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Effects of nitric oxide, spermidine, and salicylic acid signaling and their crosstalk with each other in the production of commercially important stevioside content and drought stress responses in *Stevia rebaudiana* bertoni

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Abstract

The leaves of *Stevia rebaudiana* Bertoni contain steviol glycosides (SGs), which provide the sweet taste of stevia. However, drought can have a negative impact on the plant's growth and development. To address this issue, signaling molecules such as sodium nitroprusside (SNP), spermidine (SPD), and salicylic acid (SA) are often applied to increase plant tolerance. However, the combined effects of these molecules have not been extensively studied. This research aimed to investigate the effects of controlled elicitation with SA, SNP, SPD, and their combinations on plant performance, SG content, and drought stress mitigation in *Stevia rebaudiana* under drought stress. The elicitor treatments were found to result in a significant increase in SG content, with 0.1 mM SA being the most effective treatment. Additionally, the treatments were able to reduce the stress effects on growth parameters to non-stress levels. The use of SPD, SA+SNP, and SPD+SNP on stressed plants significantly increased CAT and SOD activity, resulting in a more active antioxidant defense system that lowered MDA contents and H_2O_2 generation. These findings suggest that stevia cultivation with controlled elicitation could be used to improve plant growth, tolerance, and SG production under drought stress conditions.

Introduction

Stevia (*Stevia rebaudiana* Bertoni), a perennial herb of the family Asteraceae, is increasing in popularity as a non-caloric natural sweetener that could be utilized as a replacement for manufactured sweeteners and as a natural medication (Vasquez-Hernandez *et al.*, 2019). Diabetes, cancer, obesity, hypertension, and dental decay have been treated using the leaf extract of stevia (Gantait *et al.*, 2015; Álvarez-Robles *et al.*, 2016; Singh *et al.*, 2017). The requirements for steviol glycoside (SG) sweeteners were determined by the Joint FAO/WHO Expert Committee on Food Additives in 2007 (JECFA, 2007). These chemicals are responsible for plant–environment interactions as well as the plant's low caloric content and sweet taste (Jain *et al.*, 2014). The key ingredients in most SG products are rebaudioside A (Reb A) and stevioside (Yoneda *et al.*, 2018). Stevia can be propagated using a variety of plant parts, including seed, leaf, inter-node, node and shoot tip, and stem explants (Uddin *et al.*, 2006; Seyis *et al.*, 2017; Sichanova *et al*. 2022). The stem-cutting procedure has limitations because only a small number of new plants may be reproduced from a single plant. When the seed is utilized, the plants have a low magnitude of sweetness variation (Tadhani *et al.*, 2007; Mathur & Shekhawat, 2013; Moharramnejad *et al.*, 2019).

Climate change and water scarcity issues have affected the productivity of many plant species in semi-arid locations in recent years. Furthermore, due to the anticipated water shortage as a result of global warming, as well as increased competition from agricultural and industrial water consumers, efficient water resource management has become of great importance (Gholami Zali & Ehsanzadeh, 2018). Drought stress (DS), a major constraint to plant growth and development, causes a sharp decline in relative water content and stomatal conductance, limiting plant biomass, yield, and metabolic productivity (Gunes *et al.*, 2007). To protect themselves from dehydration and maintain cell volume, plants undergo an osmotic adjustment process through the accumulation of solutes in their cells (Rahdari & Hoseini, 2012).

Some physical and chemical factors (elicitors) that are commonly used for the synthesis of phenols, flavonoids, stevioside, and other useful chemicals can induce secondary metabolite production in plants, resulting in a protective state against stress factors without limiting plant performance (Mathur & Shekhawat, 2013; Cardenas-Manrquez *et al.*, 2016; Vázquez-Hernández *et al.*, 2019). Elicitors, which set off a cascade of complicated responses at the molecular, biochemical, and physiological levels, have been shown to stimulate certain biosynthetic pathways, resulting in increased levels of stress metabolites, all geared toward environmental acclimatization and adaptation (Giri & Zaheer, 2016).

Exogenous application of signaling molecules such as sodium nitroprusside (SNP) (a commonly used nitric oxide (NO) donor), spermidine (SPD), and salicylic acid (SA) to enhance stress tolerance by triggering plant response is a well-known stress mitigation strategy (Chavoushi *et al.*, 2019). In plants, SA is a key signaling molecule that promotes tolerance to a wide range of biotic and abiotic stressors (Horváth *et al.*, 2007). SA is also involved in the control of a number of physiological processes in plants, including membrane permeability, growth and development, and ion absorption and transport (Raskin, 1992). Under stress conditions, exogenous SA has an effect on the rate of reactive oxygen species (ROS) formation. Furthermore, SA affects antioxidant enzyme activity and boosts plant tolerance to abiotic stressors (Horváth *et al.*, 2007). SA has been discovered to have varied impacts on plant development and stress adaptation depending on the technique, concentration, application time, and plant species (Metwally *et al.*, 2003; Miura & Tada, 2014; Khan *et al.*, 2015).

Some research has highlighted the role of polyamines, particularly SPD (Alcázar *et al.*, 2010; Li *et al.*, 2016; Sequera-Mutiozabal *et al.*, 2017). Because of its cationic nature, SPD interacts with biological molecules and encourages cellular multiplication and morphogenesis when added to culture media (Kevers *et al.*, 2002). Exogenous SPD was reported to partially mitigate the DS-induced loss in photosynthetic efficiency in previous investigations (Yin *et al.*, 2014).

NO, another signaling molecule, is associated with a number of physiological processes including iron availability, growth, germination, and adaptive response to external stresses (Hancock, 2020; Ageeva-Kieferle *et al.*, 2021). From the perspective of abiotic stresses, it has been demonstrated that using the SNP which is NO donor lessens the negative effects of salt, drought, and heavy metals in plants (Arasimowicz & Floryszak-Wieczorek, 2007; Kazemi *et al.*, 2010). However, since NO is a reactive nitrogen species, investigations have revealed that its effects on various cells can be either protective or harmful, depending on the dosage and location of activity. NO also acts as an antioxidant and a signaling molecule, triggering changes in antioxidative gene expression, and protecting plant cells from oxidative damage (Arasimowicz & Floryszak-Wieczorek, 2007).

Exogenously applied elicitors increase secondary metabolite production as a response to stress. Strategies aimed at increasing the SG content of stevia (e.g., employing controlled elicitation) would contribute to enhancing the sweetness and therapeutic benefits of this species (Vasquez-Hernandez *et al.*, 2019). Various relieving agents, including SNP, polyamines, and SA have been used to treat different effects of abiotic stress, including DS, but the combined use of SNP, SPD, and SA under drought stress in stevia has not been reported (Shehab *et al.*, 2010; Bidabadi *et al.*, 2012; Kumar *et al.*, 2012; Yildiztugay *et al.*, 2014; Singh *et al.*, 2017). The vast majority of the scientific literature concentrated on a single application of these agents. Therefore, in the current study, SNP, SPD, SA, and various combinations of two of these signaling molecules (SNP+SA, SPD+SA, SPD+SNP) were exogenously applied to plant cultures. The contents of oxidative stress indicators including malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were measured to investigate the roles of these signaling molecules. The quantity of stevioside as well as the antioxidant enzyme activities like superoxide dismutase (SOD) and catalase (CAT) was investigated to determine the plant's response to stress. The aim of the present study was to assess the impact of controlled elicitation using SA, SNP, SPD, and their combinations on SG content and plant performance under DS and the potential effects of elicitors on DS mitigation in *Stevia rebaudiana*.

Materials and Methods

Plant Materials

Stevia rebaudiana plantlets were obtained from single-seed descendant seedlings. To obtain a sufficient number of plants, shoot tips of 4-week-old plantlets were transferred to culture vessels containing well-watered vermiculite. The plantlets with well-developed roots were carefully removed from the vessels and transferred to pots containing a soil:vermiculite mixture (3:1). Before drought stress was applied, the plants were given a 5-day pretreatment with stress relievers, which included SNP (0.1 mM), SPD (0.1 mM), and SA (0.1 mM), as well as SA + SNP (0.1 mM + 0.1 mM), SA + SPD (0.1 mM + 0.1 mM), and SPD+SNP (0.1 mM + 0.1 mM). The required concentrations of elicitor were prepared in sterile distilled water containing 1% Tween 20 (V/V) (Merck, Darmstadt, Germany). All pots were watered every two days until the beginning of the drought stress (DS), which was imposed by restricting water supplies for 12 days. The stress relievers were applied three times by foliar spray (using 10 mL per plant) to each group's 23-day-old plant, until all of the leaves were fully soaked on both sides. After 12 days, the shoot dry weight (SDW), shoot fresh weight (SFW), root dry weight (RDW), root fresh weight (RFW), shoot length (SL), root length (RL), and leaf number (LN) were recorded. Leaves at the same location on many stems were taken from the base, middle, and top parts of the plant in all experiments to avoid confounding variations in the results related to leaf age. The experiment consisted of five biological replicates, each containing six plants kept in a culture room (16-h light/8-h dark photoperiod at 24 ± 2 °C). The treatment concentrations of stress relievers were determined using literature and early experimental efforts (Moharramnejad *et al.*, 2019; Pradhan *et al.*, 2020).

Quantification of Stevioside Contents

The stevioside content of the samples was determined by a previously described method (Kolb *et al.*, 2001). The extracts were made by dissolving exactly 20 mg of dried leaf powder in 20 mL of analytical grade ethanol (70%, V/V). HPLC was performed using a Shimadzu system (LC-8A; Kyoto, Japan) for the quantification of Reb A and stevioside. The ethanol extracts (each 20 µl of the leaf samples) or standard samples were loaded into an HPLC C18 column (Nucleodur HILIC, 250 × 5 mm) under isocratic conditions. Analytical grade acetonitrile: water (80:20, V/V) was used as mobile phase at a flow rate of 2 mL min^{-1}. The peaks were integrated at 200 nm to quantify stevioside content. The results are given in mg/g of dry weight for each sample.

Enzyme Extraction and Protein Determination for Enzyme Assays

Enzyme extraction and protein determination for analyses of CAT and SOD activities were performed as previously described by Şahin (2019). For the enzyme analysis, 0.5 g of fresh weight (FW) of the leaves were homogenized in 2 mL of ice cold 50 mM K-phosphate buffer containing 2 mM Na-EDTA and 1% polyvinylpyrrolidone (PVP); then the homogenate was centrifuged at 4 °C and 12,000 rpm min^{-1} for 10 min. Tissue extracts were kept at –80 °C for determination of CAT and SOD activities. The protein content was determined according to Lowry *et al.* (1951).

Catalase Activities

The CAT activity was determined spectrophotometrically by monitoring the consumption of H_2O_2 at 240 nm (extinction coefficient at 38.9 mM^{-1} cm^{-1}) over a 2-min interval (Chance and Maehly 1955). The values are expressed in units (U) mg^{-1} protein^{-1}, FW.

Superoxide Dismutase Activities

The technique described by Giannopolitis and Ries (1977) was used to evaluate SOD activity, which is based on the enzyme's capacity to prevent the photochemical reduction of nitro blue tetrazolium (NBT). The SOD activity was determined spectrophotometrically by measuring absorbance at 560 nm in both the control and main groups. The values are expressed in units (U) mg^{-1} protein^{-1}, FW.

H_2O_2 Contents

As previously detailed by Şahin (2019), the H_2O_2 level was detected spectrophotometrically at 415 nm. An H_2O_2 standard curve was used to compute the H_2O_2 content, which was represented as mmol g^{-1} FW.

Malondialdehyde Contents

Trichloroacetic acid (TCA) (1%, W/V) was used to homogenize the leaves. The homogenate in TCA was combined with 0.5% (W/V) thiobarbituric acid (TBA) and incubated at 95 °C in a water bath for 30 min before quick cooling in an ice bath. The absorbance of the supernatant was measured spectrophotometrically at 532 nm. The MDA concentration was determined using an extinction coefficient of 155 mM cm^{-1} after deducting the nonspecific absorbance at 600 nm (Heath & Packer, 1968).

Statistical Analysis

All data were evaluated by analysis of variance (ANOVA), and mean values were compared via Duncan's multiple range tests using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Values of $P \leq 0.05$ indicated significance between the treatments and control at a given time. The experiments for the analysis of growth parameters were performed in five replicates. The analyses to determine the contents of oxidative stress indicators, the quantity of stevioside, and antioxidant enzyme activities were performed in three replicates.

Results and Discussion

The SDW, SFW, RDW, RFW, SL, RL, and LN of *Stevia rebaudiana* Bertoni were all measured under different experimental conditions to see how drought stress affects the development and growth of stevia (Figure 1). DS significantly increased RL (from 9.75 to 15.0 cm) but decreased SL (from 18.15 to 10.13 cm) (Figure 2B, C). Compared to the control (non-stressed plants), RFW (from 0.78 to 1.35 g) and RDW (from 0.051 to 0.063 g) were both enhanced.

Figure 1. The effects of different elicitors on morphological changes in *S. rebaudiana* cultivars: (a) SA, (b) DS, (c) control, (d) SA+SPD, (e) SNP, and (f) SDP under drought stress.

The use of SA significantly increased RFW and RDW under drought conditions, compared to non-stressed plants (Figure 2A). Additionally, when compared to drought-stressed plants, the exogenous application of SA resulted in a significant increase in LN, SL, SFW, and SDW, while RFW and RDW remained similar to those of drought-stressed plants.

These findings indicate that the SA treatment had a positive impact on plantlet growth (LN, SL, RL, RFW, and RDW) under drought stress in the current study (Figure 2), which is consistent with its effects on *Phaseolus vulgaris*, *Carthamus tinctorius*, and other plants (Kazemi *et al.*, 2010; Sadeghipour & Aghaei, 2012; Chavoushi *et al.*, 2020). Previous studies have suggested that SA regulates plant growth and development by influencing growth regulators such as auxin, abscisic acid (ABA), and ethylene (Zhu, 2001; Pacheco *et al.*, 2013).

The exogenous application of SPD to drought-stressed plants decreased LN, SL, SFW, SDW, RFW, and RDW when compared to the control. When compared with drought-stressed plants, the SPD application to the plants under drought stress caused no significant effect on LN, but it significantly decreased RL. de Agazio *et al.* (1995) reported that spermidine (Spd) treatment induced 50% inhibition of root extension in maize seedlings. The inhibition of root growth observed during Spd treatment of maize seedlings was observed to be associated with a lignification of the cell wall and a reduction of both the mitotic index and cell elongation.

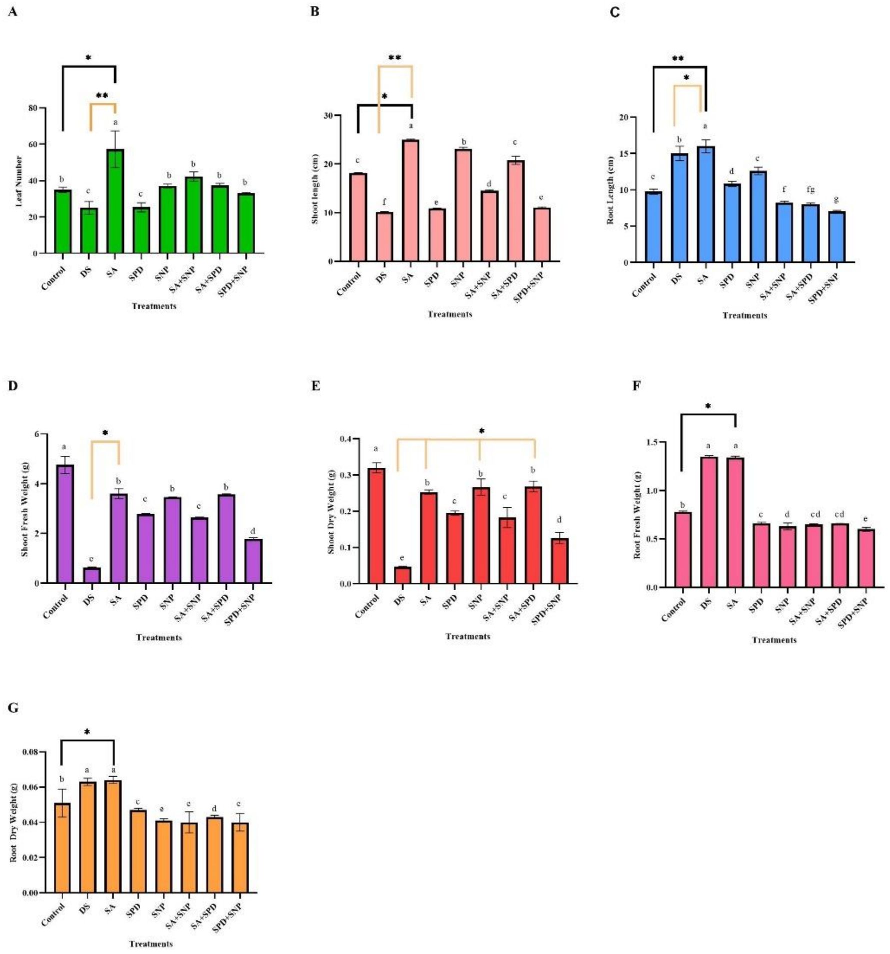

Figure 2. Effects of stress alleviators on growth parameters of plants under drought stress, A: leaf number, B: shoot length, C: root length, D: shoot fresh weight, E: shoot dry weight, F: root fresh weight, G: root dry weight. All mean values which share the same letter are not significantly different; otherwise, they differ significantly at P≤0.05. Values expressed as means ± SD of five replicates.

The application of exogenous SNP to drought-stressed plants resulted in enhanced SL and RL compared to the control plants. Furthermore, it also caused an increase in SFW and SDW when compared to the drought-stressed plants. (Figure 2B, E). A similar result was also reported in a review article (Popova & Tuan, 2010). The study also found that the SNP treatment decreased RDW and RFW, compared with non-stressed and drought-stressed plants. Chavoushi *et al.* (2019) suggest that this effect may be due to the ability of NO to alter the cell cycle and expression levels of root developmental genes.

The DS stimulated root growth and therefore RL, RDW, and RFW. This finding might be linked to the shortage of water, which caused the root to extend and expand in order to efficiently absorb soil moisture (Akinci & Losel, 2009). Under drought conditions, the combined application of SA+SNP, SA+SPD, and SPD+SNP to the plants had no significant effect on LN, but it reduced RL, SDW, RDW and RFW in comparison to the control. The combined application of SA+SNP, SA+SPD, and SPD+SNP increased SFW, SDW, LN, and SL, but decreased RL, RFW, and RDW when compared to the drought-stressed plants. The application of SA+SNP, SA+SPD, and SPD+SNP to plants under drought stress mitigated the negative effects of drought stress on growth parameters (LN, RFW, SFW, RDW, SDW, and RL). The elicitor treatments examined in our study had a considerable positive impact on the morphology of Stevia plants. This observation could be attributed to the elicitation, which led to the reduction of stress effects on practically all growth parameters to a non-stressed level.

In our research, elicitor treatments resulted in significant changes in the SG contents of stevia leaves when compared to the control plants (Figure 3).

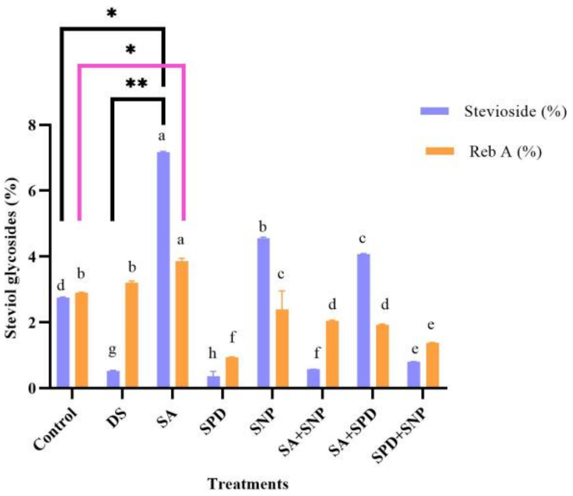

Figure 3. Effects of stress elicitor treatments on stevioside and Reb A content under drought stress. The data are means ± SD from three replicates. Values followed by different letters in the same column are significantly different (*P*≤0.05).

The best treatment that increased SG contents was 0.1 mM of SA, with 2.59-fold of stevioside and 1.33-fold of Reb A, which agrees with the findings reported by Vasquez-Hernandez *et al.* (2019), who found that SA-treated plants had a 2.42-fold increase in iso Reb B and a 1.39-fold increase in Reb A.

In addition to the SA findings, SNP and SA+SPD raised the stevioside concentration by 1.65- and 1.47-fold, respectively. The application of combinations of both SPD+SNP and SA+SNP to the plants under drought stress decreased the stevioside and Reb A content compared to the control plants. The decrease in stevioside and Reb A content under the combined treatment of elicitors may be due to a complex interaction between the different elicitors and the plant's response to stress. It is possible that when multiple elicitors are applied together, they may activate different signaling pathways that could compete with each other or interfere with each other's effectiveness. This may result in an overall decrease in the biosynthesis of Stevioside and Reb A. Our findings indicate that elicitors applied foliarly to stevia plants trigger defense mechanisms and cause glycoside accumulation and an increase in antioxidant activity. In addition to the SA results, an increase in endogenous H_2O_2 content (0.81 mmol g^{-1} FW) considerably enhanced the quantity of SGs in the leaves of stevia. These findings clearly demonstrate that elicitors applied foliarly to stevia plants trigger the defense mechanisms, which is in line with the results reported by Javed *et al.* (2018), who showed a high amount of SGs obtained in stevia leaves under various H_2O_2 concentrations. Vasquez-Hernandez *et al.* (2019) showed the relative gene expression linked with SGs biosynthesis. In this work, treatment of stevia leaves with SA (0.1 mM) increased the concentration of SGs, which correlated with the inducement of gene expression associated with the biosynthesis of these compounds and an increase in LN. Hajihashemi and Geuns (2017) clearly indicated that the elicitors examined exerted significant transcriptional control over SG production, comparable to what has been observed with gibberellin treatments on stevia plants. Consequently, the potential metabolic interaction/competition between elicitors and many phytohormonal responses appears to be involved in the physiology of growth regulation via dynamic changes. The interaction of elicitors with other plant growth regulators may be antagonistic or synergistic under optimum and stressed conditions. By signaling crosstalk with other plant growth regulators, SA can control numerous plant responses in both optimum and stressful conditions (Khan *et al.*, 2015). Iglesias *et al.* (2011) have found that SA-mediated auxin signaling inhibition. In *Solanum lycopersicum*, SA caused the production of ABA in plants under normal and saline conditions, which increased the growth properties and facilitated osmotic adaptation (Szepesi *et al.*, 2009).

SA is one of the most important phytohormones that interacts with NO, working as a secondary messenger (Nawaz *et al.*, 2017). NO has been proposed to operate as a downstream SA-signaling molecule in the mitigation of stimulated oxidation in *Triticum aestivum* seedlings that have been osmotically stressed (Naser Alavi *et al.*, 2014). SA may also promote NO production by increasing the activity of NO-producing enzymes (Zottini *et al.*, 2007). Canola plants under Ni stress treated with SA+NO showed enhanced plant growth (Kazemi *et al.*, 2010). SNP or SA treatment applied to Ni-stressed plants mitigates the harmful effects of Ni. Compared with exogenous stand-alone applications of SA and SNP to Fe-deficient *Arachis* seedlings, the combined application of SA and NO resulted in higher Fe absorption and reduced leaf interveinal chlorosis (Kong *et al.*, 2014). In our research, the application of both SA and/or SNP to the plants significantly increased LN, SL, SFW, and SDW when compared to the drought-stressed plants.

NO, in conjunction with phytohormones and secondary messengers, plays a crucial function in a variety of plant metabolic and physiological processes. Numerous synergistic and antagonistic interactions between NO and all major plant growth regulators have been identified, as summarized elsewhere (Asgher *et al.*, 2017). Bitrián *et al.* (2012) indicate that NO has a role in both stress and developmental responses caused by polyamines (PAs) such as putrescine, SPD, and spermine. It was shown that PAs can cause high NO production in *Arabidopsis* seedlings (Tun *et al.*, 2006). The fact that L-Arg is a common precursor in the production of PAs and NO adds to the evidence that these two signaling molecules are linked (Gao *et al.*, 2009). Concurrent NO and putrescine applications protected *Vigna radiata* from cadmium contamination by a number of mechanisms, including the activation of antioxidants, as well as an increase in phytochelatin production (Nahar *et al.*, 2016). In our research, the application of both SPD+SNP to the plants significantly increased antioxidant enzyme activities.

During the control of plant processes, synergistic or antagonistic interactions between SA and NO have been reported (Durner & Klessig, 1999; Manjunatha *et al.*, 2010; Gémes *et al.*, 2011; Chavoushi *et al.*, 2019). According to findings reported by Kumar and Klessig (2000), SA-stimulated protein kinase appears to work downstream of SA in the signaling pathway of NO in plant defense mechanisms in transgenic tobacco. In *Brassica napus* under Ni stress, the combined application of SA and NO had a synergistic effect (Kazemi *et al.*, 2010). SA is thought to cause stomatal closure by activating peroxidase in guard cells, leading to the generation of extracellular ROS and NO, as well as inactivating K^+_{in} channels, resulting in stomatal closure (Khokon *et al.*, 2011). In the current study, the application of both SA+SNP to the plants significantly increased antioxidant enzyme activities.

SA stimulated the generation of ROS, including H_2O_2 (Gémes *et al.*, 2011), and elevated NO synthesis in *Arabidopsis* plants in a dose-dependent manner (Zottini *et al.*, 2007). The accumulation of H_2O_2 was not consistently linked to the stress tolerance induced by SA. Mora-Herrera *et al.* (2005) investigated the sensitivities of two potato cultivars to freezing temperatures and showed that SA may promote freezing tolerance in both cultivars, but only in the less tolerant genotype was it associated by H_2O_2 accumulation. NO, as a free radical, may promote chain reactions triggered by other free radicals, and it can be protective or harmful depending on the concentrations of the reaction product (Lipton *et al.*, 1993). As a result, the cell's fate (adaptation to stress or apoptosis) is determined by the essential balance between the generation of ROS and NO. In the current study, it was found that young stevia leaves accumulated more H_2O_2, which is thought to provide functional cross-tolerance to drought stress after being exposed to SA, SNP, and SA+SPD.

The superoxide dismutase (SOD: 1.15.1.1) activity serves as the first line of defense system, while catalase (CAT: EC 1.11.1.6) is the primary enzyme involved in neutralizing H_2O_2. Compared to non-stressed plants (37.6 U mg-1 protein), the drought stress caused an increase in CAT activity (123.0 U mg^{-1} protein). However, the application of SPD (170.3 U mg^{-1} protein), SA+SNP (150.3 U mg^{-1} protein), and SPD+SNP (166.4 U mg^{-1} protein) to stressed plants resulted in a further increase in CAT activity compared to drought-stressed plants (Figure 4). This increase in CAT activity can help to reduce the high peroxide concentration that occurs under drought stress. The application of SA to plants significantly reduced CAT activity and increased hydrogen peroxide accumulation when compared to DS plants. Similar to our findings, it was found that SA treatment decreased CAT activity and increased hydrogen peroxide accumulation in soybean genotypes under water deficit conditions (Razmi *et al.*, 2017). Safari *et al.* (2022) reported that exogenous SA inhibited CAT enzyme activity in *Impatiens walleriana* under drought stress; similar findings were reported by Antonić *et al.* (2016). These results suggested that the mechanism of action of SA involves binding CAT and inhibiting its activity by increasing the level of H_2O_2.

When compared with non-stressed plants (0.025 U mg^{-1} protein), DS caused an increase in SOD activity (0.045 U mg^{-1} protein). The SOD activity of the stressed plants was increased by the application of SPD (from 0.045 to 0.072 U mg^{-1} protein), SA+SNP (from 0.045 to 0.065 U mg^{-1} protein), and SPD+SNP (from 0.045 to 0.063 U mg^{-1} protein). When compared to DS plants, SNP application to plants significantly reduced CAT and SOD activity. SOD activity was found to be lower in corn (Yildiztugay *et al.*, 2014) and safflower (Chavoushi *et al.*, 2019) after SNP treatment under drought stress, which is similar to our findings. They reported that superoxide anion and NO reacted directly, which decreased superoxide radicals and SOD activity. The SNP can prevent lipid peroxidation by inhibiting membrane peroxidation enzymes and scavenging peroxyl radicals, thus playing a protective role.

The MDA levels of the stevia plants increased significantly (from 4.73 to 14.67 mol g^{-1} FW) as a result of the drought stress, as seen in Figure 4.

When elicitors were added to drought-stressed plants, the increase in MDA content was partially alleviated. In comparison to non-stressed plants, drought-stressed plants increased CAT and SOD activities to regulate the excessive peroxide concentrations. The antioxidant defense system in SPD- ,SA+SNP-, and SPD+SNP-treated plants was more active than that of other treatments. These enhanced antioxidant activities due to SPD, SA+SNP, and SPD+SNP treatments resulted in lower MDA contents and H_2O_2 generation. The application of stand-alone or combinations of SA, SNP, and SPD to plants against drought stress led to a modest drop in MDA concentration, which helped offset the damage.

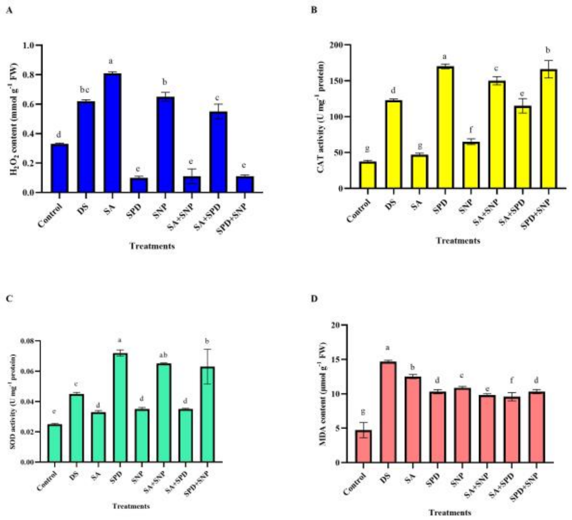

Figure 4. Effects of stress alleviators on A: H_2O_2 content, B: CAT activity, C: SOD activity, D: MDA content under drought stress. All mean values which share the same letter are not significantly different; otherwise, they differ significantly at *P*≤0.05. Values expressed as mean ± SD of three replicates.

Conclusion

Our aim was to investigate the changes in SG levels in leaves during pre-adaptation and subsequent drought stress, but we were also interested to know if changes in antioxidant enzymes, hydrogen peroxide, and MDA content could play a role in cell survival during drought stress with elicitor treatments that improved drought acclimation. Our findings demonstrated that elicitors (especially 0.1 mM SA) applied foliarly to stevia plants activated enzymatic defensive systems and stimulated the production of desired metabolites.

As a result, in abiotic stressed plants, the finer details of plant physiology, biochemistry, bioinformatics, and molecular biology techniques in conjunction with the effects of SA, SPD, and NO on plant immune-mediated defense networks can reveal new insights into how these molecules interact with other defense signaling pathways.

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