Average no. of shoots
V0 - Control	74.84	19.3±1.0
V1 - 1 mM SA	67.74	8.7±0.4
V2 - 0.5 mM SA	88.04	28.1±0.3
V3 - 0.1 mM SA	96.94	35.9±1.3

Impact of SA on shoot and root elongation

SA treatment significantly influenced both shoot and root elongation, with notable differences depending on the applied concentration. The most pronounced growth stimulation was observed in explants cultured on media supplemented with 0.1 mM and 0.5 mM SA, where shoot elongation rates exceeded those of the control group. These results suggest that SA promotes the transition from meristematic to shoot-forming tissues, accelerating shoot emergence and elongation.

Similarly, root elongation was significantly enhanced in explants exposed to 0.1 mM SA, indicating a stimulatory effect on root growth. However, at 1 mM SA, root development was severely restricted, confirming the dose-dependent influence of SA on plant morphogenesis.

The comparative analysis of shoot proliferation and elongation rates across different SA concentrations underscores the critical

importance of optimizing exogenous SA application in in vitro culture. While lower concentrations (0.1-0.5 mM) enhance plant growth and vigor, higher concentrations may trigger inhibitory responses, limiting morphogenetic potential (Figure 4).

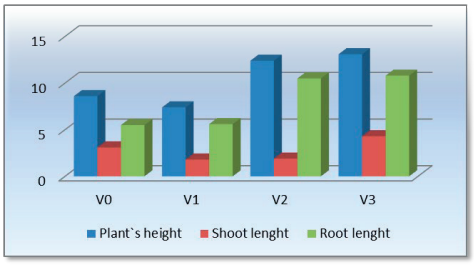

Figure 4. Biometric measurements on Brassica plants cultivated on media supplemented with different concentration of SA

Acclimatization and survival of plantlets

Following root formation, plantlets were transferred to hydroponic conditions and subjected to a hardening process to facilitate acclimatization. During the initial week, a high relative humidity (90%) was maintained, followed by gradual reductions to simulate environmental adaptation. This approach resulted in a survival rate exceeding 95%, demonstrating the effectiveness of the hardening process in ensuring successful plant establishment under hydroponic conditions.

Influence of SA on photosynthetic activity

Chlorophyll content analysis revealed a significant increase in total chlorophyll in plants treated with 0.1 mM SA compared to the control group. However, at higher SA concentrations, chlorophyll a content was either similar to or slightly lower than that of the control. These results align with previous studies (Gupta and Seth, 2021; Azeem et al., 2023), which suggest that moderate SA levels can enhance chlorophyll biosynthesis, while excessive concentrations may disrupt pigment accumulation and photosynthetic efficiency.

Overall, our findings highlight the complex regulatory role of SA in *C. annuum* development. The concentration-dependent effects observed in germination, morphogenesis, and photosynthetic pigment accumulation underscore the necessity of precise SA dosage optimization to maximize its benefits while mitigating potential inhibitory effects.

CONCLUSIONS

This study examines how varying concentrations of salicylic acid (SA) influence the growth, development, and stress tolerance of *C. annuum* under in vitro conditions by modulating physiological and biochemical responses.

The study found that high SA concentrations (1 mM) inhibited seed germination, root development, and shoot formation, while lower concentrations (0.1 mM) promoted seedling vigor and significantly enhanced shoot formation (96.94% success). These findings highlight SA's dual role in early plant development, depending on its concentration.

Low SA concentrations (0.1-0.5 mM) promoted shoot elongation, root formation, and chlorophyll accumulation, while high concentrations (1 mM) inhibited growth and reduced photosynthetic efficiency. *In vitro* derived plantlets adapted well to hydroponic conditions, achieving over 95% survival, highlighting SA's role in plant morphogenesis and greenhouse cultivation potential.

Optimizing SA concentrations is crucial to balancing its benefits and inhibitory effects. The 0.1 mM SA concentration proved most effective in enhancing growth, shoot proliferation, and stress tolerance. These findings provide insights into SA-mediated physiological responses, with practical applications for in vitro propagation and stress resilience in commercial pepper cultivation.

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