T.R.
BOLU ABANT İZZET BAYSAL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
Department of Biology

INTERACTIVE EFFECTS OF STRESS TOLERANCE-INDUCING COMPOUNDS ON PHYSIO-BIOCHEMICAL ACTIVITIES AND ANTIOXIDANT SYSTEMS OF *STEVIA REBAUDIANA* UNDER SALT STRESS

MASTER OF SCIENCE

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ACADEMIC SUPERVISOR
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BOLU, JANUARY- 2023

DEDICATION

I dedicate this work to whom my heartfelt thanks: to my father, mother, brothers, sister, my wife and my supervisor for all the support and encouragement they continually offered along the period of my post-graduation.

APPROVAL OF THE THESIS

Interactive effects of stress tolerance-inducing compounds on physio-biochemical activities and antioxidant systems of *Stevia rebaudiana* under salt stress submitted by **Arkan Alsafawi** and defended before the Examining Committe Members listed below in partial fulfillment of the requirements for the degree **Master of Science** in **Department of Biology, Institute of Graduate Studies of Bolu Abant Izzet Baysal University in 5.01.2023** by

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ABSTRACT

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Stevia rebaudiana is a plant used as a sweetener or in medical applications. This study aimed to investigate the effects of salicylic acid (SA), spermidine (SPD), and methyl jasmonate (MeJA) in specific concentrations with or without salt stress on the plant content. The result shows that the maximum fresh and dry weight was obtained when SA was combined with SPD under salt stress. At the same time, the effect of salt stress alone shows an increase in FRW. The effect of SA alone produced a higher root dry weight of *Stevia rebaudiana*. Also, we concluded that SPD with salt stress showed a better leave number, followed by SA with salt stress. We concluded that the SPD plus salt stress showed better shoot length and leaf number, followed by the SA plus salt stress. It was also concluded that CAT activity is noticeably stimulated in plants under salt stress by Spd. According to the results, the SOD antioxidant enzyme activity increased when SA was combined with salt stress, followed by MeJA under salt stress. The results showed that salicylic acid application significantly influenced and enhanced total phenolic content. Spd increased chlorophyll a content, whereas MeJA increased chlorophyll b content of plants under salt stress. While combined application of MeJA plus Spd to plants under salt stress show increasing stevioside content, application of SA increased the amount of rebaudiana A.

KEYWORDS: *Stevia rebaudiana* (Bertoni) HPLC, Salicylic acid, Spermidine, Methyl jasmonate, Superoxide dismutase, Catalase

ÖZET

BİTKİLERE STRES TOLERANSI İNDUKLEYEN BİLEŞİKLERİN, TUZ STRESİ ALTINDAKİ *STEVİA REBAUDİANA*'NIN FİZYO-BİYOKİMYASAL AKTİVİTELERİ VE ANTİOKSİDAN SİSTEMLERİ ÜZERİNE ETKİLERİ
YÜKSEK LİSANS TEZİ
ARKAN ALSAFAWİ
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ
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Stevia rebaudiana (Bertoni) tatlandırıcı olarak veya tıbbi uygulamalarda kullanılan bir bitkidir. Bu çalışma, belirli konsantrasyonlardaki salisilik asit (SA), spermidin (SPD) ve metil jasmonatın (MeJA) tuz stresi altında bitki içeriği üzerindeki etkilerini araştırmayı amaçlamıştır. Sonuç, maksimum taze ve kuru yaprak ağırlığının, tuz stresi altında SA'nın SPD kombinasyonundan elde edildiğini göstermektedir. Aynı zamanda, tuz stresinin etkisi tek başına taze kök ağırlığında bir artış göstermektedir. Tek başına SA uygulaması, *Stevia rebaudiana*'nın kök kuru ağırlığında artışa neden olmuştur. Ayrıca tuz stresi altında, SPD ve SA uygulamasının yaprak sayısında artışa neden olduğu belirlenmiştir. Tuz stresine maruz bırakılan bitkilerin, SPD ve SA uygulamaları ile daha fazla sürgün uzunluğuna ve yaprak sayısına sahip olduğu belirlenmiştir. Ayrıca, tuz stresi altındaki bitkilerde CAT aktivitesinin, SPD ile belirgin şekilde uyarıldığı sonucuna varılmıştır. Sonuçlara göre, süperoksit dismutaz (SOD) antioksidant enzim aktivitesinin, tuz stresi altında SA ve MeJA uygulaması ile arttığı belirlenmiştir. Sonuçlar, SA uygulamasının, toplam fenolik içeriğini önemli ölçüde etkilediğini ve arttırdığını göstermiştir. Tuz stresindeki bitkilerde SPD, klorofil (a) içeriğini; MeJA uygulaması ise klorofil (b)'yi arttırmıştır. Tuz stresi altındaki bitkilerde, MeJA ve SPD'nin birlikte uygulanması stevioside içeriğini arttırırken, SA uygulaması rebaudiana A miktarını arttırmıştır.

ANAHTAR KELİMELER: *Stevia rebaudiana* (Bertoni), HPLC, Salisilik asit Spermidin, Methyl jasmonate, Superoksit dismutaz, Katalaz

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LIST OF ABBREVIATIONS AND SYMBOLS

µM : Micro Mole

2,4-D : 2,4 - Dichloro Phenoxy Acetate Acid

A : Absorbance

ALG : Aminolevulinic Acid

Chl a : Chlorophyll-a

Chl b : Chlorophyll-b

CAT : Catalase

CH3 : Cycloheximide

DW : Dry Weight

FW : Fresh Weight

HPLC : High Performance Liquid Chromatography

MeJA : Methyl Jasmonate

mM : Milli Mole

NAA : Naphthalene Acetic Acid

PEC : Phosphoethanolamine

PPO : Poly Phenyl Oxidase

Px : Peroxidase

Reb A : Rebaudioside A

S : Salt stress (NaCl)

SA : Salicylic Acid

SGs : Steviol Glycosides

SOD : Superoxide Dismutase

SPD : Spermidine

ST : Stevioside

SUA : Sulforaphane

YE : Yeasentarine

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1. INTRODUCTION

1.1 *Stevia rebaudiana* (Bertoni)

The *Stevia rebaudiana* (Bertoni) plant is a dwarf perennial It has sessile leaves that are organized in pairs and face in opposite directions, and these leaves are opposite. It contains the most significant number of sweetening compounds compared to the other varieties. Growing *stevia* spp. is as easy as growing any other vegetable since it prefers a semi-humid, subtropical environment. A well-drained red or sandy loam soil with a pH between 6.5 and 7.5 is ideal. This plant should not be grown in salty soils (1).

Stevia spp. has become a more popular crop in recent years, and it is now being cultivated in several parts of the Indian states of Rajasthan, Maharashtra, Kerala, and Orissa. Farmers in India are growing Stevia spp. in massive quantities to meet the demand for natural sweeteners. The natural sweeteners found in *Stevia* spp. are called diterpene glycosides. There is 9.1% stevioside, 0.6% rebaudioside C 0.3% dulcoside, and 3.8% rebaudioside A in leaves harvested from wild *Stevia* spp. (2).

The Asteraceae family includes the woody, 80-centimeter-tall shrub known as *Stevia* spp.. and, perhaps as many as 300 unique species of *Stevia* spp. exist Soejarto et al. (1982). Its native range includes both the southern United States and the Brazilian highlands. *S. rebaudiana* (Bertoni), of the several Stevia spp., has the greatest concentration of potentially sweetening chemicals (3).

Stevia rebaudiana (Bertoni) is an herbaceous perennial plant belonging to the Asteraceae family. Though it is now grown in many countries, the plant is native to Paraguay and northern Brazil. Stevia spp. leaves contain a compound called steviol glycosides (SGs) that is 300 times sweeter than sugar. Rebaudioside A and stevioside (ST) are the two most prominent steviol glycozides in *S. rebaudiana* (Bertoni) (4).

Uniform fields of *S. rebaudiana* elite plants can only be produced by cutting propagation. Nevertheless, vegetative growth of elite genotypes is time-consuming, expensive, and fraught with virus spread. A low multiplication coefficient and a limited number of original mother plants may help reduce this risk (5).

1.1.1 Chemical Composition of examined *Stevia* spp.

Currently, scientists do not have access to information on the whole chemical make-up of the *Stevia* spp. Within the last decade, the chemical content of several *Stevia* spp. has been investigated. The valuable portion of this plant is the leaves. Only 18 of the 110 species examined exhibited detectable levels of sweetness. [stevioside, rebaudiosides A-E, dulcoside A, and steviolbioside; compounds respectively], generate sweet taste. Steviol, a component of the diterpene family, is the primary building block of these glycosides. The leaves of *Stevia rebaudiana* (Bertoni), contain eight known entkaurene glycosides, the most abundant of which is stevioside (6).

Campesterol, b-sitosterol, and stigmasterol, are also present in *S. rebaudiana* (Bertoni). Steviol, a byproduct of enzymatic hydroxylation inside the plant, is present in the same species. *Stevia* contains various compounds, some of which have been discovered to be bitter. Flavonoids from *S. rebaudiana, S. nepetifolia, S. microchaeta, S. monardifolia, S. origanoides*, and *S. procumbens* (aerial parts), and sesquiterpene lactones from *S. procumbens* and *S. origanoides* (leaves) are all present in *Stevia* spp. (7).

1.1.2 Proximate composition of *Stevia*

The stevioside, a glycoside with a glucosyl and sophrossyl residue connected to the aglycon steviol. Steviol possesses a cyclopentanoperhydrophenanthrene structure. Steviol has β-glucosyl and β-sophorosyl groups attached to carbons 4 and 13, respectively. Stevioside's sophorosyl residue is swapped out for a glucosyl-(1- 3)-sophorosyl one to produce rebaudioside A. *Stevia* spp. -derived sugar alcohols all have a similar structural feature: a steviol aglycon linked at C4 and C13 to trisaccharides containing glucose and/or rhamnose residues. Stevioside is a natural sugar substitute made from *Stevia* plants (8).

More than a hundred different compounds have been defined from *S. rebaudiana* (Bertoni); the most well-known are the natural products steviol and its glycosides steviolbioside, stevioside, dulcoside A, rebaudioside A-F, dihydroisosteviol, and rubusoside. conducted an analyzed of *S. rebaudiana* (Bertoni) leaves on a dry weight basis and determined that they had an energy value of 2.7 kcal g^{-1}. The aglycone steviol, is connected to the glycoside stevioside, which has a a sophorosyl and glucosyl residue. Eighty-five to ninety-five percent of Stevia

plant is water and contains protein, fiber, and amino acids, making it a nutrient-dense plant (9).

S. rebaudiana (Bertoni) contains eight diterpenenic glycosides (rebaudioside A, B, C, D, and E, dulcoside, steviobioside, and stevioside) volatile oils, tannins, triterpenes, labdane diterpenes, and stigmasterol. Stevioside and rebaudioside A are the two most prevalent components. In addition to being the least bitter of the glycosides, rebaudioside A is the sweetest. Glycosides B, C, and G are less sweet than Stevioside, but glycoside E is as sweet as stevioside (10).

Pederson et al. (2020) state that stevioside is a white crystalline powder made from Stevia leaves. Those with more scientific curiosity may find its atomic number and percentage components stated to be that "it is non-fermentable, heat-stable to 198 °C, has zero calories, is 200 to 300 times sweeter than sucrose, is suitable for teeth, and has anti-plaque and anti-cariogenic properties." They tabulated their report on the approximate content of *S. rebaudiana* Bertoni (Table 1.1.) (11).

Table 1.1. *S. rebaudiana* (Bertoni)'s approximate chemical component

Sample Number	Constituent	Value (%)
1	Aluminum	0.0072
2	Manganese	0.0147
3	Ash	6.3000
4	Phosphorus	0.3180
5	b-Carotene	0.0075
6	Potassium	1.7800
7	Calcium	0.5440
8	Protein	11.200
9	Chromium	0.0039
10	Selenium	0.0025
11	Cobalt	0.0025
12	Silicon	0.0132
13	Fat	1.9000
14	Sodium	0.0892
15	Fibre	15.200
16	Tin	0.0015
17	Iron	0.0039
18	Vitamin	0.0110
19	Magnesium	0.3490
20	Water	82.300

1.2 Effect of salt stress (NaCl) on the plant growth

Salt stress may stifle growth in the root, leaf and stem tissues, including cell division and growth. The impact of salt stress on Stevia spp. callus culture was investigated by Gupta et al. 2014. After two weeks of growth, yellowish compact calli were recovered from in vitro-grown *Stevia* spp. leaves. They were subcultured on MS media supplemented with 2.0 mg^{-1} naphthaleneacetic acid (NAA) and four different concentrations of NaCl (0.20, 0.15, 0.10, and 0.05 %). Compared to the control, callus biomass cultivated under salt stress exhibited slower development and browning of the media (12).

Cantabella et al. (2017) investigated salt stress and discovered that it has no major impact on plant development in *Stevia rebaudiana* (Bertoni), which stores sodium (Na^+) in their roots, thereby preventing excessive Na^+ buildup in their leaves. In addition, salt stress enhanced the proline, calcium (Ca^{2+}), potassium (K^+), and chloride (Cl^-) ion concentrations in leaves, which may aid in osmotic correction. Long-term salt stress did not alter the Chl concentrations in *Stevia* leaves, indicating the presence of a mechanism to safeguard the photosynthetic process. Chl b concentration increased significantly in the oldest plants examined. In addition, it was found that NaCl caused the buildup of reactive oxygen species (ROS) in *Stevia* spp. leaves, with this accumulation being more pronounced in the presence of 5 g/L NaCl, the maximum dosage employed in the research. Despite this, *Stevia* spp. plants can produce (16 days) or sustain (25 days) antioxidant enzymes to combat the oxidative stress caused by NaCl. Low salt stress did not impact rebaudioside A and steviolbioside concentrations (13).

1.3 Steviol Glycoside (SGs)

Steviol glycosides (SGs), which are generated from *S. rebaudiana* (Bertoni), are used as a sugar substitute since they have no calories. As the need for SGs in the marketplace grows, massive *S. rebaudiana* (Bertoni) cultivation facilities will have to be built. *S. rebaudiana* (Bertoni) can be grown from seeds. Since this species is allogamous, however, the phenotypes of the plants grown from its seeds may vary. Limiting reproduction via seed and self-incompatibility causes a large proportion of sterile seeds and a poor germination rate (14).

The plant (2n = 22), a medicinal plant endemic to Paraguay, contains the active compound steviol glycosides (SGs) mostly in its leaves (15). *Rubus sauvissimus, Stevia phlebophylla*, and *Stevia rebaudiana* are the most common

sources of steviol glycosides (sweet Chinese tea). As the name implies, they are sugar substitutes with a reduced calorie count. *Stevia rebaudiana* (Bertoni)*'s* secondary metabolites, including its steviol glycosides, alkaloids, phenols, and flavonoids, qualify it for medicinal use and as a nutritional supplement (16). Figure (1.1) shows the chemical structure of steviol glycoside, stevioside and rebaudioside A.

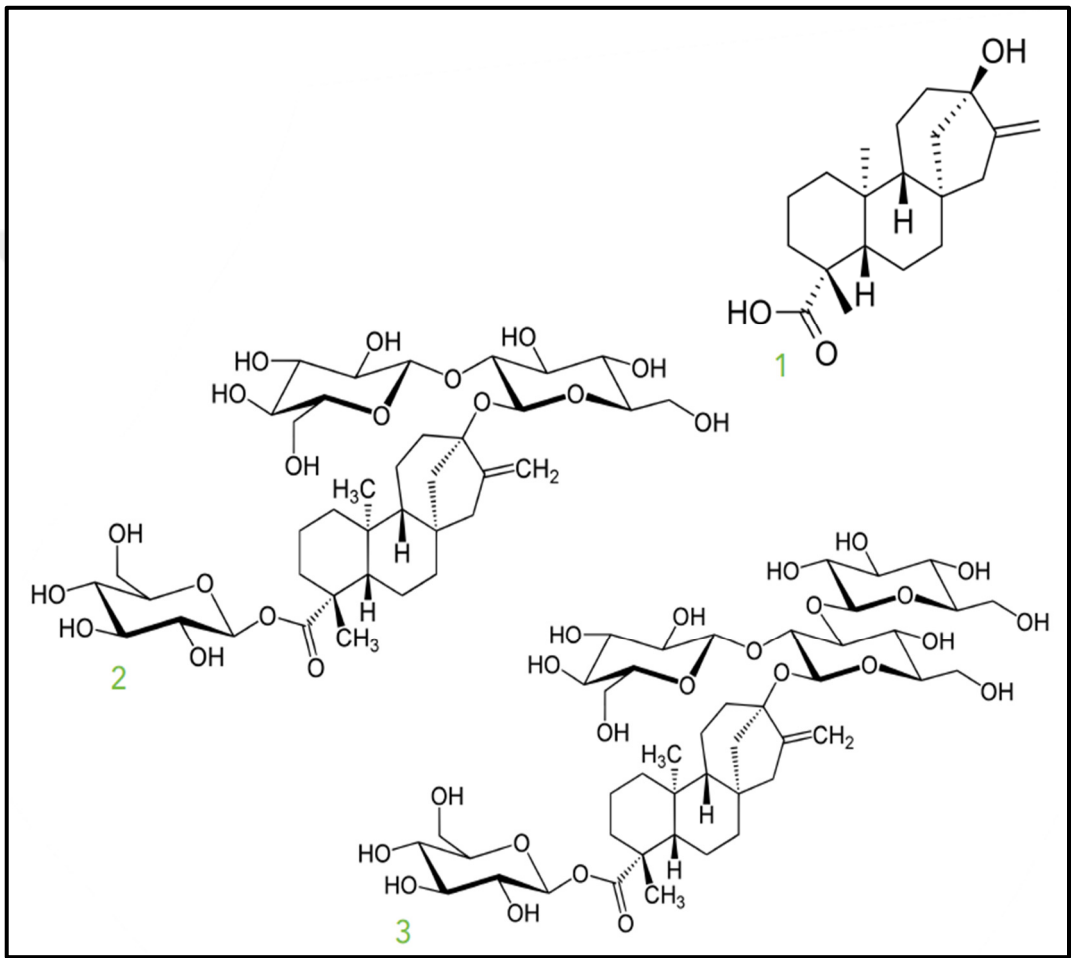


Figure 1.1. (1) Structure of steviol glycoside, (2) stevioside, and (3) rebaudioside A.

1.4 Elicitors

In plant biology, elicitors are chemicals often connected with pests, diseases, or synergistic organisms. Elicitors may bind to specific membrane-bound receptor proteins in plant cells. These receptors can recognize elicitor chemical patterns and initiate intracellular defense signaling via the octadecanoid pathway. This reaction increases the production of metabolites that minimize damage and promote resilience to pests, diseases, and environmental stress. This immunological

response is known as pattern-triggered immunity (PTI). Necrotrophic microorganisms are susceptible to PTI (17).

Elicitors are biochemicals that cause a specific living being to undergo physiological changes (18). When exposed to these chemicals, plants grown in tissue culture produce more secondary metabolites in less time. Moreover, multiple reports have shown that SGs may be made from *S. rebaudiana* (Bertoni) cells cultured *in vitro*. Elicitors may play a significant role in improving shoot multiplication, quality, and *in vitro* SG production. Several elicitors, such as phosphoethanolamine (PEC), aminolevulinic acid (ALG), cycloheximide (CH3), sulforaphane (SUA), and yeasentarine (YE), were shown to be effective in the *in vitro* cultivation of *S. rebaudiana* (Bertoni) by Bayraktar et al. (2016). For woody plants, the best results were obtained when the growth medium was supplemented with 0.5 g/L 100 mM cycloheximide (CH), casein hydrolysate (CHY), and 1.0 g/L yeasentarine (YE). This combination resulted in the maximum values for a shoot, node and leaf number per explant and shoot length. Maximum biomass accumulation was found at a concentration of 0.5 g/L aminolevulinic acids (ALG), (19).

The elicitors are classified into two groups, biotic and abiotic elicitors (20):

- Lipopolysaccharides
- Polysaccharides: Microbial glucans and chitosan and chitin and pectin in cell walls. Yeast extract, guar gum, Arabic gum, and alginate.
- Oligosaccharides: the mannuronate, mannan, guluronate, and galacturonides.
- Cellulase, pectolyase, fish protein, lactoferrin, pectinase, cryptogein, oligandrin, pectinase, hydrolysates, and glycoproteins.
- Coronatine, microbial cell walls, fungal spores, and mycelia cell walls (pathogen toxins).
- Compounds like salicylic acid, methyl salicylate, jasmonic acid, methyl jasmonate, ethylene, cytokinin, and gibberellin
- Extracts of plants (ex: oregano, *Larrea tridentata*)
- UV-radiation, temperature shift, light and heavy metals (Copper (II) sulfate, Silver Thiosulfate, Silver nitrate, Cadmium chloride, Copper (II) chloride, vanadyl sulfate, Nickel(II) sulfate , selenium), osmotic stress

induced by PVP, cadmium chloride, potassium chloride, sodium chloride, sorbitol and mannitol

- Acetic acid
- Metal ions: Ag^{+2}, Ag^{+1}, Zn^{+2}, Pb^{+2}, Co^{+2}, Fe^{+2}, Al^{+3}, Mn^{+2}, Cu^{+2} , and Cd^{+2}
- Silicon
- Calcium prohexadione
- Ethanol and ethene
- Benzothiadazole
- Inorganic salt stress: copper sulfate ($CuSO_4$), mercury chloride ($HgCl_2$), vanadyl sulfate (VSO_4), and calcium chloride ($CaCl_2$)

1.4.1 Elicitor under stress

According to the principles of elasticity, stress or pressure is measured in terms of the force exerted per unit area. Depending on the source, plant stress can be described as a substance that blocks plant's metabolism and affects a plant growth (21).

Phytoalexins, proteins, and other substances involved in stress responses are produced, accumulated, and metabolized when the elicitor activates a membrane receptor, altering gene expression (23).

Additional signal transduction cascades and plant behavioral changes may be triggered by elicitors, short-term modulatory effects on membrane characteristics, or specific post-translational modifications of proteins. By applying environmental stimuli, it is possible to improve a crop's phytochemical composition (24).

The elicitors are detected by certain receptors in plant cells, and they initiate a series of adaptive reactions to these environmental signals defined as eustress (low stress), during which the cells are better able to withstand and acclimate to changing environments (21, 25). As illustrated in Figure 1.2., a response defined as low stress, during which the cells accumulate phytochemicals, makes the cells better able to withstand and acclimate to changing environments. Specific receptors in plant cells detect environmental stimuli under the tolerance limit, and these perceptions trigger adaptive reaction cascades. Instead, the system responds negatively or in distress when environmental stimuli, such as salinity, drought, or high irradiance, are

administered at a high intensity. In other words, the number of resources (energy and molecules) available will be limited, and the depletion of metabolic reserves prevents the production of new defensive chemicals.

As illustrated in Figure 1.2., eustress (or low stress) occurs in plants when their cells respond to environmental cues by accumulating antioxidants and other phytochemicals, making them more tolerant of and adaptable to their surroundings. If ecological stresses like high irradiance, drought, or salt stress are imposed strongly or for a lengthy time, the system may respond negatively, perhaps causing discomfort (high stress). There is a certain amount of energy and molecules available for investment. If they are used up, there won't be enough metabolic reserve to produce more protective compounds (20).

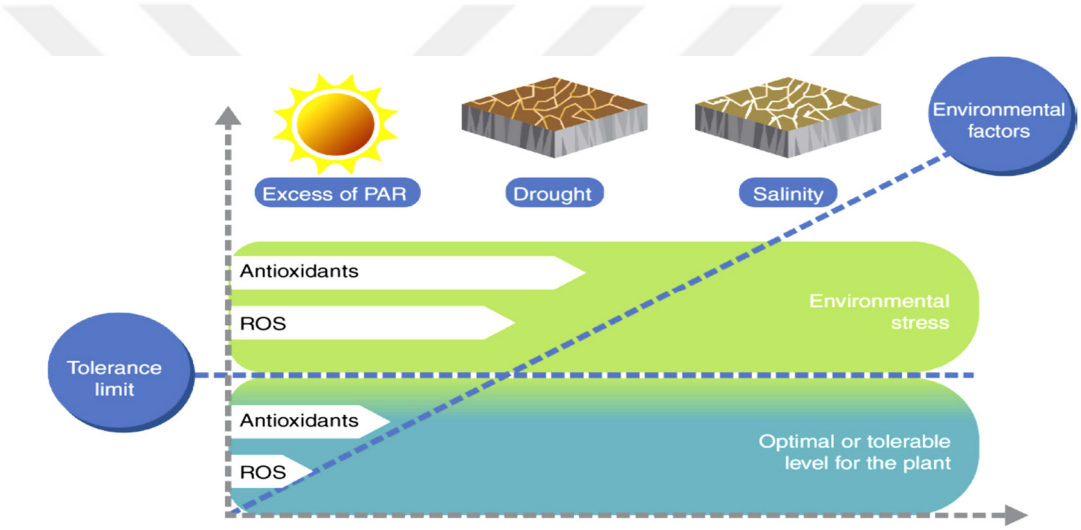


Figure 1.2. Increasing a phytochemical content by environmental stimuli (20)

As a means of coping with their surroundings, plants may produce and store a variety of phytochemicals that have nutraceutical value and boost the plant's resistance to stress. Salicylates, osmolytes, proline, phenolic compounds, glutathione, many volatile compounds, carbohydrates, proteins, and peptides, and several metabolites that increase activity of several defense molecules (26, 27). These chemicals are essential for people's health because they act as free radical scavengers against oxidative stress in all aerobic species (28).

In response to a stimulus from its environment, a cell undergoes the events shown in Figure (1.3). The left-hand alarm receptors pick up on environmental cues, which triggers a chain reaction that ultimately results in a transcriptome that can transduce, signal, and amplify the information via transcription factors and

secondary messengers that modify the expression of genes, allowing the cell to respond and adapt to its environment. Changes in the transcriptome lead to a new proteome that alters the metabolism during the acclimatization process, followed by changes in the metabolome and ionome. These phenotypic expressions are linked to cellular adaptations, environmental factors, and stress resistance. If no more changes occur in the environment, the system will return to its previous homeostatic condition and usually continue to develop and burn calories. The reaction is restarted if new changes occur in the environment, as shown by the arrow on the bottom left. Although the succeeding stages spanned distinct periods, there are significant overlaps between them, as represented by the left-hand vertical boxes (20).

Throughout the three phases of the adaptive stress response, there are continuous changes to the transcriptome, proteome, metabolome, and ionome (29). A few receptors on the surface of cells are capable of sensing environmental cues, but their actions are amplified by a network of second messengers and transcription factors. By regulating gene expression, which may include hundreds or even thousands of genes, these messengers and factors generate a transcriptome tailored to the current conditions. The mRNA encoded by the updated transcriptome can be utilized in the production of a wide variety of proteins.

This is made possible by various mechanisms, such as alternative RNA editing (which could increase the number of resulting proteins by 10 or more) and splicing. Other posttranscriptional changes, including phosphorylation, acetylation, methylation, ubiquitination, and myristoylation, are then used on the resultant proteins, many of which express themselves only under specified climatic circumstances. Considering this amplification, it is challenging to anticipate the nutraceutical value and physiological behavior using just genetic or transcriptome data. Several isolated proteins have been linked to improved stress tolerance (including chaperonins and other antioxidant proteins). The metabolome is the collection of low molecular weight molecules. However, many of them are components of metabolic pathways, and when these processes are manipulated or activated, a broad range of phytochemicals can be produced. Plants' nutraceutical qualities, stress responses, and physiological behaviors are all closely tied to the metabolome's complexity (Figure 1.3) (23).

Figure 1.4 depicts the schematic model that *Rhizoctonia solani* uses to explain the role it plays in the activation of soybean defensive systems. Pathogen elicitors set in motion a chain reaction of processes at the transcriptome, proteome, and metabolome levels, which leads to changes in energy metabolism and responses from the body's physiological systems. Membrane, Cell Wall, Golgi Apparatus, Mitochondria, Nucleus, Plasma Membrane, Ribosomes, ER, Transporters, Vacuole, and Vacuoles (30).

Groups of transcription factors, which regulate clusters of genes, have been shown to operate in tandem during metabolic stress reactions (the core metabolic response). The expression of a genus-specific set of phytochemical is driven by gene coexpression (31). The variations between crop species in terms of relative nutraceutical value and phytochemical composition have been explained by this research. There is also evidence to imply that the same plant species subjected to diverse environmental stimuli or differential intensities of the same ecological encouragement would have varying phytochemical compositions and, thus, distinct nutraceutical benefits (32).

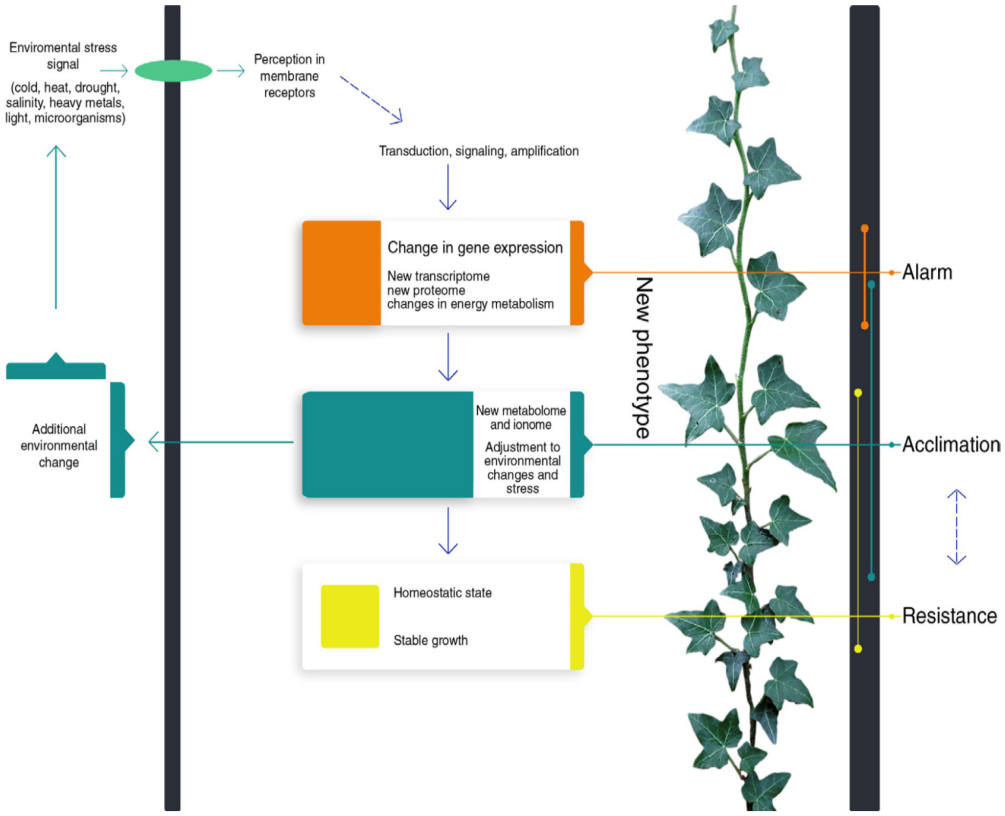


Figure 1.3. The Sequence of events occurring inside a cell in response to an external trigger (20).

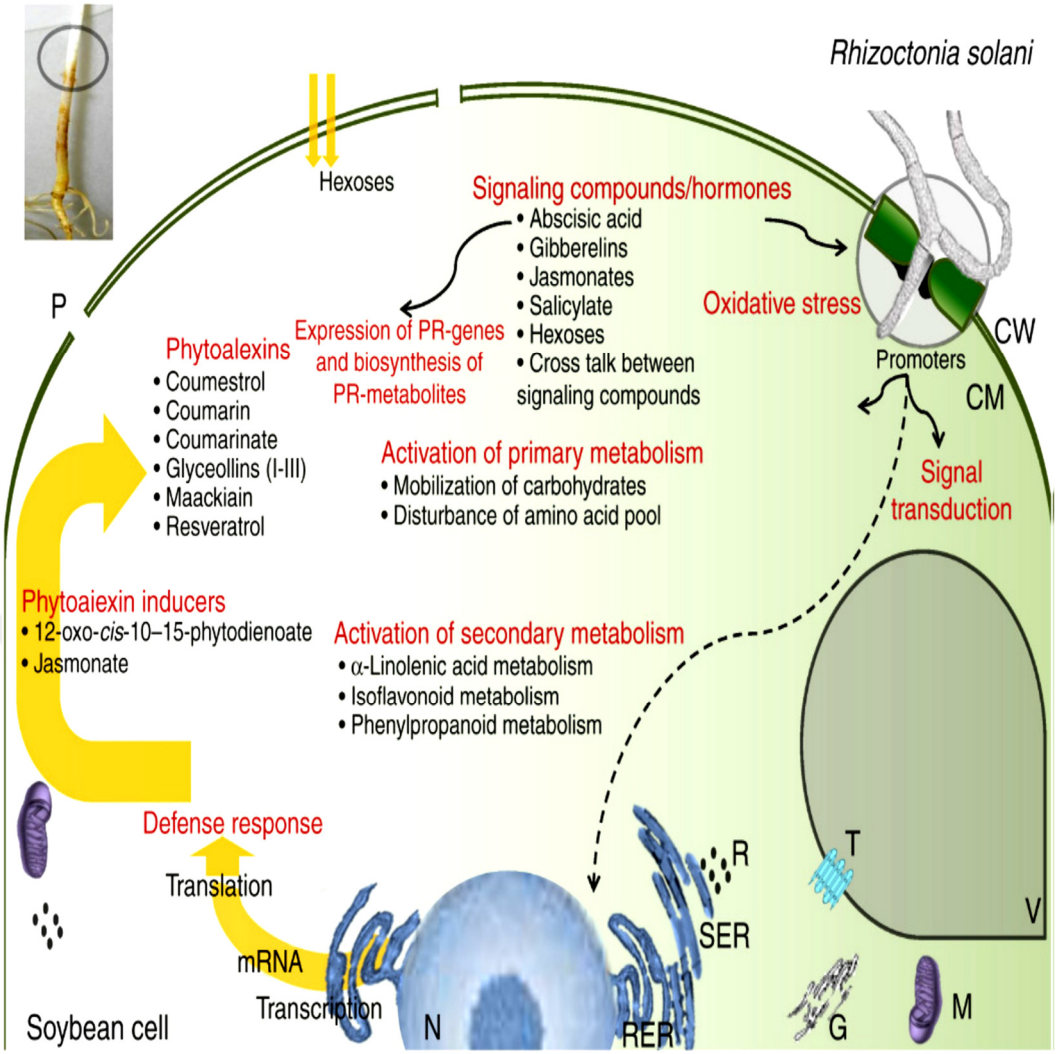


Figure 1.4. The role of *Rhizoctonia solani* in the activation of *Glycine max* Merril defense mechanisms (20).

1.4.2 Salicylic acid (SA)

Salicylic acid (SA) is found naturally in many plants (Figure 1.5). The free phenolic acid SA and its conjugate form are found in plants; the conjugate form may be produced by hydroxylation of the aromatic ring or glucosylation, and methylation. *Salix alba*, *S. daphnoides*, *S. fragilis,* and *S. purpurea* contain salicin (ß-glucoside salicylic alcohol), the most well-known natural SA derivative. In that plant, salicin concentrations are highest in the summer and lowest in the winter. The highest content of free SA was found in the branches of S. *plantifolia* and *S. purpurea* and the bark of *S. laponum* (33). From the Latin term for willow tree nowadays, SA gets its name (*Salix*) from German pharmacologist Johann Andreas Buchner who isolated 1828 a small amount of *salicin* (34). The chemical structure of *salicylic acid* is shown in Figure (1.5).

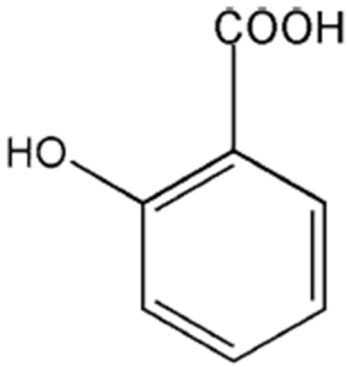


Figure 1.5. Structure of Salicylic acid (SA) (35)

Plants have developed a wide variety of remarkable chemical compounds to avoid being devoured. Among other phenolic compounds, this includes the photosynthetic machinery, flowering, membrane permeability, and enzyme activity. Here, we'll take a closer look at the physiological processes mediated by SA and try to tease out how the exogenous administration of SA influences growth and development (35).

1.4.3 Methyl jasmonate (MeJA)

MeJA is one of the most often used elicitors for increasing secondary metabolite synthesis in tissue culture research. The MeJA has been identified as a biomolecule with potential roles in signal transduction and regulating numerous responses associated with plant defense against a wide range of biotic stressors (16). The volatile organic compound known as jasmonate is used in plant defense, including seed germination, fruit ripening, flowering, root growth and senescence. The MeJA is produced when jasmonic acid is converted by the enzyme S-adenosyl-l-methionine: jasmonic acid carboxyl methyltransferase (36). Herbivory and wounding are two examples of biotic and abiotic stressors that cause plants to create methyl jasmonate which accumulates in the affected areas. The MeJA may trigger a defensive response in uninjured plants by signaling the original plant's defense systems or by spreading via direct touch or the air. The stomata or diffusion via the cytoplasm of the leaf cells allow the uninjured plants to take up the airborne it. The plants generate MeJA in response to herbivore attacks for self-defense and as a signal to nearby plants (37).

The MeJA may stimulate the production of a variety of phytoalexins (antimicrobial compounds), nicotine, and protease inhibitors in plants. The protease

inhibitors disrupt the insect's digestion to prevent it from returning to the plant for another meal. The MeJA has triggered the formation of traumatized resin ducts in *Picea abies*. As a vaccination, this may be utilized as protection against several insect pests (38). Even though the MeJA and SA have been proven to have a role in enhancing secondary metabolites in tissue cultures of different organisms (17). Their behavior is species-specific (39).

1.4.4 Spermidine (SPD)

A polyamine is an organic molecule with several amino groups. Some alkyl polyamines are manufactured, while others are found in nature. Alkylpolyamines are colorless, water-soluble, and hygroscopic. Most aromatic polyamines are crystalline at room temperature (40). They occur around neutral pH as ammonium derivatives. SPD ($C_7H_{19}N_3$) which found in living tissues and ribosomes, has several metabolic activities inside organisms. It was first extracted from sperm (41).

It is a polyamine that is aliphatic. Putrescine is converted to spermidine-by-spermidine synthase (SPDS). It's needed to make spermine and thermospermine, a structural isomer of spermine. Different biological processes (such as Ca^{2+}, Na^{+}, and K^{+} -ATPases) are coordinated by it. It manages intracellular pH and volume and preserves membrane potential. The SPD controls biological processes, including Ca^{2+} influx via the glutamatergic N-methyl-d-aspartate receptor, which has been connected to nitric oxide synthase, cGMP/PKG pathway activation and a decrease in Na^{+} and K^{+}-ATPase activity in cerebral cortex synaptosomes (42).

The SPD promotes animal lifespan via various processes that are only starting to be understood. At the molecular level, autophagy is the primary mechanism, although there is evidence for additional pathways, including regulation of inflammatory responses, lipid metabolism, cell cycle progression and apoptosis (43). It helps with RNA transcription in the lab and blocks nitric oxide synthase, both known to regulate plant growth (NOS). Additionally, the SPD serves as a precursor for the spermine and thermospermine, which support plants' resistance to salt stress and dryness. (44).

1.5 Use of Stevia plant sweeteners

1.5.1 Renal and blood pressure effects

The effects of stevioside on renal function and blood pressure control have been the subject of conflicting study findings. It enhances the fractional excretion of Na+ and K+ while decreasing renal vascular resistance and causing diuresis.

Because of this, we may conclude that stevioside vasodilates both afferent and efferent arterioles but has no impact on the glomerular filtration rate (45).

1.5.2 Medicinal potential

Stevia spp. have the potential to be used in a variety of medical contexts, including as an glucagonostic, insulinotropic, anti-inflammatory, immunostimulatory, anti-viral, chemo preventive, antihyperglycemic, antioxidant, hypotensive, anti-cancer, and anti-microbial agent, not to mention a digestive tonic and in the treatment of infectious diseases and skin and dental problems (8).

1.5.3 Antimicrobial activity

Research shows that *Stevia* may effectively combat the germs that cause tooth decay and gum disease, making it a great complement to dental care products like toothpaste and mouthwash. According to the studies, growing *Streptococcus* mutans on stevioside medium results in slower growth and less acid secretion than growing it on sucrose, glucose, or fructose media (46).

1.5.4 Anti-carcinogenic agent

Inhibitory effects on tumor formation have been seen in the presence of polyphenolic components in *Stevia* spp. leaf extracts. By inhibiting Epstein-Barr virus early antigen induction and decreasing tumor production in a two-stage mouse skin carcinogenesis model following exposure to isosteviol, steviol, stevioside, leaf aglycones, 12-O-tetradecanoylphorbol-13-acetate, 7,12-dimethylbenz[a] anthracene and other metabolites are known to inhibit tumor formation (47).

1.6 Literature review

According to Sharma et al. (2006), between 80 and 85 percent of the fresh *Stevia* spp. leaves are composed of water. Glycosides that were used extensively include stevioside, steviol, as well as rebaudiosides A and B. *Stevia* spp. contains a wide variety of essential nutrients, including vitamin C, chromium, thiamine, potassium, iron, zinc, cobalt, magnesium, tin, phosphorus, riboflavin, β-carotene, and many more. *Stevia* spp. also contain a wide variety of additional chemicals, such as caryophyllene, avicularin, austroinulin, cynaroside, chlorophyll, gibberellin, b-sitosterol, compesterol, caffeic acid, chorogenic acid, foeniculin, centaureidin, daucosterol, formic acid, dulcosides A and B, apigenin, and di-terpene glycosides, (9).

In a 2010 study by Korsangruang et al. (57), the buildup of isoflavonoids in cell suspension cultures of *Pueraria candollei* was hypothesized to be increased by

biotic and abiotic elicitors. Similarly, Portu et al. (2016) evaluated the usage of YE, CH, and MeJA as three elicitors for enhancing the phenolic content of grapes and wine by foliar spray. MeJA and YE enhanced the phenolic content substantially. By activating the secondary biosynthetic pathway, elicitors may enhance the secondary metabolite production in plants. Multiple growth metrics are elicitor responsive. In *in vitro* studies using elicitors to improve reproduction have been conducted on a broad range of species (58).

In a 2022 by Al-Mushhin et al. (109), the piling up of flavonoids and phenols enhanced significantly due to applying of SPD. Salinity significantly increased Na, however K and Spd application significantly decreased Na while simultaneously increasing K content, which resulted in a decreased Na/K ratio. The results suggest that exposure to K and Spd upregulates antioxidant status, secondary metabolite production, osmolyte accumulation and ion homeostasis to protect photosynthesis and growth from oxidative damage induced by salinity. The study's findings, according to Mohamadian et al. (2018) (110), demonstrated that methyl jasmonate, salinity stress, and their interactions with salinity and methyl jasmonate had a crucial impact on the catalase enzyme activity, total chlorophyll, proline germination rate, and germination percentage. When applying 5 mM methyl jasmonate at a salinity level, salt stress rose and methyl jasmonate enhanced the CAT's activity. Salinity decreased the germination index while boosting the activity of the enzymes superoxide dismutase and peroxidase. To counteract the impacts of salt stress, methyl jasmonate seed priming enhanced seed germination through seed vigor index and germination rate.

Karlidag et al. (2009) (110), external apply of SA stimulate the root and shoot length under salt stress. In NaCl-stressed plants SA treatment raised the root and shoot dry weight.

2. AIM AND SCOPE OF THE STUDY

The current study aims to investigate the effects of exogenous applications of SA, SPD, and MeJA, either alone or in combination, on the reduction of salinity stress impacts on *Stevia rebaudiana* (Bertoni) plants. Additionally, to assess the impact of SA, SPD, and MeJA-controlled elicitation on plant performance, SGs Steviol glycoside concentration, and the possible contribution of elicitors to the development of stress tolerance in *Stevia rebaudiana* (Bertoni) under salt stress.

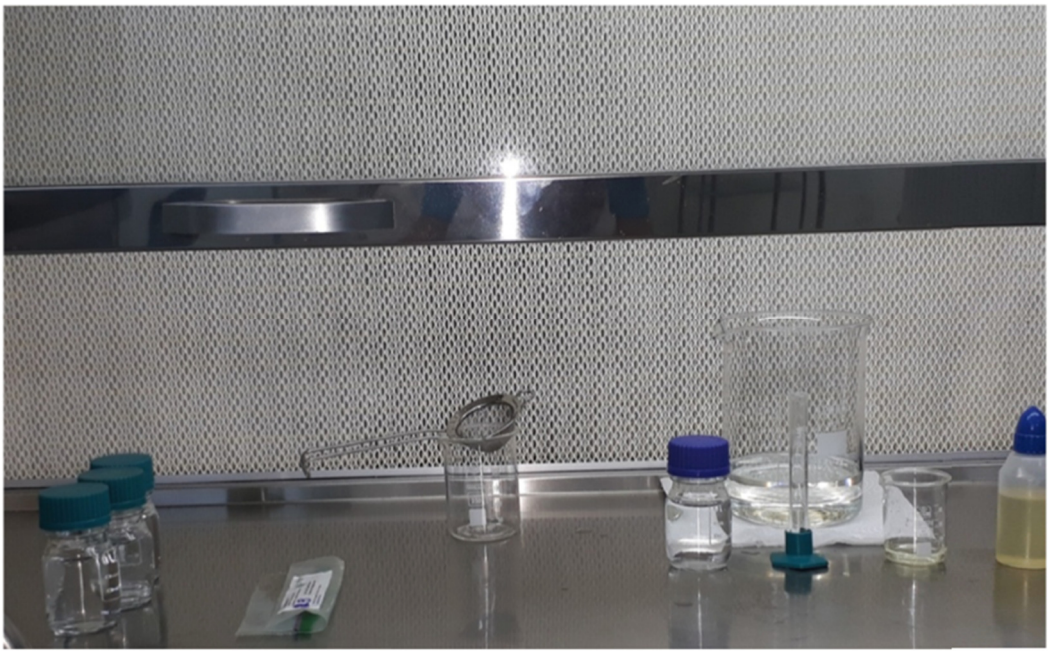
The scope of this study is:

- Effect of stand-alone or combined applications of SA, MeJA and SPD on growth and quality of *Stevia rebaudiana* (Bertoni) under salt stress
- Effect of SA, MeJA and SPD on *in vitro* growth and stress defense system in *Stevia rebaudiana* (Bertoni).
- Effect of stand-alone or combined applications of SA, MeJA and SPD on stevioside content of *Stevia rebaudiana* (Bertoni) under salt stress.

3. MATERIALS AND METHODS

3.1 Equipment sterilization

Glassware was soaked in chromic acid (5%) for a whole night before being cleaned in running water and then rinsed with dH_2O. After being washed and rinsed, the glassware was dried in a forced draft hot air oven at 121 °C teamperature and put away in dust-free cabinets. All tissue culture experiments in this investigation were performed under sterile conditions using clean, dry glassware. This method was developed to eliminate pathogens without impairing the plant's growth potential. Soil (in glass dishes) was sterilized by autoclaving at 121 °C for 1 h (Picture 3.2)



Picture 3.1. Equipment sterilization

Picture 3.2. Prepearing soil sterilization

3.2 Plant sample and culture condition

Stevia rebaudiana (Bertoni) plantlets were obtained from single-seed descendent seedlings. 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 pots containing a soil: vermiculite mixture (3:1) were categorized into ten groups: Group 1 (Control group), Group 2 (MeJ foliar spray), Group 3 (SPD foliar spray), Group 4 (SA foliar spray), Group 5 (Salt stress (SS) + MeJ foliar spray), Group 6 (SS + SPD), Group 7 (SS + SA), Group 8 (SS + SA + MeJ: Salt stress and the combined foliar spray of SA and MeJ), Group 9 (SS + SPD + SA: Salt stress and the combined foliar spray of SPD and SA), Group 10 (SS + MeJ + SPD: Salt stress and the combined foliar spray of SPD and MeJ), Group 11 (Salt stress: SS). All pots were watered every two days until the beginning of the salt stress. The stress relievers, MeJA (0.05 mM), SPD (0.75 mM), SA (1 mM), SA + MeJA (1 mM + 0.05 mM), SA + SPD (1 mM + 0.75 mM) and SPD + MeJA (0.75 mM + 0.05 mM) were applied three times as a foliar spray to each group's 23-day-old plant until all of the leaves were fully soaked on both sides. Before being stressed, the plants were given a 14-day pretreatment with stress relievers. The required concentrations of elicitor were prepared in sterile distilled water containing 1% Tween 20 (v/v) (Merck, Darmstadt, Germany). The literature study and early experimental efforts were used to identify the treatment concentrations of stress relievers (59).

The cultures were kept in a 23 ± 2°C air-conditioned culture environment with a 16:8 photoperiod:dark cycle supplied by cool white fluorescent lights.

The following steps were taken for plant preparations:

After receiving the *Stevia* plant samples, we planted them in pots, as shown in the picture (3.3). The plants received were already prepared for cultivation. Then, the samples were moved into a controlled climate room, as shown in picture (3.4).

Picture 3.3. Potting of the *Stevia* spp. Plants

Picture 3.4. All plants were placed in a controlled climate room

3.2.1 Stress conditions

To impose salt stress, *S. rebaudiana* (Bertoni) plants were irrigated with 50 ml of dH_2O, which contained 60 mM of NaCl for 10 days. The experimental solution was renewed every 2 days. An equal quantity of distilled water was given to the non-stressed groups. After 10 days of salt stress on the plants, samples from

each replication and treatment were taken. The harvested root and leaf tissues from stressed and unstressed plants were used to study growth parameters (shoot dry (SDW) and fresh weight (SFW), root dry (RDW) and fresh weight (RFW), shoot (SL) and root length (RL), and leaf number (LN)), physiological parameters, and changes in activities of antioxidants were recorded. In all experiments, leaves at the same location on many stems were excised from the top, the middle, and the base parts of the plant to avoid confounding variations in the results related to leaf age.

3.2.2 Preperation of SA, MeJA and SPD solutions

Ethanol (96%) was used to make solutions of the SA (Sigma-Aldrich), MeJA and SPD. PH levels of both solutions adjusted to 7. The necessary concentrations of both elicitors and the stock solutions of MeJA, SPD, and SA were autoclaved. The plants were treated with MeJA, SPD and SA and their combinations. In this experiment, we spray the Stevia plant with 0.05 mM, 0.75 mM, and 0.1 mA, respectively (Picture 3.5). The time of spraying was once on the first day, two times from 2 – 8 days, and we returned to 1 time per day from 8 – 14 days.

Picture 3.5. Preparing MeJA, SPD and SA solutions

3.2.3 Total phenolic content (mg/g)

After centrifuging the fresh *Stevia rebaudiana* (Bertoni) (each weighing 50 mg) in 80% acetone for 10 minutes at 10.000 x g, 20 µl supernatant was mixed with

2 ml of sterile water and 1 ml of Folin-Ciocalteu phenol reagent. After 5 min, we added 5 mL of a 20% Na_2CO_3 solution to a total volume of 10 mL of distilled water. A 750 nm absorbance reading was taken after the mixture was well stirred, and the concentration of phenolics was calculated. The phenolic content was reported in terms of gallic acid (GA) equivalents per gram of dry matter. Figure 3.1. shows the standard curve of gallic acid.

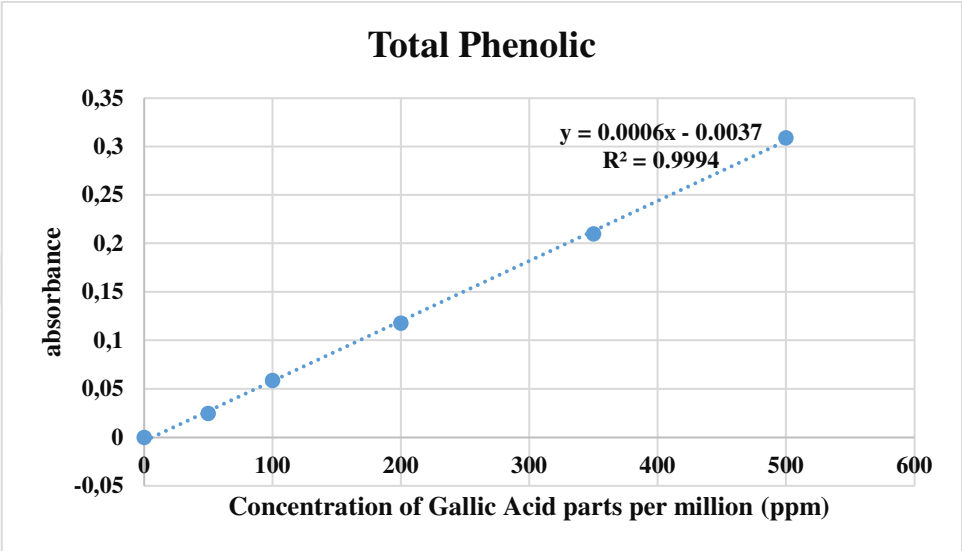


Figure 3.1. The standard curve of the gallic acid

3.2.5 Enzyme extraction method

We homogenized 0.1 g of plant material in 2 ml of ice-cold 50 mM K-phosphate buffer (PH= 7.0) containing 1 % (w/v) polyvinyl-pyrrolidone (PVP) and 2 mM Na-EDTA, then the homogenate was centrifuged at 12000 rpm min^{-1} and 40 °C for 10 minutes. The extracted tissue was either stored at -80 °C for future SOD and CAT studies or used right away.

3.2.6 Lowry Method

Using the Lowry technique (65), the amount of soluble protein in each sample was measured. 0.2 ml was extracted from each sample and placed in a test tube. The extract was supplemented with 2 ml of an alkaline copper sulfate reagent. After 10 minutes of room temperature incubation, the absorbance was measured by a spectrophotometer at 660 nm against a blank. The concentrations of bovine albumin (BSA) employed for the protein standard curve were 0, 50, 100, 200, 600, and 800 mg/l, respectively, as shown in Figure 3.2.

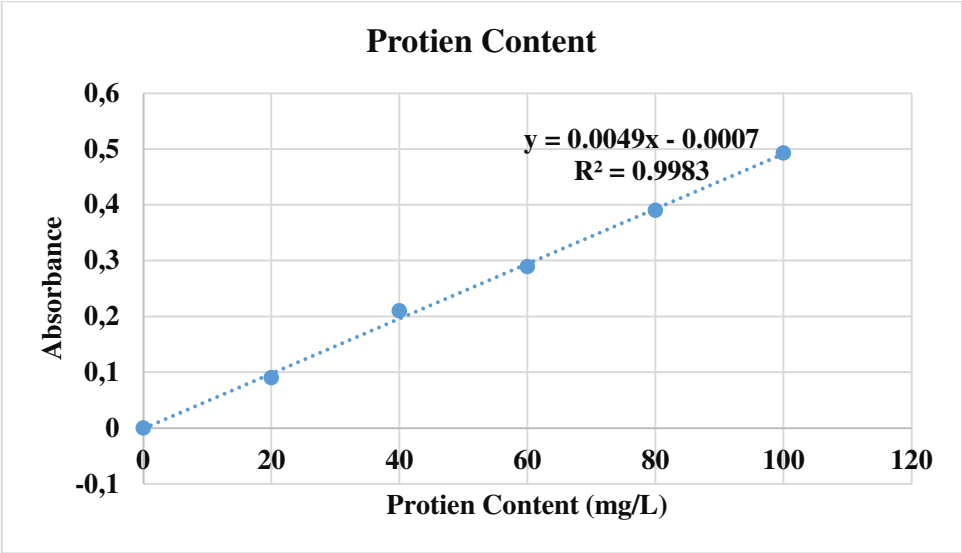


Figure 3.2. The standard curve of BSA

3.2.7 Determination of Catalase (CAT) activity

The catalase activity was measured using the Lartillot et al. technique (1988) (66). Following the breakdown of H_2O_2 in the CAT, the absorbance at 240 nm decreased. A 3 ml reaction mixture containing 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and 37 °C was used. Each tube was then filled with 20 µl of enzyme extract. The addition of enzyme extract started the reaction. Following consumption of H2O2 (extinction coefficient, 38.9 mM^{-1} cm^{-1}), at 240 nm, for 2 minutes, was used to measure the CAT activity. With the addition of 0.5 ml of 1 M HCI solution, the reaction was stopped (Ar). The absorbance of a solution containing 2.5 ml of the reaction mixture and 0.5 ml of 1 M HCI was measured spectrophotometrically to identify the initial absorbance of H_2O_2 (As). The absorbance of a solution containing 20 µl of enzyme extract, 2.5 ml of buffer, and 0.5 ml of 1 M HCI is used to get the protein absorbance value (At). Absorbance value change caused by enzymatic activity was calculated below:

$$A = (As + At) - Ar$$

Enzyme activity in terms of U ml/mg protein was calculated below:

$$\text{Activity} = (A \times V_t) / (\varepsilon \times t \times V_e) \times (1/ \textit{(mg protein)})$$

V_t = *The total volume of reaction (ml)*

V_e = *Volume of Enzyme extract (ml)*

ε = *Extinction coefficient of H202*

t = *Reaction time (min)*

3.2.8 Determination of SOD (superoxide dismutase) activity

The SOD activity was assessed using Sun et al. (2004) methodology (67). The SOD enzyme test reagent was mixed in a 200 ml baker and included the following substances: 40 ml of a 0.3 mmol/l xanthine solution, 20 ml of a 0.6 mmol/l EDTA solution, 20 ml of a 150 µmol/l nitroblue tetrazolium solution, 12 ml of a 400 mmol/l Na_2CO_3 solution, and 6 ml of BSA (1g/l). 0.05 ml enzyme extract was applied to each test tube containing 1.425 ml of the SOD assay reagent. After 20 min of incubation with 0.025 µl xanthine oxidase solution at 25 °C, the reaction was terminated by adding 0.05 ml of 0.8 mmol/l $CuCl_2$ solutions to each test tube. The formazan synthesis was evaluated by spectrophotometric analysis at 560 nm. To eliminated protein absorbance the tubes were read spectrophotometrically at 560 nm against dH_2O. The quantity of protein that inhibits the rate of NBT degradation by 50% is defined as one unit of the SOD. The percentage of inhibition was computed using the following formula:

Activity (U/ml) = (% Inhibition) / (50 x 0.1)

Specific Activity = (Activity (U/ml) / (mg/ml protein).

3.2.9 HPLC Analysis

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.

3.2.10 Determination of chlorophyll and total carotenoid content (mg/L)

The leaves were broken down into fine powder using a mortar and pestle. Then, we added 100 mg of fresh leaves material to 1 ml of methanol in a microcentrifuge tube and stirred it in the dark for 1 min. The tubes were centrifuged for 10 minutes at 4 °C and 12.000 rpm. The methanolic extracts were analyzed

through UV/VIS spectrophotometer at 470, 653 and 668 nm. The concentrations of chl a, chl b and carotenoids are calculated as follows (69).

Chlorophyll a (mg/L) = 15.65 Abs_{666} – 7.340 Abs_{653}

Chlorophyll b (mg/L) = 27.05 Abs_{653} – 11.21 Abs_{666}

Total carotenoids (mg/L) = 1000 Abs_{470} - 2.860 C_a - 129.2 C_b / 245

3.3 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.

4. RESULTS

4.1 Growth parameters

The LN, SL, RL, LFW, LTW, LDW, RFW, RDW and %RWC of *Stevia rebaudiana* Bertoni were all measured under different experimental conditions to see how salt stress (SS) and elicitors affects the development and growth of Stevia (Table 4.1).

SS decreased LN (from 33.20 to 23.00 cm), SL (from 11.24 to 8.56cm), LFW (from 1.27 to 1.14 cm), LTW (from 1.68 to 1.65 cm), LDW (from 0.17 to 0.15 cm) and % RWC (from 72.51 to 66.34 cm). Compared to the control, RFW and RDW were not significantly changed. The exogenous application of SPD under salt stress resulted in 15.26 cm longer SL and 51.80 LN. Significant effects of elicitation on LN, SL and % RWC were observed with SPD under *in vitro* salt conditions, compared to the control and SS groups. When compared to the SS and control group, significant effects of elicitation on SA + SPD under salt stress caused a significant increase in LFW, LDW and RFW. The exogenous application of SA significantly enhanced SL when compared to the control plants.

Table 4.1. Effects of stress alleviators on (LN), (SL), (RL), (LFW), (LTW), (LDW), (RFW), (RDW). All mean values which share the different letters are significantly different ($p < 0.05$). Values are presented as the means ± SD of three replicates

Treatment	LN	SL (cm)	LFW (g)	LTW(g)	LDW (g)	RFW (g)	RDW (g)	% RWC
Control	33.20^{bc}±12.51	11.24^{cde}±1.46	1.272^{bcde}±0.24	1.687^{bc}±0.30	0.170^{cd}±0.04	1.03^{ab}±0.41	0.23^{ab}±0.12	72.51^{bcd}
MeJA	31.40^{bd}±13.44	12.80^{abc}±3.27	0.704^{de} ±0.29	0.979^{cd}±0.35	0.090^{d}±0.03	0.57^{c}±0.15	0.09^{c}±0.03	67.64^{bcd}
SPD	34.00^{bc}±7.07	9.60^{de}±2.21	0.617^{e} ±0.20	0.915^{d}±0.26	0.081^{d}±0.02	1.05^{ab}±0.51	0.20^{ab}±0.10	63.28^{d}
SA	23.00^{c}±7.77	13.00^{abc}±0.79	0.698^{de} ±0.20	0.994^{cd}±0.25	0.112^{cd}±0.02	1.23^{a}±0.29	0.25^{a}±0.07	64.94^{cd}
MeJ+SS	31.71^{bd}±11.75	10.66^{cde}±2.16	1.325^{bcd}±0.61	1.658^{bc}±0.63	0.179^{db}±0.06	0.94^{abc}±0.10	0.16^{bc}±0.01	74.93^{bcd}
SA+SS	43.20^{ab}±14.97	14.80^{ab}±1.44	1.682^{abc}±0.69	1.873^{b}±0.48	0.219^{bc}±0.05	0.93^{abc}±0.31	0.17^{abc}±0.04	86.71^{ab}
SPD+SS	51.80^{a}±16.39	15.26^{a}±3.86	1.941^{ab}±0.53	2.004^{ab}±0.41	0.249^{b}±0.05	1.21^{ab}±0.20	0.20^{ab}±0.03	96.25^{a}
SA+MeJA+SS	35.20^{bc}±14.34	8.34^{e}±1.25	1.104^{cde}±0.78	1.395^{bcd}±0.95	0.155^{bcd}±0.09	0.80^{bc}±0.06	0.15^{bc}±0.02	68.53^{bcd}
SA+SPD+SS	38.20^{abc}±9.88	11.74^{bcd}±1.75	2.267^{a}±0.47	2.566^{a}±0.57	0.373^{a}±0.22	1.25^{a}±0.01	0.20^{ab}±0.02	87.39^{ab}
MeJA+SPD+SA	27.00^{bd}±7.58	8.96^{de}±2.18	1.509^{bc}±0.44	1.753^{b}±0.44	0.184^{bcd}±0.04	0.96^{abc}±0.14	0.17^{abc}±0.01	84.08^{abc}
SS	23.00^{c}±7.38	8.56^{de}±2.63	1.142^{cde}±0.20	1.652^{bc}±0.32	0.159^{bcd}±0.02	1.28^{ab}±0.49	0.20^{ab}±0.06	66.34^{cd}

4.2 Total protein content (mg/g)

The total protein content of *Stevia rebaudiana* (Bertoni) was all measured under different experimental conditions to analyze antioxidant enzymes CAT and SOD. The results of the total protein content are shown in Table (4.2).

Table 4.2. The total protein content (mg/g)

Treatment (µM)	Protein Content (mg/ml)
Control	14.52
MeJA	20.23
SPD	31.95
SA	17.68
MeJA + SS	13.36
SA + SS	11.83
SPD + SS	28.52
SA + MeJA +SS	21.66
SA + SPD + SS	14.14
MeJA + SPD + SS	22.75
SS	17.77

4.3 Superoxide dismutase (SOD) and catalase (CAT)enzyme activity (U/mg protein)

Table 4.3. presents the results of an investigation of the antioxidant enzymes, SOD and CAT in *Stevia rebaudiana* (Bertoni) treated with SPD, MeJA, SA, and their combinations under salt stress. SS increased the CAT activity from 21.15 to 26.82 U/mg protein in the control plants. The application of exogenous SA and MeJ to stressed plants increased CAT activity. When compared to SS plants, the application of MeJA, SA and SA + SPD to stressed plants dramatically reduced the CAT activity. The use of MeJA, SPD and SA on non-stressed plants raised the CAT activity from 21.15 to 37.28, 50.19, and 27.20 U/mg protein, respectively. The use of SPD, SA + MeJA, SA + SPD and MeJA + SPD on stressed plants significantly enhanced the CAT activity. When compared with the control groups, SS caused an increase in SOD activity. The SOD activity of the non-stressed plants was increased by the application of MeJA (from 0.015 to 0.048 U/mg protein), SA (from 0.015 to 0.031 U/mg protein), and SPD from (0.015 to 0.030 U/mg protein). The use of SA, MeJA, SA + MeJA, SA + SPD and MeJA + SPD on salt-stressed plants raised SOD activity significantly.

Table 4.3. The effect of elicitors on SOD and CAT activities

Treatments	SOD specific activity (U/mg protein)	CAT Activity (U/mg protein)
Control	0.015^{h}±0.0005	21.15^{i} ± 0.030
MeJA	0.048^{e}±0.0003	37.28^{d} ± 0.073
SPD	0.030^{g}±0.0001	50.19^{a} ± 0.116
SA	0.031^{g}±0.0026	27.20^{f} ± 0.111
MeJA + SS	0.088^{a}±0.0003	20.42^{j} ± 0.028
SA + SS	0.089^{a}±0.0021	19.74^{k} ± 0.043
SPD + SS	0.015^{h}±0.0007	46.39^{b} ± 0.060
SA + MeJA +SS	0.065^{b}±0.0004	33.87^{e} ± 0.078
SA + SPD + SS	0.053^{d}±0.0008	23.12^{h} ± 0.051
MeJA + SPD + SS	0.038^{f}±0.0001	39.22^{c} ± 0.529
SS	0.059^{c}±0.0018	26.82^{g} ± 0.064

4.4 Total phenolic content of the *Stevia rebaudiana* (Bertoni) (mg/g GA)

Table 4.4. presents the total phenolic content in *Stevia rebaudiana* (Bertoni) treated with SPD, MeJA, and SA and their combinations under salt stress. When compared with the control groups (24.78 mg/g GA), the phenolic content of the non-stressed plants was increased by the application of SA (126.28 mg/g GA) significantly. However, the phenolic content of the non-stressed plants was decreased significantly by the application of MeJA (2.67 mg/g GA), and SPD (6.01 mg/g GA). The use of SA, MeJA, and SPD on salt-stressed plants raised the phenolic content significantly while their combinations decreased the phenolic content.

Table 4.4. The effects of elicitors on the total phenolic (mg/g GA)

Treatment (µM)	Total phenolic (mg/g GA)
Control	24.78^{d} ± 0.17
MeJA	2.67^{j} ± 0.25
SPD	6.01^{i}± 0.25
SA	126.28^{a} ± 0.29
MeJA + SS	26.39^{b} ± 0.10
SA + SS	25.84^{c} ± 0.19
SPD + SS	22.89^{e} ± 0.25
SA + MeJA +SS	17.01^{f} ± 0.10
SA + SPD + SS	1.46^{k} ± 0.02
MeJA + SPD + SS	8.34^{h} ± 0.10
SS	8.73^{g} ± 0.25

4.5 The content of photosynthetic pigments

Table 4.5. presents the results of an investigation of the Chl (a), Chl (b) and total carotenoid content in *Stevia rebaudiana* (Bertoni) treated with SPD, MeJA, SA and their combinations under salt stress. SS significantly decreased the

chlorophyll pigments and the carotenoid content. Moreover, the exogenous stand-alone applications of SA, SPD and MeJA to *Stevia* plants resulted in reduced chlorophyll pigments and total carotenoids.

At a 60 mM level of NaCl, the contents of chlorophyll (a), chlorophyll (b) and total carotenoid content were increased by using MeJA, SPD, SA + SPD. Thus, the interaction of a low level of NaCl (60 mM) and application of MeJA, Spd, SA + SPD resulted in a greater increase rate in the traits studied compared to the control sample. SA + MeJA increased Chl (a) significantly whereas, it decreased chlorophyll b and total carotenoid content.

Table 4.5. Effect of SA, SPD, MeJA, and salt stress on the contents of photosynthetic pigments

Treatment (µM)	Chlorophyll (a) (mg/g)	Chlorophyll (b) (mg/g)	Total carotenoids (mg/g)
Control	$1.05^{d} \pm 0.53$	$0.61^{d} \pm 0.31$	$84.09^{d} \pm 0.05$
MeJA	0.66i ± 0.33	$0.33^{h} \pm 0.17$	$46.59^{k} \pm 0.058$
SPD	$0.94^{e} \pm 0.47$	$0.51^{f} \pm 0.25$	$73.27^{f} \pm 0.056$
SA	$0.94^{e} \pm 0.47$	$0.41^{g} \pm 0.20$	$66.62^{g} \pm 0.062$
MeJA + SS	$1.30^{b} \pm 0.65$	$0.85^{a} \pm 0.42$	$100.46^{b} \pm 0.044$
SA + SS	$0.72^{g} \pm 0.36$	$0.33^{h} \pm 0.16$	$52.49^{i} \pm 0.057$
SPD + SS	$1.55^{a} \pm 0.77$	$0.81^{b} \pm 0.40$	$119.51^{a} \pm 0.051$
SA + MeJA +SS	$1.10^{c} \pm 0.55$	$0.54^{e} \pm 0.27$	$80.62^{e} \pm 0.115$
SA + SPD + SS	$1.29^{b} \pm 0.65$	$0.68^{c} \pm 0.34$	$91.94^{c} \pm 0.007$
MeJA + SPD + SS	$0.85^{f} \pm 0.42$	$0.33^{h} \pm 0.16$	$57.93^{h} \pm 0.035$
SS	$0.71^{h} \pm 0.35$	$0.30^{i} \pm 0.15$	$50.68^{j} \pm 0.107$

4.6 Stevioside and Rebaudioside A content

The association between elicitors and stress responses was also shown to cause glycoside accumulation. The application of stand-alone or combinations of MeJA, SPD and SA, and salt stress on certain concentrations of stevioside and rebaudioside A for *Stevia rebaudiana* (Bertoni) is shown in Table 4.6. and Figure 4.2.

The best treatment for increasing the stevioside content of plants was MeJA + SPD under salt stress compared to the control (from 1.48 to 2.18 g/100 g) and the best combination raised the content of rebaudioside A compared to the control by SA + SPD under salt stress (from 1.77 to 2.42 g/100 g).

Table 4.6. Effect of SPD, MeJA, SA and salt stress on stevioside and rebaudioside A content in *Stevia rebaudiana*

Treatment (µM)	Stevioside g/100 g	Rebaudioside A g/100 g
Control	$1.48^{d} \pm 0.01$	$1.77^{h} \pm 0.005$
MeJA	$1.72^{c} \pm 0.017$	$1.40^{j} \pm 0.015$
SPD	$1.03^{h} \pm 0.011$	$1.98^{g} \pm 0.012$
SA	$0.74^{j} \pm 0.017$	$2.55^{a} \pm 0.017$
MeJA + S	$1.15^{g} \pm 0.01$	$2.38^{c} \pm 0.007$
SA+S	$0.64^{k} \pm 0.007$	$1.72^{i} \pm 0.005$
SPD + S	$1.25^{e} \pm 0.015$	$2.36^{d} \pm 0.011$
SA +MeJA +S	$1.19^{f} \pm 0.005$	$2.10^{f} \pm 0.01$
SA + SPD + S	$1.02^{i} \pm 0.006$	$2.42^{b} \pm 0.015$
MeJA + SPD + S	$2.18^{a} \pm 0.019$	$1.98^{g} \pm 0.0054$
Salt stress (S)	$1.73^{b} \pm 0.015$	$2.28^{e} \pm 0.012$

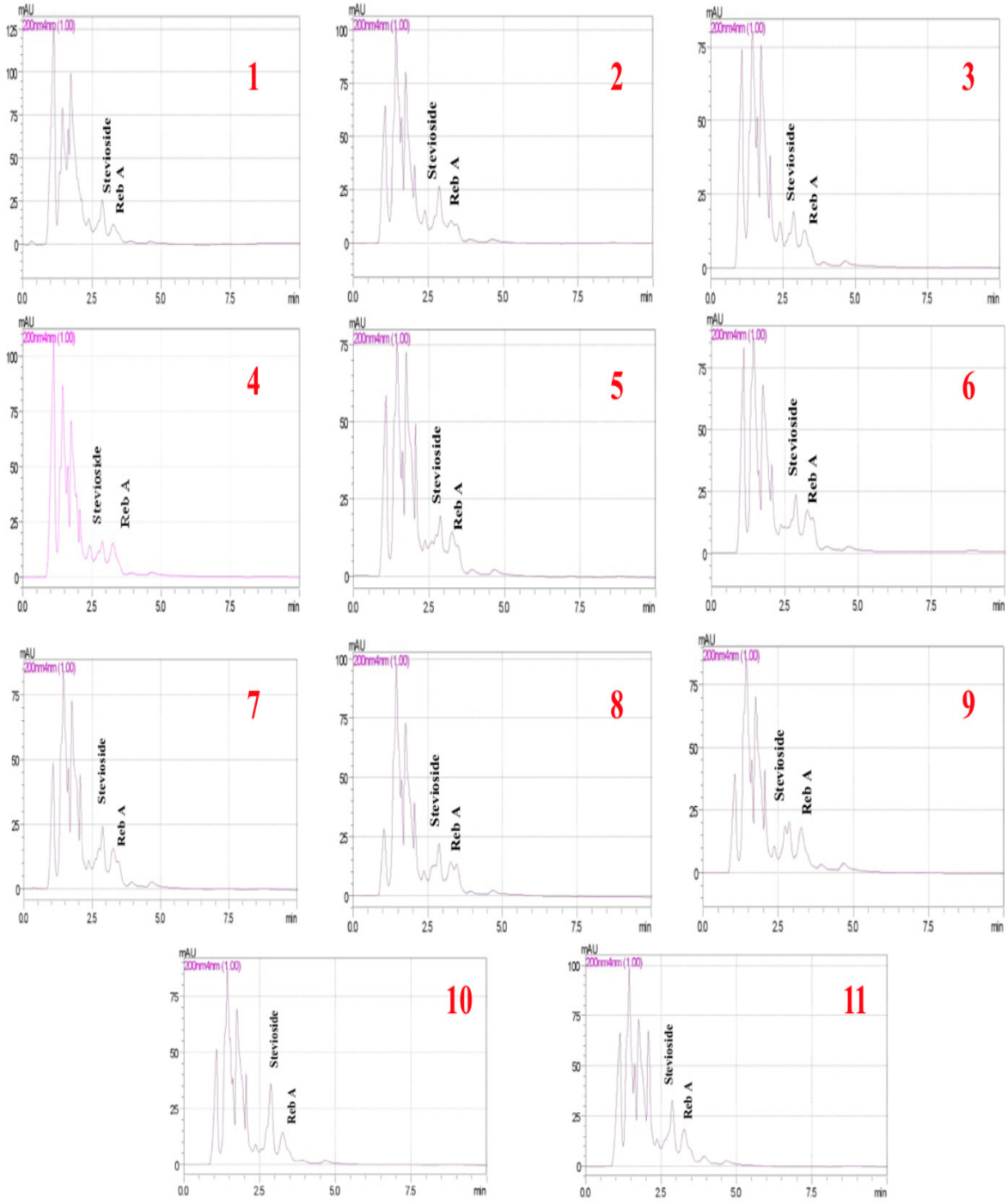


Figure 4.1. Steviol glycosides chromatograms of *S. rebaudiana* Bertoni (1) Control, (2) MeJA, (3) SPD, (4) SA, (5) MeJS+SS, (6) SA+SS, (7) SPD+SS, (8) SA+MeJA+SS, (9) SA+SPD+SS, (10) MeJA+SPD+SS, (11) SS.

5. DISCUSSION

According to studies, the plant's height decreased as the content of NaCl increased (100). One of the first effects of NaCl stress is the stimulation of an osmotic effect in the soil, which restricts plant water intake (101). This occurs as plant height and pigments decrease. In the present study, plant growth parameters such as plant (LFW), (LDW), and (SL) were inhibited by salt stress.

The decrease in Chl (a), Chl (b), and carotenoids under NaCl stress has been observed in other plant species and is attributed to an increase in chlorophyllase activity that encourages its breakdown. During the stressful phase, the *Stevia* spp. plant may show a decrease in evapotranspiration and stomatal conductance (102). Our findings support the deleterious impact of NaCl stress on Chl (a), Chl (b), and carotenoids in the current investigation (Table 4.5).

Photosynthesis is significantly impacted by an increase in salt (NaCl) by changes to the organelle ultrastructure and pigment content (Ashraf and Harris, 2013). Prior studies by Zeng et al. (2013) and Cantabella et al. (2017) also noted a considerable drop in the amount of chlorophyll in the *Stevia* plant.

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The SA is one of the important elicitors that increases in response to environmental stress, and eventually increases metabolite levels in *Stevia* spp. as an elicitor (103). In our research, salt stress and elicitor treatments led to a considerable change in the SG content of *Stevia* plant leaves when compared to a control group. When compared with the control groups, the application of SA significantly increased the Reb A (2.55 g/100 g) content of the non-stressed plants whereas it decreased the stevioside content. Our findings, which are in line with the 2020 (97) study, show that salicylic acid is the best way to increase Reb A in *Stevia rebaudiana* (Bertoni). Rebaudioside A having the greatest rates in the HPLC results suggested that SA could increase the rate of secondary metabolites steviol glycoside and, consequently, the sweetness attribute in *stevia* plant leaves.

The best treatment for increasing the stevioside content of plants was MeJA+SPD under salt stress compared to the control (from 1.48 to 2.18 g/100 g)

and the best effect combination raised the content of Reb A compared to the control by SA+Spd under salt stress (from 1.77 to 2.42 g/100 g). MeJA and SA supplementation enhanced stevioside of *Stevia* as mentioned in previous research (77).

MeJA and SA are two of the most significant hormones that signal plants and function as elicitors, triggering intracellular signals. These responses typically lead to the buildup of secondary metabolites in tissues and gene transcription for the manufacture of terpenoids (104). All plant tissues, including roots, leaves, flowers, and fruits, express *DXS* and *DXR* genes (105). Studies have demonstrated that signal molecules and phytohormones have a significant impact on the expression of *DoDXS* and *DoDXR* in the biosynthesis pathway of *IPP* and *DMAPP*.

Several studies have demonstrated that MeJA acts on the expression of the *SrDXS* and *SrDXR* genes to influence the production of *SGs* (106). SA's signals and modes of action are likely to interact with other hormones, and it appears that it can either boost or inhibit some physiological processes in plants (107). This implies that further information is required to fully understand the impact of MeJA on the expression of the *Stevia* spp. genes. Studies on the MEP pathway genes, however, have revealed that MeJA has a favorable impact on the genes that are associated with expression. Additionally, some research suggests that MeJA may stimulate the expression of the genes involved in the ent-KA and stevioside biosynthesis pathways, increasing the amount of stevioside produced (109). This shows that MeJA may have a favorable influence on the gene expression of SGs and may be effective as an elicitor. Most studies on elicitors show that applying different concentrations of methyl jasmonate increased stevioside synthesis when compared to control *in vitro* plantlets (95); this is also consistent with our study and is shown in Table (4.6).

Zheng et al., (2013) said that plant growth reduces under salt stress due to the loss of photosynthesis associated with the reduction of carbon intake and the closure of stomata (63). The dry weight, leaf area, and number of leaves, has all been affected by saline stress in various plant species, as found in the research of Ahmad et al. (2012) (64) and Sabra et al. (2012) (65). The induced osmotic stress from salt stress reduces plant water uptake (66). One of the unintended consequences of salt stress is a decrease in the plant's ability to take up nutrients from the soil due to its dependence on the osmotic potential of both water and the

plant itself. Furthermore, the results of the current experiment are compatible with those of various other research that have validated the findings (68).

The bioregulatory effects of SA on physiological processes, such as photosynthetic activity, sink and cell elongation, protein synthesis, cell differentiation, source regulation, ion uptake, cell division, enzymatic activities, and, as well as an increase in the plant's antioxidant capacity, could be responsible for the stimulatory effect of SA in plants (69,70). For instance, the ability of salicylic acid to act as an anti-stress agent in plants has improved those plants' capacity to withstand stress (71). In a study on corn (*Zea mays*) and soybeans (*Glycine max*) Borsani et al. (2001) (92) showed similar results. El-Khallal et al. (2009) (74), Delavar et al. (2010) (75), and Ben et al. (2009) (73) observed similar results when they used the SA on maize. Tadhani et al. (2007) (16) and Megeji et al. (2005) (15) studied the effects of SA on different plant species. When the concentration of SA was increased in yellow maize plants, the vegetative character values increased. When plants develop resistance to environmental stimuli, signaling molecules like MeJA and SA play a crucial role (19); and MeJA stimulates lignocellulose production and cell growth in plants (17). In many earlier studies, including those conducted It has been discovered that methyl jasmonate and SA help plants develop (110). According to studies (50) treatment with 50 M SA stimulated *stevia* plant growth *in vitro* culture while higher doses of this PGR reduced it.

A recent study has focused on polyamines, particularly spermidine. Because of their capacity to disrupt cellular hormonal balances, synthetic plant growth retardants may change metabolic profiles (78). It was found that after spermidine treatment, hydroxycinnamic acids, total phenolic and flavonoid content were all found to be at their lowest levels, whereas they were all at their highest levels after methyl jasmonate treatment (80). They also reported noticeable changes in the phenolic profiles among treatments. Regarding the spermidine in our study, the outcomes showed that the fresh and dry weight of the leaves appeared to be improved; this is consistent with the earlier study carried out in 2021, which used 0.75 mM of SPD and resulted in an increase in (LFW) and plant (SL) as well as increases in stem root diameter (10-35%) and (LF) (35-46%) (96).

SOD, and CAT are examples of enzymatic antioxidants; total phenolic compounds, flavonoids, and others are non-enzymatic antioxidants (60) Given

Stevia rebaudiana's current broad use, studying its natural defense mechanisms is of the highest importance. But total phenolic content (TPC) in leaves did not change significantly after 96 hours in either the control or salicylic acid treatments, but it did increase in the methyl jasmonate-treated plants compared to the 24-hour time point, while it decreased in the SPD-treated plants. Furthermore, they observed that adding MeJA at 50, 100, or 200 μM did not promote the growth of *Stevia* plant cells in a petri dish. These results suggest that larger concentrations of MeJA and SA may have been ineffective in controlling *Stevia* plant growth during *in vitro* cultivation. The second possibility is that the *Stevia* plant was exposed to very high concentrations of MeJA and SA over an extended time (92).

In a salinized environment, glycophytes and salt-sensitive plant species often exhibit growth inhibition. This might be explained by the insufficient photosynthesis brought about by stomatal closure and the decrease in the carbon assimilation rate induced by salt stress (80). Harrathi et al. (2012) (81) discovered that safflower's growth was severely decreased by 50 mM NaCl, whereas Zeng et al. (2013) (63) showed that *S. rebaudiana*'s biomass output was unaffected by low (60 mM NaCl) and moderate salt stress concentrations (90 mM NaCl), indicating that *S. rebaudiana* was more salt stress resistant. Salt stress shows a more significant impact on shoot development than root development; the shoot/root ratio was lower in salt stress-treated plants than in controls. The current experimental findings corroborated this inference by demonstrating that all NaCl treatments resulted in decreased shoot/root ratios.

SS decreased LN (from 33.20 to 23.00 cm), SL (from 11.24 to 8.56cm), LFW (from 1.27 to 1.14 cm), LTW (from 1.68 to 1.65 cm), LDW (from 0.17 to 0.15 cm) and % RWC (from 72.51 to 66.34 cm). Compared to the control, RFW and RDW were not significantly changed. The exogenous application of SPD under salt stress resulted in 15.26 cm longer SL and 51.80 LN. Significant effects of elicitation on LN, SL and % RWC were observed with SPD under *in vitro* salt conditions, compared to the control and SS groups. When compared to the SS and control group, significant effects of elicitation on SA + SPD under salt stress caused a significant increase in LFW, LDW, RFW. The exogenous application of SA significantly enhanced SL when compared to the control plants.

In line with these preliminary studies, Cheong et al. (2003) (36) discovered that methyl jasmonate and SA significantly increased POX and SOD activities

while significantly reducing CAT activity in an *in vitro* culture of *Stevia rebaudiana*. The SOD is a crucial antioxidant enzyme present in all aerobic organisms, where it helps to prevent the potentially harmful consequences of stress-related reactive oxygen species, according to Ashraf et al. (2010) (60) and Mittler et al. (2002) (61). According to Ali et al. (2006), *Panax ginseng* (Korean ginseng) suspension cultures treated with methyl jasmonate, and SA showed increased SOD and POX activities (82).

According to several research, methyl jasmonate and SA treatment increased SOD and POX activity while decreasing CAT activity in *Mentha piperata* (peppermint) (72) and *Haematococcus pluvialis* (chlorphyta) (73). In reaction to stress, several secondary metabolites, such as flavonoids and total phenolics may accumulate (72).

In the Kranner et al. (2010), MeJA and SA significantly increased the total phenolic content (21). Phenolic chemicals are the most common class of antioxidants. Another way to reduce radicals is through electron donation. If antioxidants and polyphenols are present, singlet oxygen may prevent biological components from suffering from oxidative damage (85). Kim et al. (2006) (86) discovered a strong correlation between raising the antioxidant capacity and total phenolic content of *Raphanus sativus* (raphani semen) *in vitro* culture using several elicitors. After being treated with MeJ and SA, Ali et al. discovered that Panax ginseng cultivated in suspension cultures had higher levels of total phenolic activity (82).

According to research by Chung et al. (2016) (87), the total phenolic content of *Momordica charantia* in in-vitro cultures treated with 1M MeJA and SA was significantly higher than in control cultures (87).

The MeJA is produced by higher plants and plays a significant signaling role in plant defense systems, according to a study by Sharma et al. (2015) (88). Therefore, increasing the formation of secondary metabolites by giving methyl jasmonate exogenously is a feasible strategy. In comparison to control plants, *Stevia rebaudiana* plants treated with a 50 µM methyl jasmonate feed increased 8.05-fold in stevioside content.

Furthermore, in *Stevia rebaudiana*, according to Bayraktar et al. (2016), when 100 M SA was added to the growth medium, stevioside content was reported to increase to 13.84 mg/g dry weight an increase of 8.89 times greater than the

amount seen in the control plants (19). The SA is believed to be a strong defense mechanism for the organism against biotic and abiotic stresses. Its principal cultural function is therefore that of an elicitor (89). Contrarily, Lucho et al. (2019) (79) found that spermidine and SA-treated plant extracts had lower antioxidant capacities than PBZ (Paclobutrazol) and methyl jasmonate-treated extracts. The three antioxidant assay findings collectively demonstrated that SPD was unable to enhance the antioxidant capacity of stevia leaves.

Saravi et al. (2017) found that salt stress's chlorophyll fluorescence characteristics of the stevia plant were negatively affected by salt stress, suggesting that the photosynthetic system was compromised. On the other hand, the application of endophytic fungus linked with spermidine increases plant resilience to salt stress environments by improving chlorophyll fluorescence characteristics and decreasing the detrimental impact of salinity (90).

The effect of the interaction between salinity and salicylic acid was the most effective treatment for growth parameters when *stevia* plants under salt stress was irrigated by salicylic acid foliar (91). The SA as an antistress agent may enhance the plant's tolerance to environmental stresses (71); similar results were obtained by Borsani et al. (2005) (93) on corn, by Khan et al. (2003) (72) on soybeans, and by Khodary (2009) (73) on maize.

The leaf is the main component of the plant that absorbs solar energy and transforms it into compounds that the plant may use to grow and develop. The underlying cause of salt stress' tendency to slow down plant development is damage to leaf function. A recent study discovered that the number of fully expanded leaves substantially decreased as salt stress levels increased, causing older plants to age more quickly and produce fewer new leaves (93).

Plants exposed to salt stress for 28 days exhibited chlorosis, wilting, and necrosis in the leaves of plant. A salt stress concentration exceeding 90 mM NaCl was shown to be detrimental to the chloroplast structure of *S. rebaudiana*, as evidenced by the occurrence of necrosis and chlorosis in the leaves. The results supported this conclusion: as the concentration of NaCl increased, the levels of Chlorophyll (a), Chlorophyll (b), and Chlorophyll (a + b) decreased significantly. Also, if the Chlorophyll (a) / Chlorophyll (b) ratio changes, it could mean that photosystem I and II contribute differently to photosynthesis. Because the same amount reduced the contents of both chlorophyll a and chlorophyll b, the

chlorophyll (a) / chlorophyll (b) ratio was unaffected. It was hypothesized that salt stress had a comparable effect on the two photosystems in the leaves of *S. rebaudiana* (94).

6. CONCLUSIONS AND RECOMMENDATIONS

This research evaluated how salt stress and elicitors affected the development and growth of *Stevia* spp. by taking measurements of its LN, SL, RL, LFW, LTW, LDW, RFW, and RDW, as well as the percentage of RWC. LN, SL, LFW, LTW, LDW, and % RWC were all decreased by salt stress but, RFW and RDW did not significantly differ from the control. Higher SL and LN were produced because of the exogenous application of SPD under salt stress. Under the *in vitro* salty conditions, significant effects of elicitation on LN, SL, and % RWC were seen with SPD when compared to the control and SS groups. Significant effects of elicitation on SA+SPD during salt stress increased LFW, LDW, and RFW as compared to the SS and control groups. Comparing the exogenous application of SA to the control plants, SL was greatly improved.

The association between elicitors and stress responses was also shown to cause steviol glycoside accumulation. The most effective methods for increasing the stevioside and Reb A content of plants under salt stress were MeJA + SPD and SA + SPD applications, respectively. It was also discovered that salt stress and elicitors induced antioxidant molecules in *Stevia* spp. Exogenous SA, SPD and MeJ were applied to non-stressed plants to increase CAT and SOD activity. SPD, SA + MeJA, SA + SPD, MeJA + SPD dramatically increased CAT activity in stressed plants. In plants under salt stress, the use of SA, MeJA, SA + MeJA, SA + SPD, MeJA + SPD greatly increased SOD activity. The contents of chlorophyll (a), chlorophyll (b), and total carotenoids were increased by using MeJA, SPD, and SA + SPD. The phenolic content of the non-stressed plants was significantly increased by the application of SA.

We assume that additional research on stevioside and antioxidant molecules will shed light on the regulatory interactions between these molecules and the part they play in the development of stevioside production processes.

The results presented in the thesis regarding the analysis of steviol glycosides, growth parameters, and antioxidant molecules in stevia affected by salt stress and elicitors are believed to create a user base for future studies in understanding the mechanism that can be employed for the improvement of a large-scale production of steviol glycosides.

7. REFERENCES

"Vancouver citation system was used in this thesis."

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