UNIVERSIDADE ESTADUAL DO SUDOESTE DA BAHIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA
ÁREA DE CONCENTRAÇÃO: FITOTECNIA

IMPROVING CITRUS TOLERANCE: SALICYLIC ACID-MEDIATED RESPONSES TO WATER DEFICIT AND SALINITY

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VITÓRIA DA CONQUISTA
BAHIA - BRAZIL
2025

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Dissertation submitted to the State University of Southwestern Bahia, as part of the requirements of the Graduate Program in Agronomy, Area of Concentration in Crop Science, for the degree of Master.

Advisor: Prof. Dr. Paulo Araquém Ramos Cairo

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S113i

Sá, Milton Carriço.
Improving citrus tolerance: salicylic acid-mediated responses to water deficit and salinity. / Milton Carriço Sá, 2025.
49f.
Orientador (a): Dr. Paulo Araquém Ramos Cairo.
Dissertação (Mestrado) – Universidade Estadual do Sudoeste da Bahia, Programa de Pós-Graduação em Agronomia, Área de concentração em Fitotecnia. Vitória da Conquista, 2025.
Inclui referência F. 37 – 43
1. Combined abiotic stresses. 2. Oxidative damage. 3. Osmoregulation. 4. Plant growth regulators. I. Cairo, Paulo Araquém Ramos. II. Universidade Estadual do Sudoeste da Bahia, Programa de Pós-Graduação em Agronomia. III. T.

CDD: 634.3

Catalogação na fonte: ***Karolyne Alcântara Profeta – CRB 5/2134***

UESB – Campus Vitória da Conquista – BA

UNIVERSIDADE ESTADUAL DO SUDOESTE DA BAHIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA
Área de Concentração em Fitotecnia
Campus de Vitória da Conquista, BA.

DECLARAÇÃO DE APROVAÇÃO

TÍTULO: "Improving Citrus Tolerance: Salicylic Acid-Mediated Responses to Water Deficit and Salinity"

AUTOR (A): Milton Carriço Sá

Aprovado como parte das exigências para obtenção do Título de MESTRE EM AGRONOMIA, ÁREA DE CONCENTRAÇÃO EM FITOTECNIA, pela seguinte Banca Examinadora:

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ABSTRACT

SÁ, M.C. **Improving citrus tolerance: salicylic acid-mediated responses to water deficit and salinity**. Vitória da Conquista – BA, UESB, 2025. 49 p. (Dissertation: Master of Science in Agronomy; Area of Concentration: Crop Science)*.

Water deficit and salinity significantly limit the expansion of citrus plants (*Citrus sinensis*) worldwide. Although these two types of abiotic stress are harmful throughout the biological cycle, they can become critical for plant survival and establishment in the early development stages. This study aimed to investigate the effects of salicylic acid (SA) foliar application on the morphophysiological and biochemical traits of young citrus plants subjected to water deficit and salinity conditions, either alone or simultaneously. A greenhouse experiment was conducted with seven-month-old citrus plants subjected to four growing conditions - no stress, water deficit (WD), salinity (S), and WD + S - and pre-treated with SA application at 0, 1, 2, and 4 mM. Results showed that water deficit and salinity reduced gas exchange and damaged the photosynthetic apparatus by increasing reactive oxygen species (ROS) levels. Simultaneous stresses caused more photosynthetic pigment degradation and reduced gas exchange, and less biomass production than water deficit and salinity stresses alone. The application of 2 mM SA mitigated the adverse effects of stress by improving gas exchange and osmoregulation, and enhancing antioxidant enzyme activity and phenolic content. Furthermore, it prevented electrolyte leakage and ROS accumulation, whose metabolic performance varied depending on the type of stress. These findings suggest the foliar application of SA as an effective strategy to improve the young post-transplanted citrus plants' tolerance to water deficit and salinity, alone or simultaneously.

Keywords: Combined abiotic stresses; oxidative damage; osmoregulation; plant growth regulators.

***Advisor:** Prof. Dr. Paulo Araquém Ramos Cairo, UESB.

RESUMO

SÁ, M.C. **Aprimorando a tolerância dos citros: respostas mediadas por ácido salicílico à deficiência hídrica e salinidade**. Vitória da Conquista – BA, UESB, 2025. 49 p. (Dissertação: Mestrado em Agronomia; Área de Concentração: Fitotecnia)*.

Deficiência hídrica e salinidade estão entre as formas de estresse abiótico que mais afetam negativamente o crescimento e desenvolvimento das culturas agrícolas em todo o mundo. Na fase inicial do desenvolvimento dos citros (*Citrus sinensis*), a exposição aos estresses hídrico e salino compromete a sobrevivência das plantas e o estabelecimento do plantio, sobretudo quando ocorrem simultaneamente. O presente estudo objetiva avaliar os efeitos da aplicação foliar de ácido salicílico (AS) sobre características morfofisiológicas e bioquímicas de plantas jovens de citros submetidas a condições de deficiência hídrica e salinidade. O experimento foi conduzido em casa de vegetação, adotando-se um delineamento inteiramente casualizado, disposto em esquema fatorial 4 × 4, correspondente a 4 condições de crescimento: (1) ausência de estresse (regime de irrigação a 90% da capacidade de vaso, sem adição de solução salina), (2) deficiência hídrica (DH – regime de irrigação a 45% da capacidade de vaso, sem adição de solução salina), (3) salinidade (S – regime de irrigação a 90% da capacidade de vaso e adição de solução salina), e (4) DH + S (regime de irrigação a 45% da capacidade de vaso e adição de solução salina); e aplicação de AS nas concentrações de 0, 1, 2 e 4 mM. Os resultados mostraram que deficiência hídrica e salinidade reduziram as trocas gasosas e danificaram o aparato fotossintético por aumentarem os níveis de espécies reativas de oxigênio (ERO). Estresses simultâneos causaram maior degradação de pigmentos fotossintéticos e redução das trocas gasosas, e menor produção de biomassa em comparação aos estresses por deficiência hídrica e salinidade isolados. A aplicação de AS na concentração de 2 mM mitigou os efeitos adversos dos estresses por intensificar as trocas gasosas e osmorregulação, e aumentar a atividade de enzimas antioxidantes e o teor de fenólicos. Além disso, preveniu o extravasamento de eletrólitos e acúmulo de ERO, de modo que as alterações metabólicas variaram a depender da condição de estresse. Esses resultados sugerem a aplicação foliar de AS como uma estratégia eficaz em aumentar a tolerância de plantas jovens de citros aos estresses por deficiência hídrica e salinidade, isolados ou simultâneos.

Palavras-chave: Dano oxidativo; estresses abióticos combinados; osmorregulação; reguladores de crescimento vegetal.

***Orientador:** Prof. Dr. Paulo Araquém Ramos Cairo, UESB.

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LIST OF ABBREVIATIONS, ACRONYMS, AND SYMBOLS

A	CO_2 assimilation rate
AA	amino acid
A/C_i	instantaneous carboxylation efficiency
APX	ascorbate peroxidase
Car	carotenoids
Chl *a*	chlorophyll *a*
Chl *b*	chlorophyll *b*
Chl/Car	chlorophyll/carotenoids ratio
C_i/C_a	ratio of internal and external CO_2 concentration
$\Delta\Psi$	water potential difference between pre-dawn and midday
E	transpiration rate
EL	electrolyte leakage
F_0	minimal chlorophyll fluorescence
F_m	maximum chlorophyll fluorescence
F_v	variable chlorophyll fluorescence
F_v/F_0	electron transport capacity of photosystem II
F_v/F_m	maximum quantum yield of photosystem II
GPX	guaiacol peroxidase
g_s	stomatal conductance
H_2O_2	hydrogen peroxide
iWUE	intrinsic water-use efficiency
O_2^-	superoxide anion
PCA	principal component analysis
PLS-DA	partial least square-discriminant analysis
PSII	photosystem II
Ψ_w 5:00 a.m.	leaf water potential at pre-dawn
Ψ_w 12:00 p.m.	leaf water potential at midday
ROS	reactive oxygen species
RS	reducing sugar
RWC	relative water content
SA	salicylic acid
SOD	superoxide dismutase
SS	soluble sugar
Total Chl	total chlorophyll
VIP	variable importance in projection
VPD	vapor pressure deficit
WUE	water-use efficiency

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1 INTRODUCTION

Climate change has increased the frequency and intensity of abiotic stresses, especially drought and soil salinity, which significantly limit crop growth and development. Water deficit is known to cause cell dehydration and induce stomatal closure, limiting gas exchange and reducing photosynthetic rate (Ozturk et al., 2020). In turn, high soil salinity causes Na^+ and Cl^- accumulation in plant cells, negatively affecting biosynthetic processes and mineral nutrient uptake (Zahra et al., 2022). Furthermore, both water deficit and salinity cause enhanced generation of reactive oxygen species (ROS) in plants, resulting in the degradation of cellular components, including photosynthetic pigments. These combined effects severely limit the growth and yield of major crops worldwide.

Some effects of salinity stress alone are often similar to those of water deficit stress, such as reduced shoot growth and inhibited leaf expansion due to cellular dehydration (Hao et al., 2021). Conversely, under field conditions, where water deficit and salinity often co-occur, the physiological responses of plants to these simultaneous stresses can vary greatly compared to the effects of either one alone. When water deficit and salinity occur simultaneously, salt accumulation in the root zone causes an osmotic gradient that reduces root water uptake, intensifying water deficit stress (Xue et al., 2021). As a result, cellular hydration becomes lower, causing enhanced protein denaturation, membrane degradation, and ion toxicity. Moreover, ROS accumulation under simultaneous stresses becomes significantly higher, exacerbating oxidative damage (Kumar et al., 2022). While many studies have been published regarding the effects of both water deficit and salinity stress alone, to the best of our knowledge, no one has yet investigated possible physiological responses of tolerance or plasticity of young citrus plants when they are subjected to these stresses simultaneously.

Since citrus species are known to be sensitive to water deficit and salinity, grafting has often been used as a strategy to improve plant tolerance to abiotic stress. Typically, the rootstock in a grafted tree provides better tolerance to challenging abiotic conditions compared to a fruit tree with its own roots (Fu et al., 2020). However, this cultivation management alone has not proven effective in mitigating plant growth inhibition, especially when water deficit and salinity occur simultaneously as soon as seedlings are transplanted into the field. Additionally, the frequent use of only one rootstock genotype can favor the endemic occurrence of diseases and pests, so new strategies have been tested to improve the resilience of citrus plants to abiotic stress. In this scenario, the use of growth regulators emerges as an alternative, although further studies are required to confirm their effectiveness.

Salicylic acid (SA) is a phenolic plant hormone with multiple roles, either under optimal conditions or for acclimation to environmental stress (Sharma et al., 2023). It is well known to modulate plant metabolic resource partitioning, stimulating adventitious root formation in response to stress, and to interact with abscisic acid to induce stomatal closure, regulating water loss through

transpiration and increasing water-use efficiency (Iqbal et al., 2022). Furthermore, other relevant roles of SA include: regulating ionic balance in plant tissues; activating the synthesis of protective metabolites and stress-tolerant proteins (Ghassemi-Golezani and Samea-Andabjadid, 2022); attenuating electrolyte leakage, helping to maintain cell membrane integrity and stability; and upregulates the expression of genes encoding antioxidant enzymes, helping to mitigate oxidative damage (Wang et al., 2022).

It is important to strengthen strategies for water deficit and salinity stress tolerance in citrus plants for the development of the citrus industry, and this includes strategies to mitigate occasional environmental stresses in young plants. However, it is still largely unknown how mechanisms such as gas exchange, CO_2 assimilation, osmoregulation, and antioxidant system regulation - mediated by exogenous SA - are associated with improved tolerance to combined water deficit and salinity stresses (Vital et al., 2022). In this study, we hypothesized that the occurrence of simultaneous water deficit and salinity stresses may aggravate the negative effects of each stress alone on young citrus plants, and that exogenous salicylic acid (SA) plays a regulatory role in mechanisms such as stomatal control of gas exchange, CO_2 assimilation, osmoregulation, and antioxidant system, thus enhancing plant tolerance to these simultaneous environmental stresses. Therefore, we aimed to evaluate the effects of foliar application of SA on the morphophysiological and biochemical characteristics of young citrus plants subjected to water deficit and salinity conditions, alone or simultaneously.

2 THEORETICAL FRAMEWORK

2.1 Orange cultivation in Brazil: socioeconomic aspects, botany, and management

The sweet orange [*Citrus sinensis* (L.) Osbeck] belongs to the family Rutaceae, which includes various species of fruit-bearing plants, such as citrus fruits (oranges, lemons, limes, tangerines, and other related species) (Montenegro et al., 2023). Orange is the most widely cultivated citrus fruit worldwide, with 47.5 million tons (M t) produced during the 2023/24 harvest season, compared to 36.6 M t of tangerines, 9.3 M t of lemons and limes, and 6.8 M t of grapefruits (USDA, 2024).

Orange trees are medium-sized, reaching approximately 4 meters in height. Their leaves and flowers are aromatic, and the fruits are rich in vitamin C, containing notable amounts of vitamins A and B, along with minerals such as calcium, potassium, sodium, phosphorus, and iron (Yulistyarini and Hadiah, 2020). Native to the tropical and subtropical regions of Southeast Asia, the distinctive flavor and aroma, along with their adaptability to various soil and climate types, have contributed to their widespread cultivation (Gentile et al., 2020).

Brazil is the second-largest citrus producer in the world and the leading producer of oranges and orange juice. The country's citrus industry is predominantly focused on orange production, both in terms of cultivated area and production volume (USDA, 2022). During the 2023/24 harvest season, Brazil accounted for 34.7% of global orange production (16.5 million tons) and 68.7% of global orange juice production (1.1 million tons), representing approximately three-quarters of global orange juice exports (USDA, 2024).

The main producing states are São Paulo (12,519,500 t), Minas Gerais (1,091,402 t), and Paraná (800,000 t), contributing 74.87%, 6.53%, and 4.78% of national production, respectively. Bahia ranks as the fourth-largest orange producer in the country and the largest among the northeastern states, with a production of 653,549 t from 50,000 ha harvested, representing 3.9% of national output and 52.20% of the Northeast's production (IBGE, 2023).

Orange cultivation plays an important role in income generation and job creation in producing regions. It is an activity with strong participation from small and medium-sized producers and employs a substantial workforce (Vidal, 2022). In addition to fresh fruit and orange juice, citrus pulp meal – an ingredient in animal feed - and essential oils extracted from the fruit peel are widely traded by-products, particularly in the domestic market. These oils are highly valued and used in the food, pharmaceutical, cosmetics, and cleaning product industries (Mergulhão, 2018).

Due to the lower incidence of high-risk endemic pests and diseases affecting citrus, Bahia stands out as an important escape area and a region with productive potential (Lima et al., 2017). However, the crop's productivity in the state (13.07 t ha^{-1}) is low compared to the national average

(28.68 t ha^{-1}), ranking among the lowest in the country (IBGE, 2023). This is primarily attributed to the limited adoption of technology, an issue closely linked to the technical expertise of citrus growers. The scarcity of rainfall directly affects flowering, compromising crop productivity, especially since most citrus plants are cultivated without irrigation, under persistent water deficit conditions (Vidal, 2022).

Citrus plants also do not tolerate saline soils, with root development being limited by this condition (Othman et al., 2023). The threshold salinity for citrus is, on average, 1.4 dS m^{-1}, so orchard productivity decreases by 13% for every 1.0 dS m^{-1} increase in soil electrical conductivity above the threshold value (Darshan and Shukla, 2021). In general, salinity negatively affects plant growth, development, and productivity due to osmotic stress, caused by reduced water absorption, as well as toxic effects and nutritional imbalances resulting from the accumulation of Cl^- and Na^+ ions in the cells (Aparicio-Durán et al., 2021; Darshan et al., 2022).

In citrus cultivation, the grafting technique has become widely used as an alternative to ensure disease tolerance and higher production under adverse conditions (Sajid et al., 2020; Brito et al., 2021). In Bahia, the predominant combination is the 'Cravo' lemon as rootstock and the 'Pera' orange as scion. The 'Cravo' lemon is the main rootstock used in Brazil, showing tolerance to drought and salinity, with a threshold salinity of 2.5 dS m^{-1}. However, despite this tolerance, these abiotic factors still lead to significant production losses, compromising orchard maintenance, especially when they occur during the early stage of seedling development, after field transplanting (Vidal, 2022). Furthermore, the predominance of a single genetic material poses a risk for the endemic occurrence of pests and diseases (Wan et al., 2022).

2.2 Abiotic stresses

Abiotic stresses are disadvantageous influences exerted by environmental conditions that prevent the plant from reaching its full genetic potential (Gull et al., 2019). Citrus plants are highly sensitive to water deficit and salinity, so these abiotic stresses are important limiting factors for the crop development worldwide, including in Bahia (Ziogas et al., 2021; Abobatta, 2022).

2.2.1 Physiological effects of water and salt stresses

Water deficit, characterized by insufficient water availability in the soil to meet the plants' needs, occurs in most natural or agricultural habitats and results from prolonged periods without precipitation (Leite et al., 2021). Rainfed cultivation, common in the Northeast region of Brazil, subjects plants to periods of water deficit, compromising productivity (Vidal, 2022).

Water deficit causes a series of negative physiological effects that impair growth, development, and production (Rustioni and Bianchi, 2021). The cellular dehydration resulting from

water deficit leads to reductions in leaf water potential and relative water content (RWC), which cause stomatal closure, resulting in reduced gas exchange and negatively affecting photosynthesis and growth. This reduction in cellular turgor leads to lower plant height, stem diameter, leaf area, and dry matter production (Al-Selwey et al., 2023).

Furthermore, water stress results in an increase in the synthesis of reactive oxygen species (ROS), which reach a toxic threshold for the plant, degrading various cellular components, including photosynthetic pigments. Common forms of ROS in plant cells include hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) (Ahsan et al., 2023).

In addition to water deficit, water and soil salinity are among the main challenges faced by the agricultural sector in recent decades. The main economically important crops are classified as moderately or severely sensitive to salinity, with reported losses ranging from 20 to 50% of production due to exposure to salt stress. It is estimated that over 33% of agricultural land is affected by salinity due to factors such as irrigation with saline water and high soil water evaporation (El-Beltagi et al., 2023).

Salt stress has two components: osmotic stress, with effects similar to water deficit; and ionic effects, resulting from the accumulation of toxic ions, which interfere with nutrient absorption and cause cytotoxicity (Abdeldym et al., 2020).

In the osmotic phase of salt stress, there is a decrease in shoot growth, with reduced leaf expansion and inhibition of lateral bud formation. Protein denaturation and membrane destabilization are also observed due to reduced hydration of these macromolecules (Afzal et al., 2023). In the ionic effects phase, toxic amounts of Na^+ and Cl^- ions accumulate in the leaves, leading to inhibition of photosynthesis and biosynthetic processes, as well as negatively affecting the absorption of most mineral nutrients, such as nitrogen, phosphorus, potassium, calcium, and magnesium (Abdeldym et al., 2020).

When present in large quantities, Na^+ ions begin to compete for the binding sites of proteins necessary for the transport of other essential nutrients, such as potassium, impairing their absorption by the plant and causing deficiency symptoms. Although Na^+ reaches toxic concentrations before Cl^- in most plants, some species, such as citrus, grapevines, and soybeans, are highly sensitive to excess Cl^- (Madani et al., 2022; Vives-Peris et al., 2023).

2.2.2 Acclimation mechanisms to stresses

Being sessile, plants are unable to move to a more favorable environment in order to avoid abiotic stress. As an alternative, they have developed the ability to compensate for stressful conditions through physiological or morphological changes, which enable their survival and reproduction under

adverse conditions or allow the plant to endure the stress until more favorable conditions return (Adil and Quraishi, 2023).

Acclimation is the process by which plant performance is improved due to changes in its physiology or morphology, after repeated exposure to environmental stress (Wu, 2023). Plant acclimation mechanisms involve morphological changes, stomatal closure, osmotic adjustment, activation of antioxidant defenses, and exclusion and internal tolerance to toxic ions. The effects of these changes aim to achieve cellular homeostasis, so that the plant's life cycle can be completed under the new environmental regime (Negi et al., 2022).

Due to phenotypic plasticity, plants are capable of altering their phenotype in response to abiotic stress. This plasticity can result in morphological changes that enable plants to avoid or mitigate the harmful effects of stress (Assaeed et al., 2023). As an example, there is an increase in the root/shoot biomass ratio, reducing the transpiring surface area and carbon and energy consumption, while increasing the capacity for water and nutrient absorption. Plants can reduce their leaf area through a decrease in leaf cell division and expansion or by foliar senescence and abscission, so that a greater proportion of assimilates is allocated to the root system, supporting continued root growth (Prisa, 2023).

Osmotic adjustment refers to the ability of plant cells to accumulate solutes and use them to lower the water potential during periods of osmotic stress, so that the relative water content (RWC) remains practically the same (Verbeke et al., 2022). This alleviates the adverse effects of water stress on the plant, particularly on plant growth and stomatal regulation (stomatal opening and closing). This reduction in water potential facilitates the influx of water into the cell, leading to the maintenance of cellular turgor (Mendes et al., 2023).

Proline is a compatible osmolyte that plays a role in osmoregulation. The increase in proline concentration under stress conditions helps in osmotic adjustment, allowing for a reduction in water potential and the maintenance of cellular turgor. Furthermore, proline also has an osmoprotective function, reducing the degradation of cellular components by reactive oxygen species (Mendes et al., 2023). In addition to the accumulation of compatible osmolytes, another acclimation mechanism is the activation of hydrolytic enzymes that participate in the hydrolysis of reserve macromolecules, where starch and proteins are converted into soluble sugars and amino acids to increase solute concentration inside the vacuole, promoting osmotic adjustment (Ghassemi-Golezani and Samea-Andabjadid, 2022).

Environmental stresses can disrupt plant metabolism through various mechanisms, most of which result in the accumulation of ROS in the cells (Ahsan et al., 2023). ROS are detoxified by antioxidant enzymes through the process of ROS inactivation. Superoxide dismutase (SOD), which oxidizes and reduces $O_2^{\bullet -}$, and catalase (CAT), which catalyzes the detoxification of H_2O_2 into water

and oxygen, are examples of antioxidant enzymes that act in ROS inactivation (El-Beltagi et al., 2023).

Therefore, plants are capable of acclimating through morphological changes (phenotypic plasticity), stomatal closure, osmotic adjustment (via the hydrolysis of storage macromolecules or the accumulation of compatible solutes), increased activity of antioxidant enzymes, and mechanisms of exclusion and internal tolerance to toxic ions. Together, these anti-stress mechanisms enable plants to acclimate to stresses such as water deficiency, salinity, heavy metal toxicity, heat, cold, and their possible combinations.

2.2.3 Combinations of abiotic stresses

In field conditions, plants are often subjected to combinations of simultaneous abiotic stresses. Such combinations of stresses can have effects on plant physiology that differ from those observed when these stresses act individually. Therefore, the physiological acclimation mechanisms of plants to a combination of abiotic stresses may also differ from those when these stresses are applied separately (Vital et al., 2022).

When occurring individually, salinity stress shares certain similarities with water stress, including a reduction in shoot growth and inhibition of leaf expansion due to cellular dehydration (Abdeldym et al., 2020). However, water deficit and salinity are commonly associated under field conditions, and the physiological responses to the combination of both stresses are substantially different from those observed when water deficit and salinity act separately (Dugasa et al., 2019).

Under salinity stress, ionic toxicity occurs due to the accumulation of Na^+ and Cl^- ions in plant cells at toxic levels to the tissue. The cytotoxic effects resulting from the buildup of these ions lead to photosynthesis inhibition and interfere with the uptake of essential nutrients, with symptoms of nutrient deficiencies being observed. Under water deficit conditions, this situation is exacerbated, as cellular dehydration reduces turgor pressure and increases ionic toxicity (Kumar et al., 2022). Therefore, it is important to investigate the combined stresses to uncover which mechanisms confer greater plasticity or tolerance to the combination of water and salt stresses.

2.3 Plant hormones: mobile signals in stress response

Plants constantly perform metabolic adjustments in response to the environment, primarily to ensure their survival under unfavorable conditions. These adjustments to environmental conditions occur through sensory systems that react to external stimuli. Plant hormones are mobile signals (chemical messengers) that form part of the plant's sensory systems, transmitting information and triggering responses to stimuli (Parwez et al., 2022).

Environmental stresses alter many plant physiological processes, causing primary disturbances that signal changes in environmental conditions to the plant. These stimuli induce plant responses through modifications of existing pathways or activation of stress response pathways, which involve the accumulation of phytohormones (Khan et al., 2023).

Plant hormones, at nanomolar concentrations, can activate reactions in target cells, regulating a wide range of responses to abiotic stress. They are, therefore, essential for the plant's ability to respond to stresses (Zheng et al., 2023). Salicylic acid plays an important role in plants' response to environmental stimuli and is highly relevant to the plants' acclimation capacity under abiotic stress conditions.

2.3.1 Salicylic acid as an attenuator of abiotic stresses

Salicylic acid (SA) is a phenolic phytohormone that regulates many aspects of plant growth and development, as well as responses to biotic and abiotic stresses (Yang et al., 2023).

SA is found in various plant organs (leaves, fruits, stems, and roots), playing an important role in defense responses and acclimation to stress. SA triggers the production of defense proteins that help combat pathogens and prevent the spread of infections. It is also involved in the induction of systemic acquired resistance (SAR), enabling plants to respond more effectively to future pathogenic attacks (Ullah et al., 2023).

In addition to pathogens, SA is also involved in plant responses to abiotic stresses such as extreme temperatures, oxidative stress, salinity, and drought. Under stressful conditions, SA regulates the expression of genes encoding antioxidant enzymes, reducing oxidative stress; activates the synthesis of stress tolerance proteins, contributing to the acclimation of plants to adverse conditions (Han et al., 2023); interacts with abscisic acid, inducing stomatal closure and regulating water loss through transpiration; modulates growth and development, influencing resource allocation to different parts of the plant and stimulating adventitious root formation in response to stress (Bernabé-Antonio et al., 2023); and attenuates electrolyte leakage, helping to maintain membrane integrity and stabilization (Koche et al., 2021).

The application of exogenous SA has been studied as a possible strategy for mitigating the adverse effects of abiotic stresses, such as water deficit and salinity. Under such stresses, SA application regulates stomatal closure, reduces water loss through transpiration, improves water-use efficiency, and regulates ion balance in plant tissues (Tayyab et al., 2020; El-Beltagi et al., 2023). It also stimulates the synthesis of antioxidant enzymes and protective metabolites, helping to mitigate oxidative damage (Mohi-Ud-Din et al., 2021).

Although salicylic acid is best known for its role in pathogen response, its function in responses to abiotic stresses is increasingly being recognized as a key part of plants' ability to cope

with a variety of environmental challenges (Koche et al., 2021; Han et al., Bernabé-Antonio et al., 2023).

3 MATERIAL AND METHODS

3.1 Site description and experimental design

A greenhouse experiment was conducted at the State University of Southwestern Bahia, in Vitória da Conquista, Bahia State, Brazil (14°53'08" S, 40°48'02" W; 881 m asl) from March to May 2024. According to the Köppen-Geiger classification, the local climate is of the *Cwb* type (dry-winter subtropical highland climate), with an average annual precipitation of 733.9 mm, concentrated between November and March. The region's maximum and minimum temperatures are 26.4 ºC and 16.1 ºC, respectively, with an average annual temperature of 20.2 ºC. During the experimental period, the average temperature and relative humidity inside the greenhouse were 21.8 ºC and 83.1%, respectively (Fig. 1A). Vapor pressure deficit (VPD) data (Fig. 1B) were obtained following the method proposed by Abtew and Melesse (2013).

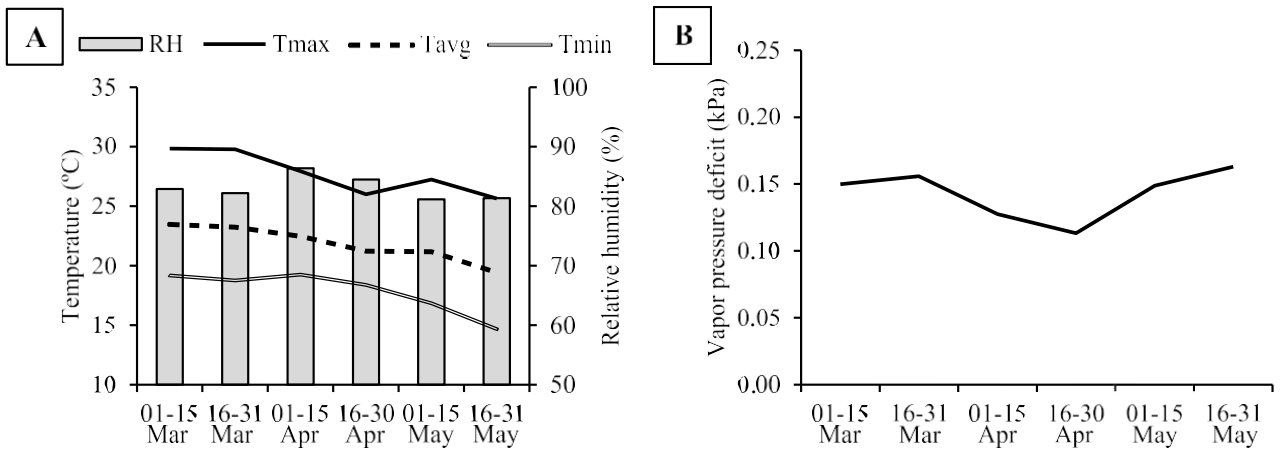

Figure 1. (**A**) Maximum (Tmax), average (Tavg), and minimum (Tmin) temperatures and relative air humidity (RH); and (**B**) vapor pressure deficit inside the greenhouse during the experimental period.

A completely randomized design was used, in a 4 × 4 factorial scheme, with four replicates and one plant per pot, thereby totaling 64 experimental units. One factor consisted of the following growing conditions: no abiotic stress (C, irrigation at 90% of pot capacity, without added saline solution), water deficit (WD, irrigation at 45% of pot capacity, without added saline solution), salinity (S, irrigation at 90% of pot capacity, with added saline solution), and WD + S (irrigation at 45% of pot capacity, with added saline solution). The other factor was SA application at 0, 1, 2, and 4 mM (Fig. 2).

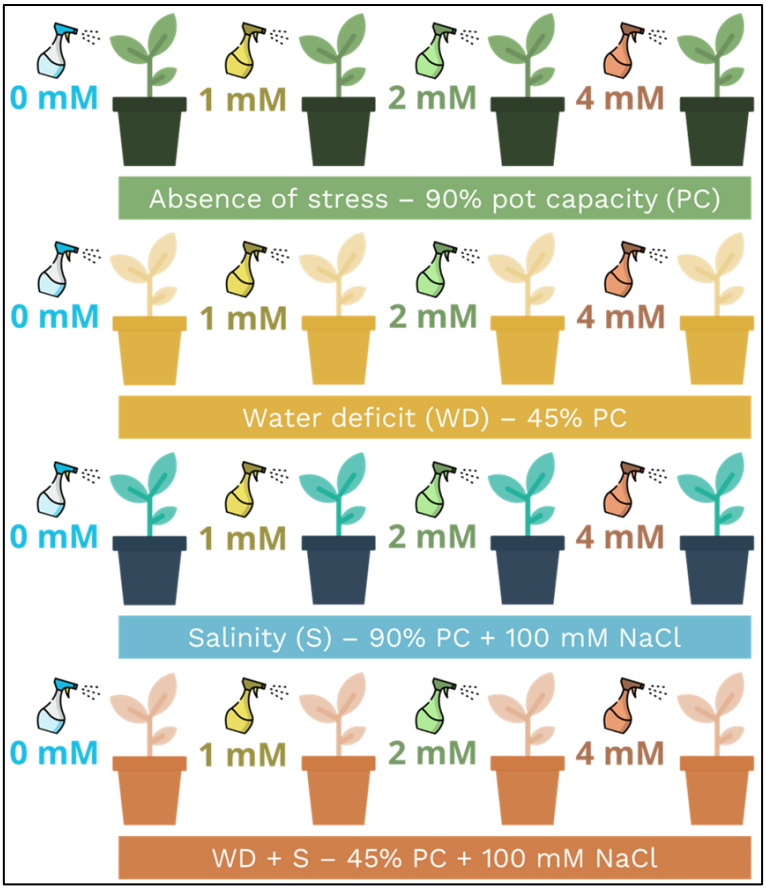

Figure 2. Treatment arrangement: young citrus plants pre-treated with SA application at 0, 1, 2, and 4 mM under different growing conditions.

3.2 Plant material and growing conditions

Seven-month-old citrus plants (*Citrus sinensis* orange tree scions, cv. 'Lima', grafted onto Swingle citrumelo rootstock) with approximately 60 cm in height, were planted in 25 dm^3 pots containing 20 kg of substrate prepared from Sandy Clay Loam soil and vermiculite (2:1). The substrate used had the following composition: pH (H_2O) – 5.1; P – 3 mg dm^{-3}; K^+ – 0.21 $cmol_c$ dm^{-3}; Ca^{2+} - 0.5 $cmol_c$ dm^{-3}; Mg^{2+} - 0.3 $cmol_c$ dm^{-3}; Al^{3+} - 0.4 $cmol_c$ dm^{-3}; H^+ - 2.4 $cmol_c$ dm^{-3}; Soil Base Saturation (SB) – 1.0 $cmol_c$ dm^{-3}; Cation Exchange Capacity (CEC) – 3.1 $cmol_c$ dm^{-3}; organic matter – 8 g dm^{-3}. When the seedlings were transplanted into the pots, fertilization was performed using a nutrient solution (Hoagland and Arnon, 1950) at 50% ionic strength to allow seedling acclimation to the new substrate. Substrate moisture was initially maintained at 90% of pot capacity, through daily irrigation.

The stress treatments were implemented at 35 days after transplanting (DAT). Irrigation regimes were established based on well-watered plants, with 90% of pot capacity, and water-deficit plants, based on stopped irrigation until 45% of pot capacity. Deionized water was supplied every 2 days, in an amount sufficient to maintain both irrigation regimes, using the gravimetric method (Soares et al., 2022). Salinity was induced by irrigation based on saline solution (100 mM NaCl) with

electrical conductivity maintained at 10.70 ± 0.06 dS m^{-1}. Soil electrical conductivity was also monitored by a conductivity meter (Tecnal-TEC 4MP) to ensure the same salinity levels for both well-watered and water-deficit plants.

Foliar SA sprayings at 0, 1, 2, and 4 mM (30 mL $plant^{-1}$) were applied starting at 20 DAT (before starting stress treatments) and replicated every seven days, totaling 10 sprayings. SA was previously dissolved in 5% (v/v) ethanol to increase the solubility in water. We concluded the experiment at 95 DAT, i.e., at 60 days after starting stress treatments, when plants were then completely removed from the pots for morphophysiological and biochemical measurements and analyses.

3.3 Leaf gas exchange and chlorophyll *a* fluorescence

Gas exchange measurement was performed on fully expanded and physiologically mature leaves of the shoot middle third at 8–10 a.m. to determine CO_2 assimilation (*A*) and transpiration (*E*) rates, stomatal conductance (g_s), and the ratio of internal and external CO_2 concentration (C_i/C_a). Water-use efficiency (*A*/*E*), intrinsic water-use efficiency (A/g_s), and instantaneous carboxylation efficiency (A/C_i) were subsequently estimated. The measurements were performed using an infrared gas analyzer (IRGA, LI-6400, LI-COR®, Nebraska/USA), based on constant photosynthetically active radiation (PAR) at 1000 µmol m^{-2} s^{-1} and CO_2 air concentration (reference air) at 420 µmol mol^{-1}. Chlorophyll *a* fluorescence was measured using the IRGA coupled to a leaf chamber fluorometer (6400xt, LI-COR®, Nebraska/USA). The minimum, maximum, and variable chlorophyll fluorescence (F_0, F_m, and F_v, respectively), electron transport capacity of photosystem II (PSII) (F_v/F_0), and maximum quantum yield of PSII (F_v/F_m) were recorded after 30 min of dark adaptation.

3.4 Plant water status

Leaf water potential (Ψ_w) was measured at ~5:00 a.m. (pre-dawn) and ~12:00 p.m. (midday) on physiologically mature leaves from the middle third of the shoot, using a Scholander pressure chamber (Model 1000, PMS) (Scholander et al., 1965). The difference between measured water potentials at 5:00 a.m. and 12:00 p.m. ($\Delta\Psi$) was calculated. The relative water content (RWC) was calculated using the formula: RWC (%) = [(FM – DM)/(TM – DM)] × 100, where FM, DM, and TM were the fresh, dry, and turgid masses of leaf blade discs, respectively (Weatherley, 1950).

3.5 Soluble and reducing sugar, amino acid, proline, and starch

The content of soluble sugar (SS), reducing sugar (RS), amino acid (AA), and proline was extracted from 200 mg of dried and ground material from physiologically mature leaves. The extraction was performed by adding 5 mL of 0.1 M KH_2PO_4 buffer (pH 7.4) three times, totaling a

final volume of 15 mL. After each buffer addition, the mixture was centrifuged for 45 minutes at 2,500 *g*, and the supernatant was collected for SS, RS, and AA quantifications. The resulting pellet was dried in a drying oven at 80 ºC for 48 hours for starch measurement.

Quantification of SS was carried out by adding 0.1 mL of the supernatant and 0.9 mL of water to 4.0 mL of anthrone reagent. The mixture was heated in a water bath at 100 ºC for 3 min. After cooling, absorbance was measured using a spectrophotometer at 620 nm, and the results were expressed as mmol SS g^{-1} dry mass (Yemm and Willis, 1954). For RS determination, an aliquot of 0.5 mL of the supernatant was added to a reaction medium containing 0.5 mL of dinitrosalicylic acid (DNS) and heated in a water bath at 100 ºC for 15 min. After cooling, 4.0 mL of water was added for dilution. Absorbance was read using a spectrophotometer at 540 nm, and the results were expressed as mmol RS g^{-1} dry mass (Miller, 1959).

Amino acid and proline content was determined by adding 0.1 mL of the supernatant and 0.9 mL of water to a reaction medium containing 0.5 mL of 0.2 M $Na_3C_6H_5O_7$ buffer (pH 5.0), 0.2 mL of ninhydrin (5% in methyl cellosolve), and 1 mL of 0.01 M KCN (2% in methyl cellosolve). The mixture was heated in a water bath at 100 ºC for 15 min. After cooling, 1.3 mL of 60% ethanol (v/v) was added. Amino acid and proline content was determined in a spectrophotometer at 570 nm and 440 nm, respectively. Results were expressed as mmol g^{-1} dry mass (Yemm and Coccking, 1955).

Starch content was obtained by the anthrone-sulfuric acid colorimetric method. A total of 5 mL of 0.5 M H_2SO_4 was added to 125 mg of the dry, ground material, which had been previously treated with 0.1 M KH_2PO_4 buffer (pH 7.4) to remove soluble sugar, and heated at 100 ºC for 1 hour. The volume was then adjusted to 250 mL with water. An aliquot of 1 mL of this solution was mixed with 5 mL of 5 mM anthrone reagent under cooling. The final solution was then heated at 100 ºC for 11 min. Absorbance was read by using a spectrophotometer at 620 nm. Results were expressed as g 100 g^{-1} dry mass (Soares et al., 2022).

3.6 Photosynthetic pigment

Photosynthetic pigment was extracted from leaf disc samples after immersion in 4 mL of dimethyl sulfoxide (DMSO) saturated with $CaCO_3$ (5% w/v) for 48 hours in the dark. The content of chlorophylls *a* and *b* and carotenoids was quantified using a spectrophotometer with absorbance readings at 665, 649, and 480 nm, respectively. The results were expressed as µg cm^{-2} (Wellburn, 1994).

3.7 Electrolyte leakage

Membrane permeability was measured by the electrolyte leakage rate. Leaf disc samples were immersed in 20 mL of deionized water at 25 ºC for 24 hours. The initial electrical conductivity of the

solution (C_1) was determined using a conductivity meter (Tecnal-TEC 4MP). Samples were heated at 100 ºC for 20 minutes and the final electrical conductivity (C_2) was measured after cooling to room temperature. Electrolyte leakage (EL) was calculated using the formula: EL (%) = (C_1/C_2) × 100 (Valentovic et al., 2006).

3.8 Reactive oxygen species

Hydrogen peroxide (H_2O_2) content was measured from samples of fresh foliar tissues (0.1 g) homogenized in a cold bath with 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The extract was centrifuged at 11,000 *g* for 15 min at 4 ºC. Then, an aliquot of 0.2 mL of the supernatant was added to 0.4 mL of 50 mM KH_2PO_4 buffer (pH 7.0) and 0.8 mL of 1 M KI, in the dark, for 10 min. Absorbance readings were taken using a spectrophotometer at 390 nm, and the results were expressed as mmol g^{-1} fresh mass (Velikova et al., 2000).

The quantification of superoxide anion (O_2^-) was performed using fresh leaf samples (0.1 g) mixed with 1.7 mL of 50 mM KH_2PO_4 buffer (pH 7.8). An aliquot of 0.3 mL of 1% (w/v) polyvinylpyrrolidone (PVP) was added, and the mixture was centrifuged at 11,000 *g* for 15 min under cooling (4 ºC). Next, 0.3 mL of the supernatant was added to 0.3 mL of 1% PVP and diluted with 0.7 mL of deionized water. After 30 min at room temperature, absorbance was read at 530 nm, and the results were expressed as µmol g^{-1} fresh mass (Yang et al., 2011).

3.9 Antioxidant enzyme activities and phenolic content

Superoxide dismutase (SOD; superoxide:superoxide oxidoreductase; EC 1.15.1.1), catalase (CAT; hydrogen-peroxide:hydrogen-peroxide oxidoreductase; EC 1.11.1.6), ascorbate peroxidase (APX; L-ascorbate:hydrogen-peroxide oxidoreductase; EC 1.11.1.11), and guaiacol peroxidase (GPX; phenolic donor:hydrogen-peroxide oxidoreductase; 1.11.1.7) activities were determined from fresh leaf tissue samples (0.2 g) homogenized in 1 mL of 50 mM KH_2PO_4 buffer solution (pH 7.8). The homogenates were centrifuged at 11,000 *g* for 20 minutes at 4 ºC. SOD activity was determined by defining one unit of SOD activity as the amount of enzyme required to inhibit nitroblue tetrazolium (NBT) photoreduction by 50% in the enzymatic extract of leaf tissues. An aliquot of 40 µL of the enzymatic extract was transferred to a reaction medium in the dark containing 50 mM NaH_2PO_4 buffer (pH 7.8), 1 mM EDTA, 13 mM L-methionine, and 1 mM NBT. The reaction was initiated by adding 1 mM riboflavin, and the reaction medium was exposed in a chamber under 15 W fluorescent lamps for 15 minutes. SOD activity was measured using a spectrophotometer at 560 nm and expressed as unit mg^{-1} protein (Beauchamp and Fridovich, 1971).

For CAT activity measurement, 20 µL of the enzymatic extract was transferred to a reaction medium containing 160 µL of 50 mM NaH_2PO_4 buffer (pH 7.0) and 20 µL of 0.3 M H_2O_2. CAT

activity was calculated as the rate of H_2O_2 decomposition at 240 nm over 3 minutes at 25 ºC (Madhusudhan et al., 2009). APX activity was measured using 10 µL of the enzymatic extract added to a reaction medium containing 50 mM NaH_2PO_4 buffer (pH 7.0), ascorbic acid, and 0.5 M EDTA. APX activity was calculated as the rate of ascorbate oxidation at 290 nm per minute at 25 ºC (Nakano and Asada, 1981). GPX activity was estimated in a reaction medium containing 50 mM Na_3PO_4 buffer (pH 6.0), 9 mM guaiacol, and 30% (v/v) H_2O_2. The kinetic evolution was measured over 1 min. One unit of GPX was defined as the amount of enzyme required to form tetraguaiacol per minute at 470 nm (Lin and Kao, 1999).

Phenolic content was measured using 200 mg of fresh leaf samples homogenized in 15 mL of 50% (v/v) methanol in an ultrasonic bath at 40 kHz for 20 minutes at room temperature. The mixture was centrifuged at 11,000 *g* for 15 min. After collecting the supernatant, 15 mL of 70% (v/v) acetone was added, and the previous process was repeated. Water was added to the combined supernatants to bring the volume to 50 mL. An aliquot of 1 mL of the obtained extract was mixed with 1 mL of Folin-Ciocalteu reagent, 2 mL of 20% (w/v) $CaCO_3$, and 2 mL of water. Absorbance readings were taken at 700 nm, 30 min after the addition of reagents (Larrauri et al., 1997).

3.10 Biometric attributes

The number of leaves was determined by counting all the leaves on each plant. The total leaf area (TLA) was estimated as the sum of the areas of all leaves on each plant, calculated by multiplying the length (L) by the maximum width (W) of each leaf, using the formula: $TLA = (L_1 \times W_1) + (L_2 \times W_2) + ... + (L_n \times W_n)$, following the method proposed for citrus (Mazzini et al., 2010). Foliar abscission was calculated as the percentage of leaves that underwent abscission, and leaf area reduction (LAR) was determined using the formula: LAR = average leaf area × number of abscised leaves.

The total root volume was measured with a graduated cylinder. Shoot (leaves and stem) and root dry mass and total biomass were determined after drying the plant material in an oven at 65 ºC until a constant mass was achieved. The root/shoot ratio was subsequently calculated.

3.11 Statistical analysis

The data were evaluated for homogeneity and normal distribution of residuals using the Shapiro-Wilk test. Subsequently, the data were statistically analyzed using analysis of variance (ANOVA) and multiple comparisons of means by Scott-Knott's test ($p<0.05$) with SISVAR statistical software (version 5.8). All data were further analyzed by multivariate analysis using the MetaboAnalyst platform, normalized through logarithmic and auto-scaling transformations.

Parameters with Variable Importance in Projection (VIP) scores greater than 1 were considered significant contributors to the PLS-DA model.

4 RESULTS

4.1 Leaf gas exchange

The results showed that stress conditions reduced *A* (Fig. 3A), *E* (Fig. 3B), and g_s (Fig. 3C), notably under S and WD+S, compared to the control. However, this reduction was attenuated by the application of 2 mM SA under WD and S, and 4 mM SA only under WD. Under WD+S, though, SA did not exhibit an attenuating effect on the reduction of *A*, *E*, and g_s. The C_i/C_a ratio decreased under WD and S compared to the control (Fig. 3D), but the SA application under WD alleviated this reduction. Under S, 2 mM and 4 mM SA led to a greater C_i/C_a ratio than 0 mM and 1 mM SA. Conversely, under WD+S, the C_i/C_a ratio was lower with SA application (1, 2, and 4 mM) compared to its absence (0 mM).

WUE and iWUE increased under WD and S individually, but remained stable under combined stresses (Fig. 3E, F). Under WD+S, the 2 mM SA application enhanced both WUE and iWUE (107.1% and 57.2%, respectively). Under S and WD+S, the instantaneous carboxylation efficiency decreased compared to no stress (Fig. 3G). However, under S, 2 mM SA increased the A/C_i ratio.

4.2 Plant water status

Pre-dawn measured Ψ_w had a decrease under all stress conditions, especially under WD (~150%) and WD+S (~85%), compared to no stress (Fig. 4A). Under WD, 2 mM and 4 mM SA application attenuated the Ψ_w decreasing, keeping it similar to Ψ_w under WD+S (−1.7 MPa). However, 1 mM SA application was not effective in preventing a sharp decrease in Ψ_w, neither under WD (−2.8 MPa) nor with no stress (−1.0 MPa). Midday measured Ψ_w was higher under S, compared to no stress, but reached its lowest level under WD (−2.9 MPa) (Fig. 4B). Measured Ψ_w variation from pre-dawn to midday ($\Delta\Psi$) showed a smaller Ψ_w decrease under S and WD+S, compared to WD and no stress (Fig. 4C). Under WD and no stress, SA application reduced $\Delta\Psi$ but had no effect under S and WD+S.

RWC decreased under all stress conditions, notably under WD (14.0%) (Fig. 4D). Under no stress, SA application reduced RWC. Moreover, no differences in RWC were observed in plants under S and WD+S treated with SA application compared to no stress.

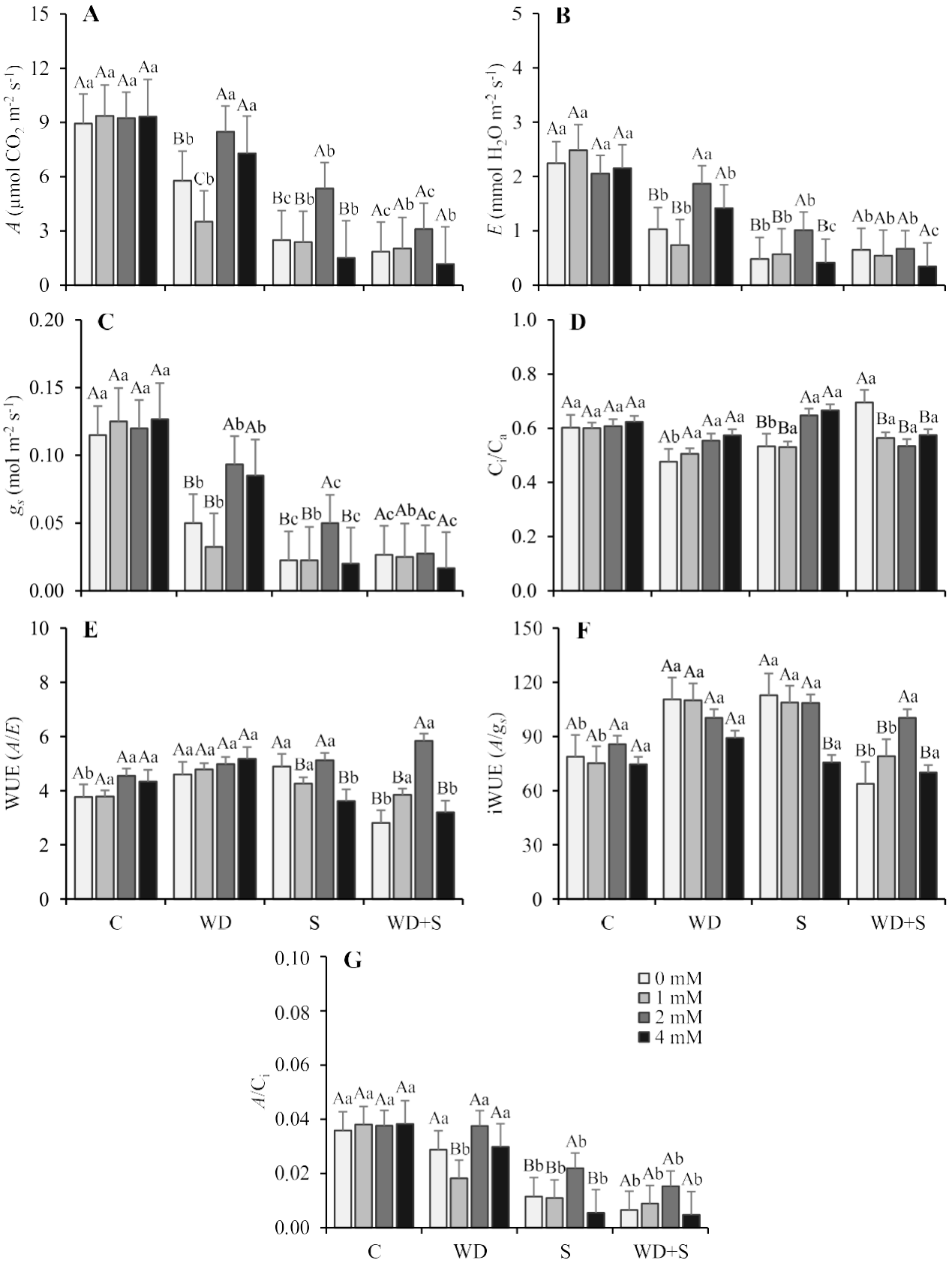

Figure 3. (**A**) CO_2 assimilation rate (*A*), (**B**) Transpiration rate (*E*), (**C**) Stomatal conductance (g_s), (**D**) Ratio of internal and external CO_2 concentration (C_i/C_a), (**E**) Water-use efficiency (WUE = *A*/*E*), (**F**) Intrinsic water-use efficiency (iWUE = A/g_s), and (**G**) Instantaneous carboxylation efficiency (A/C_i) of young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with different salicylic acid concentrations. Bars in each column indicate the standard error of means (n = 5). Uppercase letters compare salicylic acid concentrations within each

growing condition, while lowercase letters compare the growing conditions within each salicylic acid concentration, by Scott-Knott's test (p<0.05).

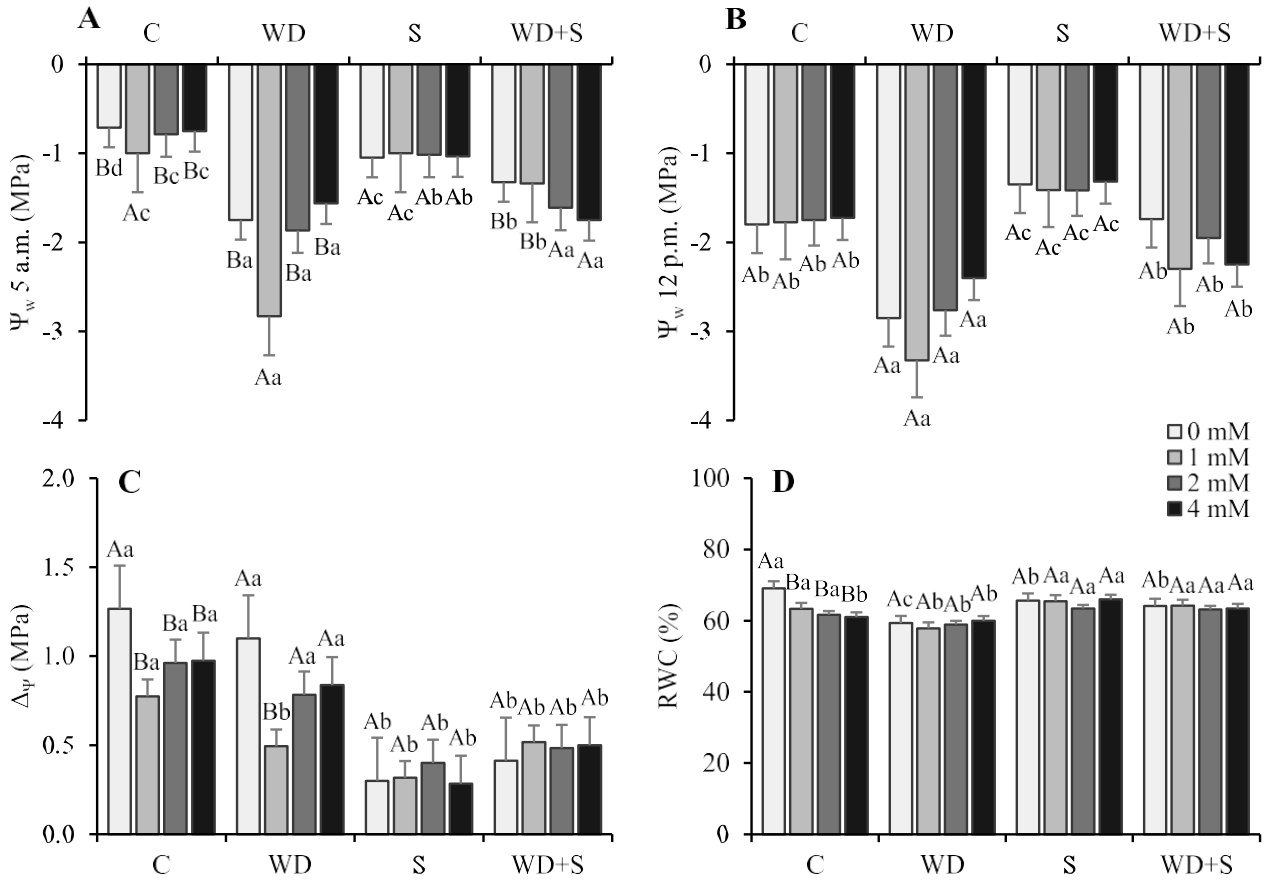

Figure 4. (**A**) Leaf water potential at pre-dawn (Ψ_w 5:00 a.m.), (**B**) Leaf water potential at midday (Ψ_w 12:00 p.m.), (**C**) Water potential difference between pre-dawn and midday ($\Delta\Psi$), and (**D**) Relative water content (RWC) of young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with different salicylic acid concentrations. Bars in each column indicate the standard error of means (n = 5). Uppercase letters compare salicylic acid concentrations within each growing condition, while lowercase letters compare the growing conditions within each salicylic acid concentration, by Scott-Knott's test (p<0.05).

4.3 Starch, soluble sugar, reducing sugar, amino acid and proline content

Starch content decreased under WD and WD+S (52.6% and 43.6%, respectively) (Supplementary Fig. S1A), but this decrease did not occur with 4 mM SA application under WD and 1 mM SA application under WD+S. Conversely, under S, SA application, regardless of concentration, decreased starch content. SS content increased under all stress conditions, compared to no stress (Supplementary Fig. S1B). This increase in SS content was enhanced by 2 mM SA application under WD (28.6%), WD+S (100.0%), and no stress (25.0%), but no effect of SA application was observed under S. RS content increased under WD and WD+S, notably under WD (1.6 mmol g^{-1} DM) (Supplementary Fig. S1D). This increase in RS content was enhanced by 2 mM SA application under no stress (37.5%) and WD+S (77.8%). Under S, 1 mM SA application elevated RS content to its

highest level (1.1 mmol g^{-1} DM). In contrast, under WD, RS content decreased with SA application, notably 4 mM SA.

Under all stress conditions, there was a decrease in both the starch/SS ratio (Supplementary Fig. S1C) and the starch/RS ratio − the latter notably under WD (76.6%) (Supplementary Fig. S1E). However, the 4 mM SA application prevented this decrease in both starch/SS and starch/RS ratios under WD and WD+S.

Amino acid content increased under WD (44.7%) and WD+S (63.2%) (Supplementary Fig. S1F). Under no stress, the highest amino acid content (5.6 mmol g^{-1} DM) was achieved with 2 mM SA application. Under WD, SA application reduced amino acid content, notably 4 mM SA. Under WD+S, amino acid content increased with 1 mM SA, whereas it decreased with 4 mM SA. Under S, amino acid content increased with SA application, notably 1 mM SA and 2 mM SA.

Proline content increased under all stress conditions, notably under WD+S (172.9%) (Supplementary Fig. S1G). Under S and no stress, the increase in proline content was enhanced by SA application, notably 4 mM SA and 2 mM SA, respectively. In contrast, under WD and WD+S, proline content was decreased with SA application, notably 4 mM SA.

4.4 Chlorophyll *a* fluorescence and photosynthetic pigment

Under S and WD+S, F_0 increased and F_v/F_m decreased (Fig. 5A, E). F_m, F_v, and F_v/F_0 decreased under all stress conditions, notably S and WD+S, compared to no stress (Fig. 5B, C, D). However, the 2 mM SA application prevented this reduction in F_m and F_v, whereas 4 mM SA simply prevented the reduction in F_m. Under WD and S, 2 mM SA prevented a reduction in F_v/F_0, but this application had no effect under WD+S. Under S, 1 mM and 2 mM SA application reduced F_0 and allowed the highest F_v/F_0 and F_v/F_m.

Under all stress conditions, a decrease in Total Chl (Fig. 6A), Chl *a* (Fig. 6B), Chl *b* (Fig. 6C), and Chl/Car ratio (Fig. 6E) was observed. These decreases were more prominent under stresses that included salinity (S and WD+S), in which a decrease in Car content was also observed (Fig. 6D). However, SA application prevented this decrease in Car content, in addition to preventing decreases in contents of Total Chl and Chl *b* under WD+S. Under WD+S and no stress, SA application increased the Chl/Car ratio. Under all stress conditions, 2 mM and 4 mM SA application prevented decreases in contents of Total Chl and Chl *a*. Under S, 2 mM SA application allowed the highest Chl *b* content (0.011 μg cm^{-2}).

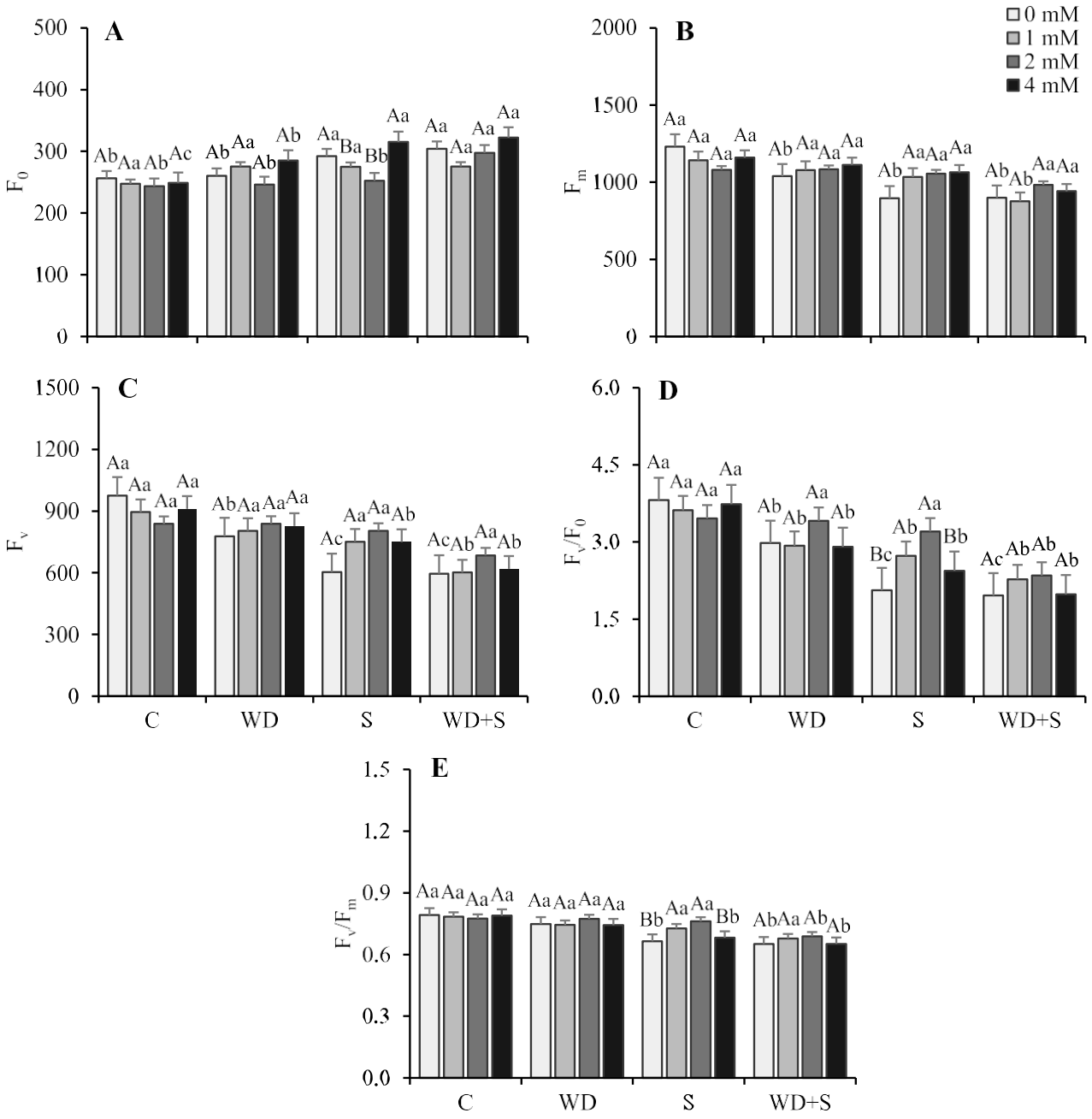

Figure 5. (**A**) Minimal chlorophyll fluorescence (F_0), (**B**) Maximum chlorophyll fluorescence (F_m), (**C**) Variable chlorophyll fluorescence (F_v), (**D**) Electron transport capacity of photosystem II (PSII) (F_v/F_0), and (**E**) Maximum quantum yield of PSII (F_v/F_m) of young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with different salicylic acid concentrations. Bars in each column indicate the standard error of means (n = 5). Uppercase letters compare salicylic acid concentrations within each growing condition, while lowercase letters compare the growing conditions within each salicylic acid concentration, by Scott-Knott's test (p<0.05).

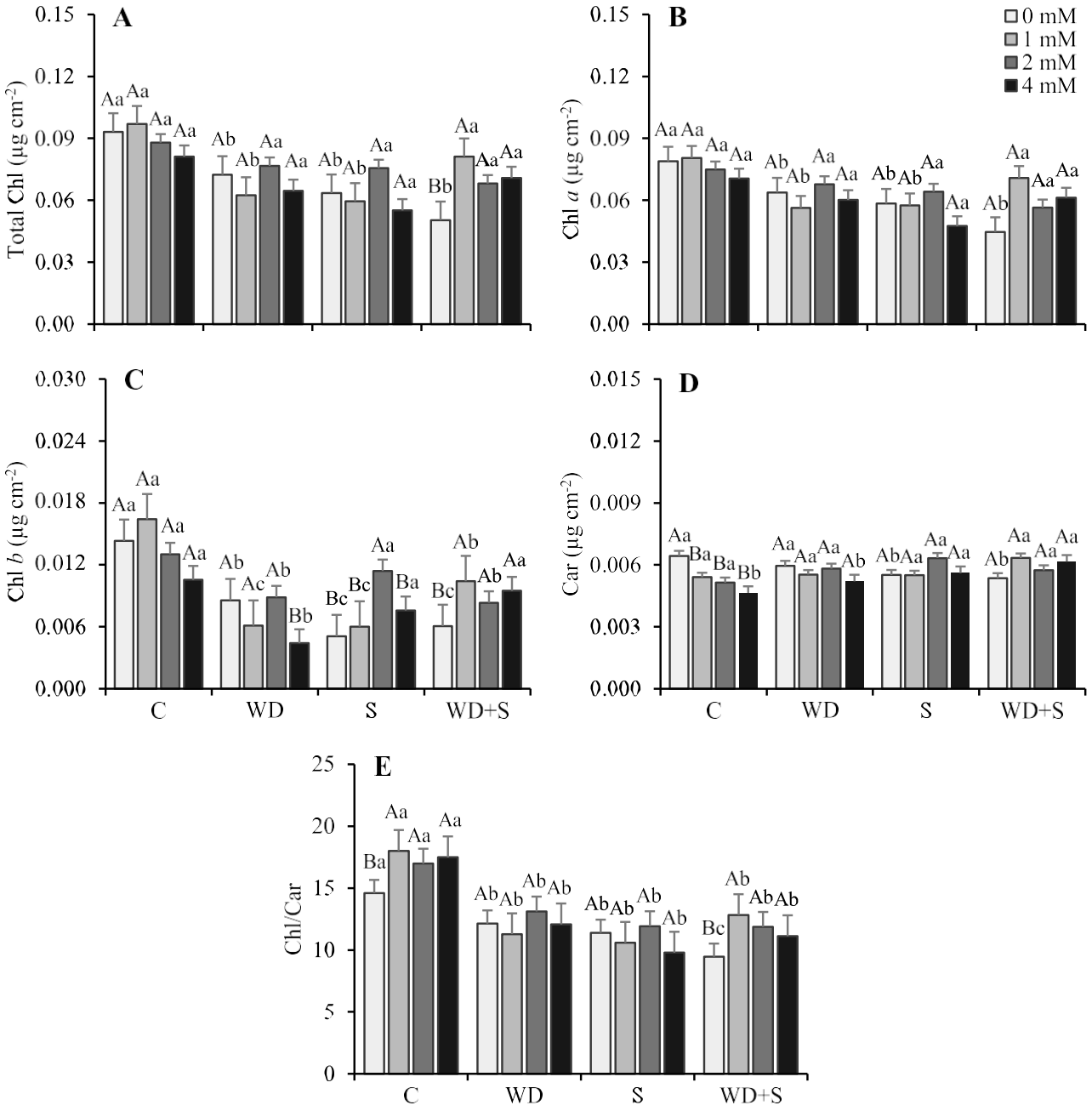

Figure 6. (**A**) Total chlorophyll (Total Chl), (**B**) Chlorophyll *a* (Chl *a*), (**C**) Chlorophyll *b* (Chl *b*), (**D**) Carotenoids (Car), and (**E**) Chlorophyll/Carotenoids ratio (Chl/Car) of young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with different salicylic acid concentrations. Bars in each column indicate the standard error of means (n = 5). Uppercase letters compare salicylic acid concentrations within each growing condition, while lowercase letters compare the growing conditions within each salicylic acid concentration, by Scott-Knott's test (p<0.05).

4.5 Electrolyte leakage and reactive oxygen species accumulation

Electrolyte leakage increased under S and WD+S. Under S, this increase was smaller (59.2%) with 2 mM SA application and larger (83.2%) with 4 mM SA. Under WD+S, electrolyte leakage was attenuated by 2 mM and 4 mM SA application (Fig. 7). O_2^- content increased only under WD (Fig. 8A), and this increase was attenuated by SA application, notably 4 mM SA. H_2O_2 content increased

under WD and S (Fig. 8B). Under WD, this increase was attenuated by 2 mM SA and, notably, 4 mM SA, whereas under S, 2 mM SA prevented an increase in H_2O_2 content.

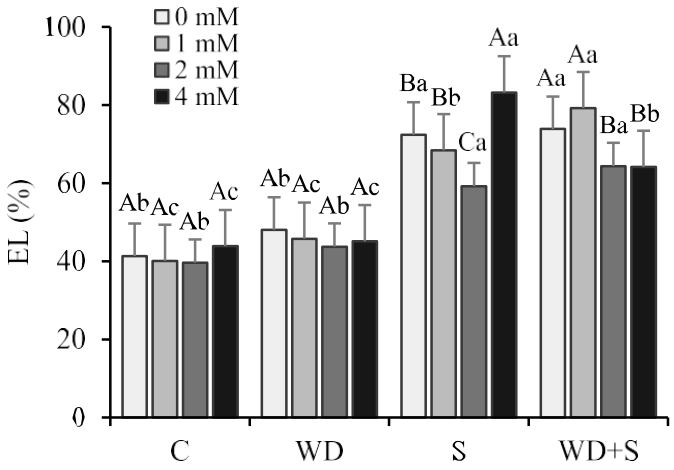

Figure 7. Electrolyte leakage (EL) of young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with different salicylic acid concentrations. Bars in each column indicate the standard error of means (n = 5). Uppercase letters compare salicylic acid concentrations within each growing condition, while lowercase letters compare the growing conditions within each salicylic acid concentration, by Scott-Knott's test (p<0.05).

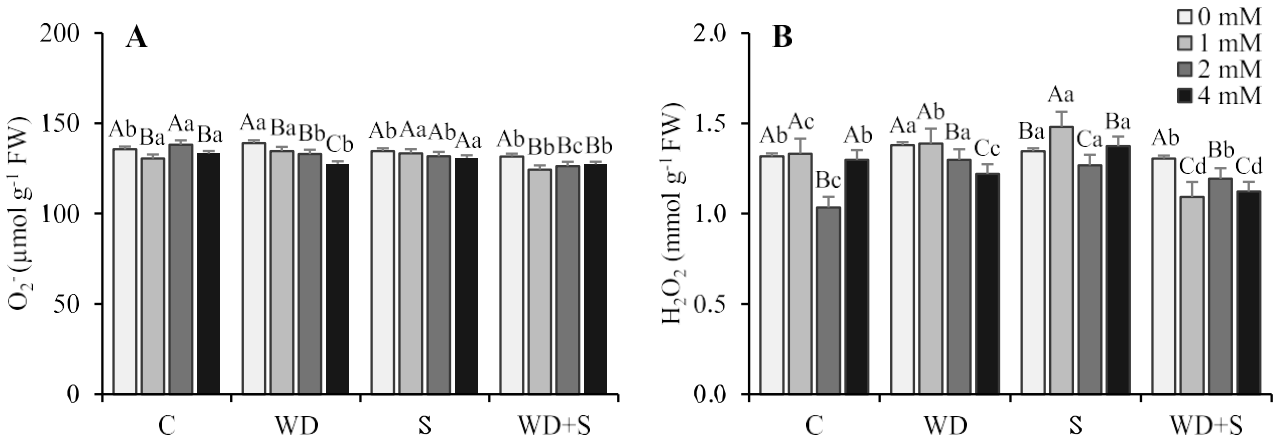

Figure 8. (**A**) Superoxide anion (O_2^-) and (**B**) Hydrogen peroxide (H_2O_2) of young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with different salicylic acid concentrations. Bars in each column indicate the standard error of means (n = 5). Uppercase letters compare salicylic acid concentrations within each growing condition, while lowercase letters compare the growing conditions within each salicylic acid concentration, by Scott-Knott's test (p<0.05).

4.6 Antioxidant enzyme activity and phenolic content

Regarding antioxidant enzyme activity, under WD, there was an increase in SOD and APX; under S, APX and GPX were increased; and under WD+S, only SOD increased (Fig. 9A, B, C). SA application enhanced increases in SOD activity under S and no stress, and in GPX activity under WD and WD+S. In contrast, SA application caused decreased GPX activity under S. Phenolic content decreased under stress conditions (Fig. 9D), but this effect was reversed with SA application. Under

WD, phenolic content increased with 2 mM and 4 mM SA, while under S, the greatest increase (6.9 mg g^{-1} FW) occurred with 2 mM SA. Under WD+S, phenolic content increased notably with 4 mM SA.

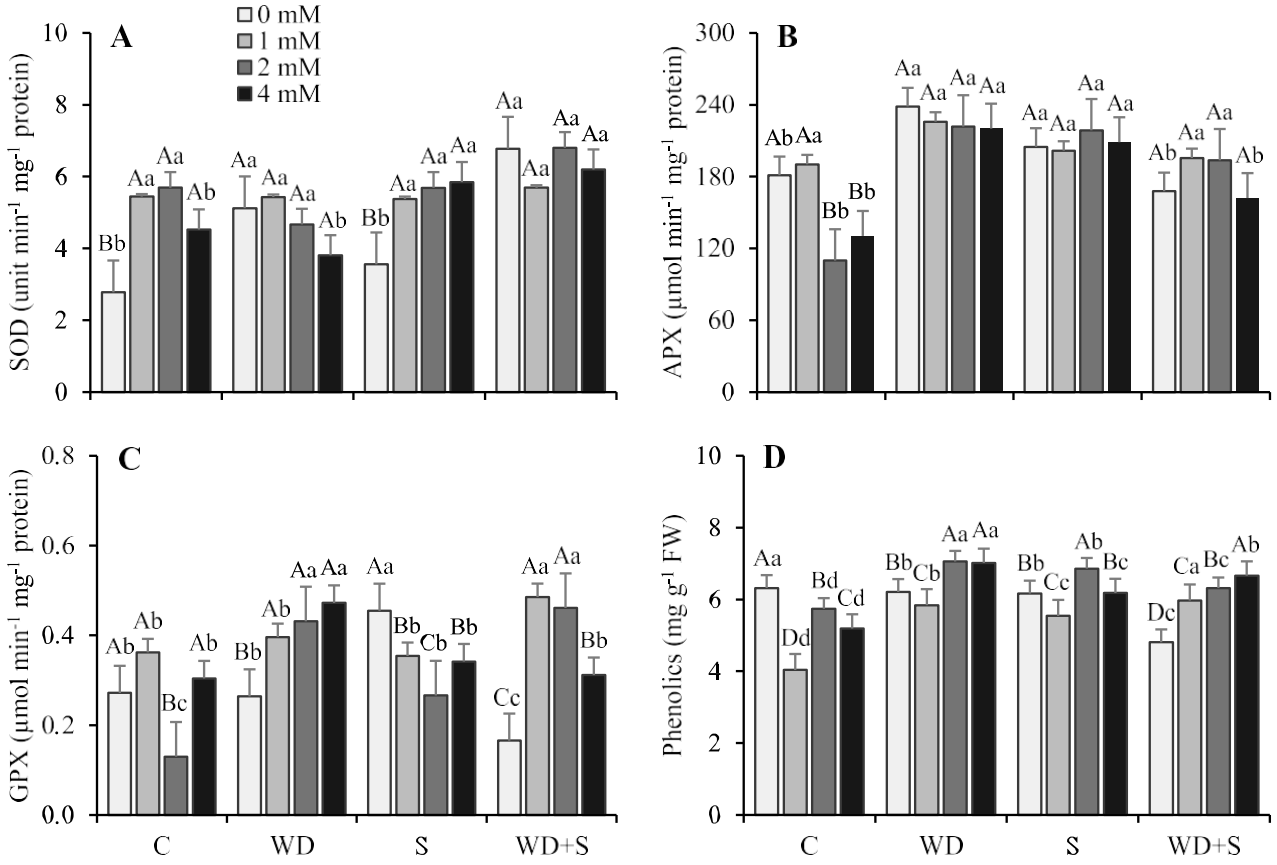

Figure 9. (**A**) Superoxide dismutase (SOD), (**B**) Ascorbate peroxidase (APX), (**C**) Guaiacol peroxidase (GPX), and (**D**) Total phenolics of young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with different salicylic acid concentrations. Bars in each column indicate the standard error of means (n = 5). Uppercase letters compare salicylic acid concentrations within each growing condition, while lowercase letters compare the growing conditions within each salicylic acid concentration, by Scott-Knott's test (p<0.05).

4.7 Multivariate analysis

Multivariate analyses combining all information provided a comprehensive view of the observed data patterns from the specific analyses. Gas exchange parameters (A, E, g_s, and A/C_i) and chlorophyll fluorescence indicators (F_m, F_v, F_v/F_0, F_v/F_m) were all reduced under stress conditions, especially under S and WD+S (Fig. 10A). The 2 mM SA application mitigated this reduction under WD and S stresses alone, but not under these stresses simultaneously. WUE and iWUE increased under WD and S alone, but not under WD+S. However, the 2 mM SA application increased WUE and iWUE under WD+S. F0 and electrolyte leakage also increased under S and WD+S, but this effect was attenuated with 2 mM SA application.

Gas exchange and chlorophyll fluorescence attributes were strongly associated with non-stressed plants, reflecting a high positive correlation between these parameters (Fig. 10B). On the other hand, WUE and iWUE are best related to WD, while F_0 and electrolyte leakage were associated with S and WD+S. Several physiological parameters, such as electrolyte leakage, gas exchange, and chlorophyll fluorescence attributes, had VIP scores higher than 1, indicating a very specific behavior under each stress condition, which improves their discrimination (Fig. 10C).

The number of leaves, total leaf area, shoot dry mass, root dry mass, root volume, and total biomass decreased under all stress conditions, while leaf abscission and leaf area reduction increased, especially under S and WD+S (Supplementary Fig. S2A). Application of 2 mM SA increased root volume, shoot and root dry mass, and total biomass in plants under no stress and WD. Conversely, under S and WD+S, there was an increase in foliar abscission and leaf area reduction with SA application, notably 4 mM SA, increasing root/shoot dry mass ratio. Foliar abscission, leaf area reduction, and root/shoot dry mass ratio were strongly associated with S and WD+S stresses (Supplementary Fig. S2B). Regarding biometric attributes, the dry mass of shoots and roots, as well as total biomass, had VIP scores greater than 1, revealing a well-separated pattern for each stress condition (Supplementary Fig. S2C).

Soluble and reducing sugar, amino acid, and proline content increased under WD and WD+S, while starch content decreased (Supplementary Fig. S3A, B). Chlorophyll and carotenoid content decreased under all stress conditions, especially under S and WD+S. H_2O_2 and O_2^- levels increased notably under WD. Each stress condition showed an increase in the activity of specific antioxidant enzymes: WD increased SOD and APX, S increased APX and GPX, and WD+S increased SOD and CAT activities, while phenolics were reduced in all stress conditions compared to the absence of stress. Application of 2 mM SA increased soluble sugar content under WD and WD+S, while decreasing ROS levels and increasing photosynthetic pigment and phenolic content under all stress conditions. SA application also caused an overall increase in antioxidant enzyme activities: SOD, under S; GPX, under WD and WD+S; and CAT, under WD and S. Biochemical traits such as soluble sugar, proline, chlorophyll, and O_2^- content, as well as CAT activity, had VIP scores greater than 1 (Supplementary Fig. S3C).

Treatments that included salinity were separated from those under WD and no stress by the first component, indicating that the adverse effects observed under WD+S are more associated with salt accumulation than with water deficit per se (Fig. 11A). However, the simultaneous occurrence of water deficit along with salinity may exacerbate metabolic damage. The most impactful morphophysiological and biochemical traits, with VIP scores greater than 1, included gas exchange (A, E, g_s, and A/C_i) and chlorophyll fluorescence (F_0, F_v, F_v/F_0, and F_v/F_m) parameters, water potential difference between pre-dawn and midday ($\Delta\Psi$), electrolyte leakage, shoot dry mass, total biomass,

and soluble sugar, proline, and $O_2^{\cdot-}$ content (Fig. 11B). They were the most determining morphophysiological and biochemical attributes for plant responses to the stress conditions established in this study. The CO_2 assimilation rate (*A*) was positively correlated with other gas exchange parameters (*E*, g_s, A/C_i, and iWUE), chlorophyll fluorescence indicators (F_m, F_v, F_v/F_0, and F_v/F_m), chlorophyll content, photosynthetic area (number of leaves and total leaf area), and biomass production (shoot and root dry mass, and total biomass), however, it was negatively associated with the increase in osmolyte content (soluble sugar and proline), photosynthetic area reduction (foliar abscission and leaf area reduction), and indicators of tissue damage (F_0 and electrolyte leakage) (Fig. 11C), thus showing that changes in these parameters strongly affect or are induced by the plant photosynthetic capacity.

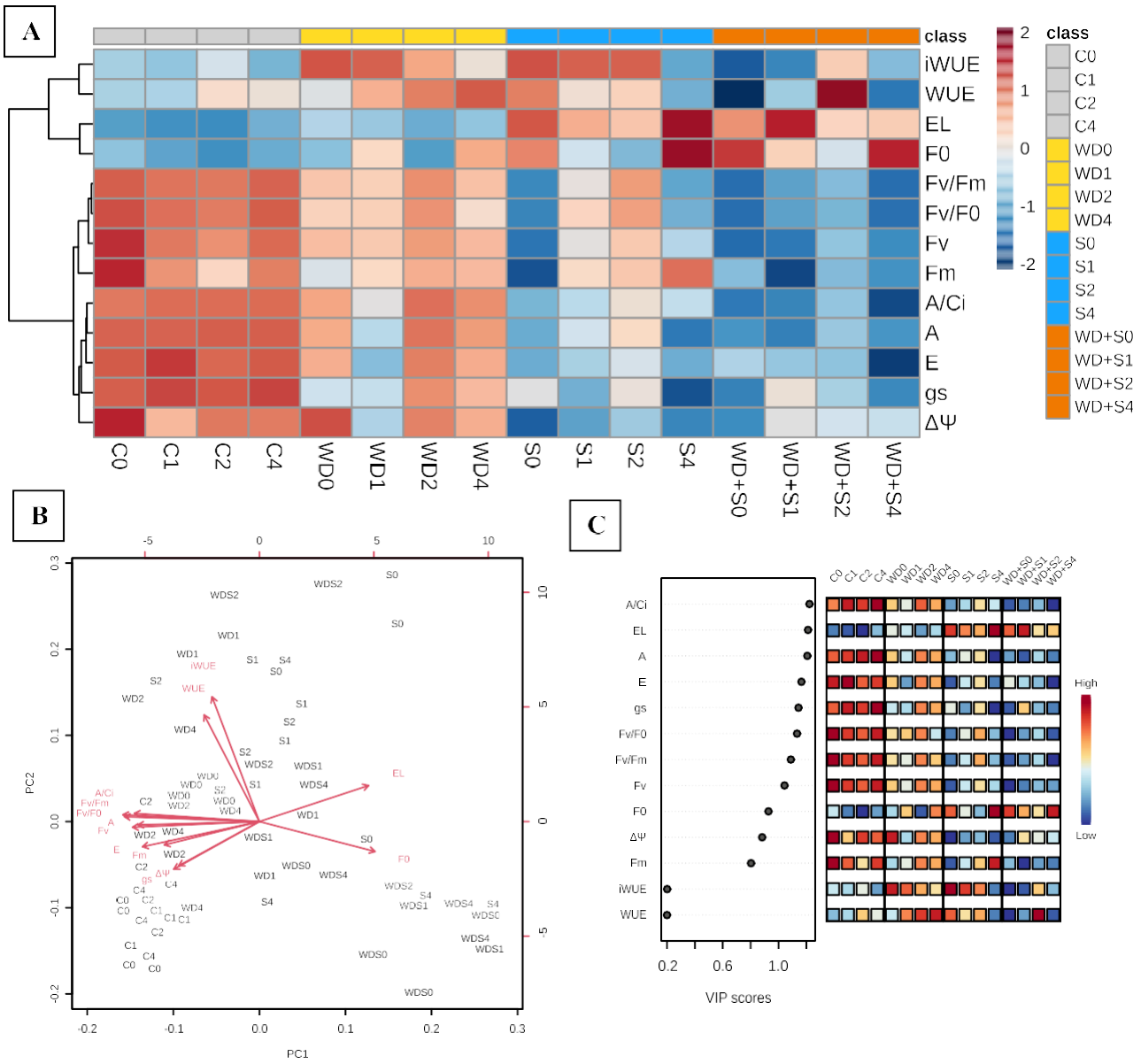

Figure 10. (**A**) Heatmap representation, (**B**) Principal Component Analysis Biplot (PCA Biplot), and (**C**) Variable Importance in Projection (VIP) scores of physiological traits in young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S,

45% pot capacity + 100 mM NaCl) conditions and treated with 0, 1, 2, and 4 mM salicylic acid concentrations. The data was normalized by using logarithmic and auto-scaling transformations in the MetaboAnalyst platform (*n* = 5).

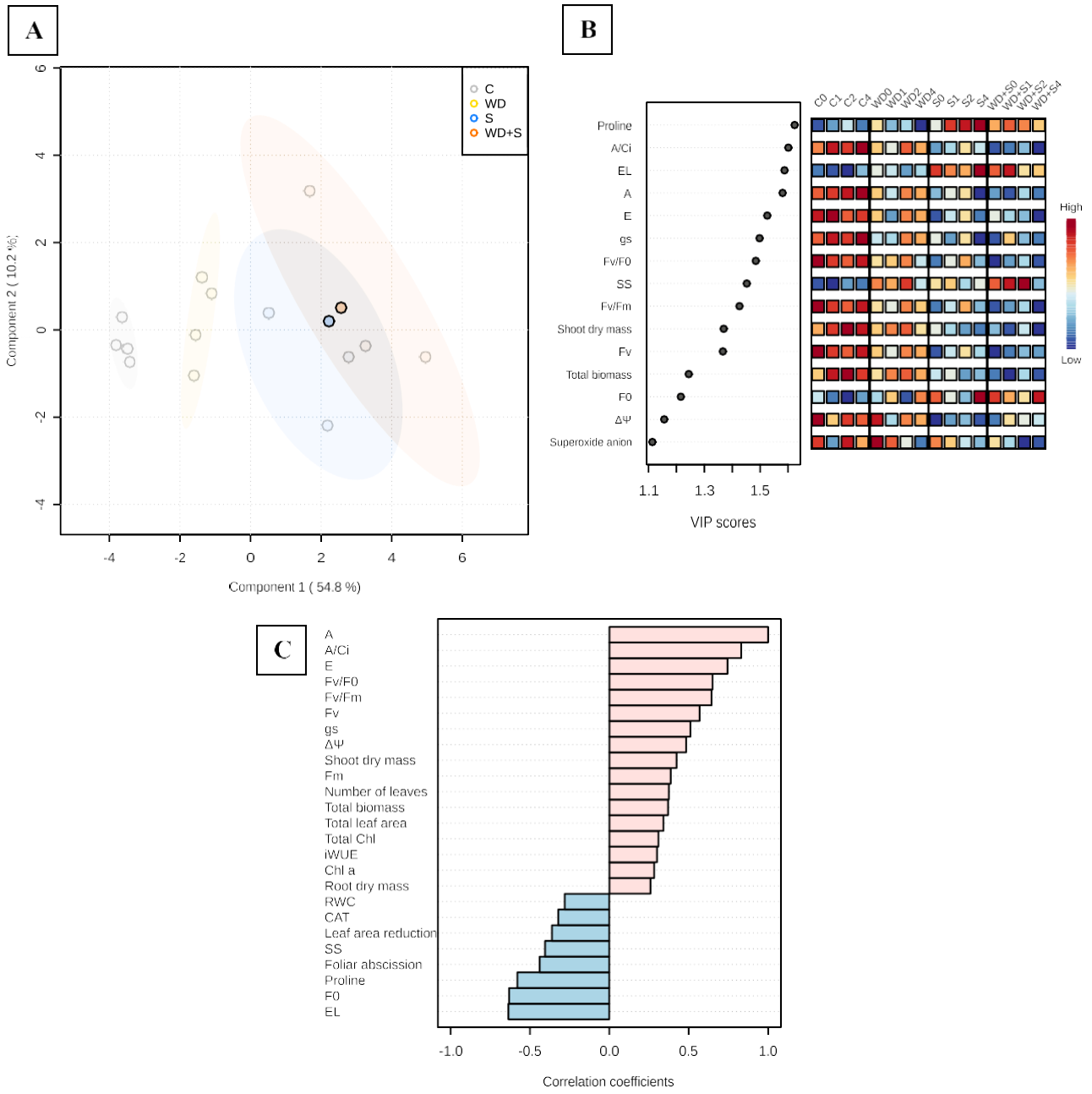

Figure 11. (**A**) Partial least square-discriminant analysis (PLS-DA), (**B**) Variable Importance in Projection (VIP) scores of the PLS-DA model, and (**C**) Top compounds correlated with CO_2 assimilation (*A*) in young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions. Only traits with VIP scores higher than 1 were considered to have an important impact on the PLS-DA model. The data was normalized by using logarithmic and auto-scaling transformations in the MetaboAnalyst platform (*n* = 5).

5 DISCUSSION

5.1 Metabolic responses of citrus plants common to both water deficit and salinity

Research on the effects of abiotic stresses on agricultural crops often focuses on maintaining high productivity under adverse conditions. In this study, we focused on reporting and understanding the morphophysiological and biochemical changes that occur during the early growth of young citrus plants exposed to water deficit and salinity – two common challenges worsened by climate changes that occasionally affect seedlings after planting in the field. Understanding metabolic adjustments and the mechanisms underlying plant acclimation to different stress conditions is essential for developing genetically enhanced materials with greater tolerance. The availability of a diverse range of tolerant genotypes enables the selection of ideal materials for specific situations while minimizing the risks of endemic outbreaks of pests and diseases. Furthermore, SA has proven effective in inducing and enhancing the acclimation responses of plants to stress, thereby mitigating the damage incurred during this critical phase of orchard establishment. Thus, SA provides a viable approach for managing crops under unfavorable edaphoclimatic conditions.

Both water deficit and salinity conditions negatively affected young citrus plants, causing osmotic stress that led to oxidative damage and reduced gas exchange. Stomata are key regulators of photosynthesis and transpiration, responding to various environmental stimuli. During gas exchange measurements, the VPD was close to the average observed throughout the experimental period. Therefore, we concluded that the VPD was not high enough to negatively influence gas exchange (Fig. 1B), which aligns with the simulation by Pieruschkaa et al. (2010) regarding gas exchange responses to varying VPD levels.

The plant's ability to absorb water and rehydrate its tissues overnight is regulated by soil water availability and osmotic potential. Beyond water deficit, salt accumulation in the root zone creates an osmotic gradient that hinders water uptake, leading to tissue dehydration and osmotic stress. All stress conditions reduced Ψ_w at pre-dawn and RWC, with a particularly pronounced reduction under WD. The decrease in guard cell turgor due to water deficit or salinity stress leads to partial or complete stomatal closure, which negatively affects gas exchange and reduces CO_2 diffusion into the leaf, thereby limiting its availability for the Calvin cycle (Chaudhry and Sidhu, 2021). Moreover, the behavior of internal CO_2 concentration is closely related to stomatal opening and the plant's capacity to perform photosynthesis. The C_i/C_a ratio decreased under isolated stress conditions, indicating that less CO_2 is being internally fixed. This primarily occurs because the reduction in stomatal conductance limits the environmental CO_2 entry into the leaf, resulting in a lower internal CO_2 concentration relative to the atmospheric concentration. Although reduced stomatal conductance negatively impacts photosynthesis, it also acts as a stress-avoidance mechanism: stomatal closure

limits the CO_2 assimilation rate but minimizes water loss through transpiration, contributing to improved WUE and iWUE.

In addition to diminished gas exchange, water deficit and salinity also caused oxidative damage. Reduced F_m and F_v/F_0 under stress conditions suggest lower efficiency in converting light energy into chemical energy, meaning that more energy is dissipated as fluorescence rather than being used in electron transport. This indicates that the plant is having difficulty capturing and efficiently using light energy in PSII, implying a reduction in the plant's photosynthetic capacity (Tang et al., 2021). This reduction occurs because PSII reaction centers are structurally damaged or partially inactive, resulting in an inability to reach maximum fluorescence under a saturating light pulse. A lower F_m can also denote that the plant is increasing energy dissipation as heat (a non-photochemical process) as a protective response to avoid greater damage to PSII components.

Damage to the photosynthetic apparatus is evidenced by decreased levels of Total Chl, Chl *a*, and Chl *b* content. Under stress, ROS accumulation compromises cellular components, including photosynthetic pigments, leading to their degradation and decreased levels. In addition to the oxidative damage caused by ROS accumulation, stress conditions also inhibit chlorophyll biosynthetic pathways due to the reduced availability of precursors, such as 5-aminolevulinic acid (ALA). The reduction in chlorophyll content impairs the photochemical reactions and reduces the photosynthetic rate (Ali et al., 2022). As chlorophylls are the primary pigments involved in the conversion of light energy into chemical energy, their degradation affects the ATP and NADPH production, an essential process for the plant's growth, development, and survival.

All stress conditions reduced the Chl/Car ratio. Chlorophylls are more susceptible to degradation, as they are directly related to the photosynthetic reaction centers and thus more exposed to oxidative damage caused by ROS accumulation. Conversely, carotenoids play a more specific role in light capture and photoprotection by acting as antioxidants, neutralizing free radicals and converting excess absorbed light energy into heat (Srivastava, 2021). As a result of this differential degradation, the Chl/Car ratio decreases, as chlorophylls are degraded faster than carotenoids. This process is characterized as an acclimation strategy that enables the plant to adjust its light-harvesting antenna to optimize light capture and reduce the risk of photoinhibition at the reaction centers by preventing the overproduction of free radicals. Therefore, this mechanism contributes to the stability and integrity of the thylakoid membrane, protecting chlorophylls and chloroplasts from oxidative damage.

5.2 Specific responses to water deficit conditions

Under conditions of water restriction (WD and WD+S), the decrease in Ψ_w was accompanied by a rise in soluble and reducing sugar, amino acid, and proline content, concomitantly with a

reduction in the starch amount. In adverse situations, starch – the carbohydrate form stored in plants – can be remobilized and degraded into simpler sugars. Soluble sugars, like sucrose, and reducing sugars, such as glucose and fructose, act as osmolytes, helping to regulate cellular osmotic potential and maintain cell turgor, thereby preserving cell integrity and preventing dehydration. Additionally, starch hydrolysis quickly provides energy to sustain metabolic processes and vital functions under stress (Liu et al., 2022). Although the reduction in starch content may be attributable to limitations in CO_2 absorption and assimilation, leading to decreased carbohydrate synthesis, it could also result from enhanced hydrolysis. The reduction in starch/SS and starch/RS ratios in stressed plants suggests that starch is being degraded into soluble sugar. This response indicates that the plants are converting long-term carbohydrate stores into more readily usable forms to try to osmoregulate and withstand stress, especially under water deficit.

Amino acid also participates in osmotic adjustment, as well as in ROS detoxification, intracellular pH regulation, and sustaining alternative mitochondrial respiratory pathways. In response to osmotic stress, proteins can be degraded into amino acids to decrease cell water potential, preventing dehydration. In addition to lowering water potential, proline acts as an osmoprotectant by stabilizing cell membranes, preventing protein denaturation, and neutralizing ROS (Heinemann and Hildebrandt, 2021). Proline was identified as the parameter with the highest VIP score in the PLS-DA analysis, exhibiting more pronounced accumulation under WD+S and proving to be a key osmoregulatory molecule and oxidative protector in the acclimation of citrus plants.

Osmoregulation refers to the capacity of plant cells to accumulate solutes and use them to lower water potential during periods of osmotic stress, which facilitates water uptake and reduces water loss through transpiration, ensuring that the relative water content remains practically unchanged (Ozturk et al., 2020). This process attenuates the adverse effects of water restriction on the plant, particularly on growth and stomatal regulation, since the reduction in leaf water potential facilitates water influx into the cell, helping to preserve cell turgor. Although the accumulation of soluble sugars, amino acids, and proline, simultaneously with the reduction in starch content, indicates an effort at osmoregulation, this response was insufficient to maintain the relative water content in stressed plants, failing to prevent stomatal closure, as evidenced by the reduction in *A*, *E*, and g_s.

5.3 Salinity conditions intensify damage to the photosynthetic apparatus

Although both individual stresses lowered gas exchange and chlorophyll content, salinity caused greater inhibition of photosynthetic parameters than water deficit, affecting photosynthesis by a combination of osmotic stress, toxicity, and nutritional imbalance, which compromises membrane stability of photosynthetic pigments and the activity of photosynthesis-related enzymes (Hao et al.,

2021). At noon, S maintained higher Ψ_w values compared to the absence of stress and a very low $\Delta\Psi$, indicating that salt accumulation in plant cells reduced water loss and minimized Ψ_w variation throughout the day, due to a lesser stomatal opening. As a result, stomatal conductance, transpiration, and CO_2 assimilation rates were reduced to a greater extent.

Beyond the reduction in stomatal conductance, the plant's biochemical capacity to fix CO_2 can be further impaired by the decreased activity of photosynthesis-related enzymes, such as RuBisCO, which catalyzes CO_2 fixation into 3-phosphoglycerate (Zahra et al., 2022). Salinity reduced the A/C_i ratio, both individually and in combination with water deficit. This indicates a reduction in the plant's photosynthetic ability, mainly due to decreased RuBisCO activity, which reflects a lower plant efficiency in converting the available CO_2 into carbohydrates. The excess of Na^+ and Cl^- in plant cells disturbs the ionic balance, impairing the absorption of essential nutrients, such as K^+ and Mg^{2+} (Liu et al., 2022). The Mg^{2+}, in particular, is a cofactor for RuBisCO enzymatic activity. Its deficiency caused by the excess of Na^+ compromises CO_2 fixation, decreasing the photosynthetic rate. Additionally, the ionic imbalance changes chloroplast stroma pH, harming RuBisCO catalytic capacity.

The oxidative damage caused by salt accumulation was also more intense, leading to a greater degradation of photosynthetic pigments, as evidenced by a larger reduction in Chl *b* content, F_v, F_v/F_0, F_v/F_m, and increased F_0. Both an increase in F_0 and a decrease in F_v, F_v/F_0, and F_v/F_m reflect diminished maximum potential efficiency of PSII and indicate photochemical inhibition due to damage to PSII components, causing energy to be released as fluorescence (Chen et al., 2021). This suggests that ion accumulation in the chloroplasts, as well as increased ROS, dissociation of key proteins, and disruption of the thylakoid membrane, affected the organization and function of chlorophyll and the antenna complex, limiting PSII photochemical efficiency in utilizing absorbed light energy for electron transport, which directly impacts photosynthesis and chemical energy generation.

Along with chlorophyll degradation, carotenoid content was also reduced under S and WD+S. Although abiotic stresses usually increase carotenoid levels as a protective mechanism, in cases of severe or prolonged stress, carotenoid degradation can occur, especially when the plant's antioxidant defense capacity is exceeded (Srivastava, 2021). In accordance with the behavior of photosynthetic pigments, electrolyte leakage – an important indicator of cell membrane integrity – increased markedly under growth conditions with salinity. ROS accumulation causes lipid peroxidation, damage to membrane proteins, and alterations in phospholipid structure, resulting in loss of membrane selectivity and increased permeability, evidenced by the leakage of ions from the cytoplasm to the extracellular medium (Sarwar et al., 2022). This contributes to the loss of cellular homeostasis and osmotic control, leading to cellular dehydration. These damages to the

photosynthetic apparatus consequently reflected in lower biomass production under salinity conditions, which allows us to conclude that citrus plants are especially sensitive to salt stress, indicating that high salinity levels are more harmful to the photosynthesis of citrus plants than a single water deficit. This idea is supported by the maintenance of A/C_i, F_0, F_v/F_m, carotenoids, and electrolyte leakage values under WD conditions.

5.4 The combination of water deficit and salinity exacerbates stress

Plants under combined stresses showed a response pattern similar to that of salinity stress alone, but its magnitude was greater. According to the PLS-DA analysis, the metabolic responses of plants under S and WD+S diverged substantially from those under WD and no stress. Therefore, the adverse effects of combined stresses were primarily due to salt accumulation rather than water deficit, suggesting that salinity is the major stress factor under WD+S conditions. Nevertheless, the occurrence of water deficit further exacerbated the adverse effects of salinity, intensifying metabolic damage. Studies on simultaneous water deficit and heat stresses in cowpea (Kumar et al., 2022) and soybean plants (Vital et al., 2022) have also identified a primary active stress that, when combined with another stressor, amplified the overall adverse effects.

5.5 ROS accumulation and antioxidant enzyme activation depend on the type of stress

Under stress conditions, the ability to balance ROS synthesis and quenching is decisive for plant tolerance to severe oxidative damage. As a defense mechanism to prevent ROS accumulation, plants are endowed with enzymatic and non-enzymatic antioxidants that mitigate oxidative damage (Kumar et al., 2022). Each stress condition induced increased activity of specific antioxidant enzymes. This differential activation of antioxidant enzymes depends on the major ROS forms generated under each type of stress, as well as the role played by each of these enzymes. This could be observed in plants under WD, where the oxidative damage caused to chloroplasts and mitochondria led to a pronounced increase in O_2^- synthesis. Highly reactive species such as O_2^- can directly damage cellular components, such as proteins, lipids, and nucleic acids, compromising the integrity of the cell membrane (Thiruvengadam et al., 2024). Since SOD catalyzes the dismutation of O_2^- into molecular oxygen and H_2O_2, which is a less reactive ROS, SOD activity increased. To quench H_2O_2, APX activity also increased, since this enzyme uses ascorbate as an electron donor to catalyze the conversion of H_2O_2 into water and oxygen.

Under S stress, the major ROS generated was H_2O_2. At moderate levels, H_2O_2 plays a role as a signaling molecule, regulating plant responses to environmental stimuli. However, under stress conditions, its accumulation causes oxidative damage (Niu et al., 2023). H_2O_2 quenching can be performed by both APX and GPX, using distinct substrates. In the present study, the activation of

these two peroxidase enzymes suggests a metabolic effort to effectively quench the H_2O_2 accumulated in various cellular compartments. Conversely, under WD+S, an increase in SOD and CAT activity was observed. SOD activation aims to prevent O_2^- accumulation, while CAT activation is decisive to decompose H_2O_2 into water and oxygen, since CAT is more effective than APX and GPX under extreme stress conditions, when ascorbate and guaiacol levels are limited (Sarwar et al., 2022). These variable enzymatic responses reveal the plasticity of the plant antioxidant system, which enhances the activation of major ROS-compatible enzymes under each type of stress. Furthermore, the occurrence of photoinhibition, evidenced by changes in chlorophyll fluorescence, photosynthetic pigment content, CO_2 assimilation rate, and ROS-related attributes corroborates damage to the photosynthetic apparatus caused by water deficit and salinity stresses.

5.6 SA enhances acclimation mechanisms and mitigates stress-related metabolic damage

SA application induced distinct plant responses depending on the stress condition, often enhancing acclimation mechanisms, thereby mitigating stress. In plants under WD or S alone, 2 mM SA application allowed for maintenance of higher *A*, *E*, and g_s. High *E* level, even in stressed plants, plays an important role as a foliar temperature regulator, preventing protein denaturation and loss of cell membrane integrity (Chaudhry and Sidhu, 2021). In contrast, under WD+S, 2 mM SA application caused a greater decrease in *E* and g_s than in *A*, resulting in higher WUE and iWUE, since the plant reduced water loss while maintaining the photosynthetic rate.

SA stimulates the synthesis and accumulation of osmolytes, such as soluble sugar and amino acid, acting as a metabolic modulator of these biochemical pathways. Under WD and WD+S, 2 mM SA application increased SS content, which led to the lowest starch/SS ratio, suggesting enhanced starch hydrolysis. SA-induced osmoregulation is usually associated with the regulation of hydrolytic enzymes acting in starch degradation, producing compatible sugars (Ozturk et al., 2020). Under S, SA application influenced amino acid and proline accumulation. By increasing nitrate reductase and glutamine synthetase activities, SA enhances nitrogen assimilation, thereby promoting the synthesis of amino acids. Increased levels of leucine, valine, alanine, and tyrosine were observed as a result of SA application in faba bean (*Vicia faba* L.) under salt stress (Ghassemi-Golezani and Samea-Andabjadid, 2022). Moreover, SA induces pyrroline-5-carboxylate synthetase (P5CS) expression, favoring proline biosynthesis from glutamate (Wang et al., 2022).

Previous studies have reported that SA acts as a precursor in the synthesis pathways of phenolic compounds through hydroxylation or oxidation-reduction reactions that modify its chemical structure. These phenolics neutralize ROS and function as signaling molecules, regulating the expression of stress-response genes (Sharma et al., 2023). In this study, SA enhanced the activity of enzymatic antioxidants (SOD, under S; and GPX, under WD and WD+S), helping to maintain ROS

balance by attenuating O_2^- accumulation under WD and WD+S, as well as H_2O_2 among all stress conditions. The 2 mM SA application also mitigated the increase in electrolyte leakage under all stress conditions and prevented a reduction in F_m, F_v, F_v/F_0, and F_v/F_m, particularly under WD and S. Conversely, SA application was ineffective in preventing these decreases under WD+S.

Under S and WD+S, 4 mM SA application intensified morphological changes, including marked foliar abscission and leaf area reduction. According to Iqbal et al. (2022), SA can interact with abscisic acid (ABA) and ethylene, accelerating foliar abscission and increasing the root/shoot ratio. The reduction in transpiring surface is a strategy to optimize water use, although it comes at the cost of reduced photosynthesizing capacity.

In summary, the exogenous SA application provided different acclimation mechanisms depending on the major plant responses to each stress condition. These mechanisms included the maintenance of the photosynthesis rate and mitigation of oxidative damage by regulating stomatal conductance and activating the antioxidant defense system (Fig. 12). Furthermore, the 2 mM SA application helped to improve most morphophysiological and biochemical responses, enhancing plant tolerance to stresses. Plant responses to simultaneous stresses, as well as the interaction with SA, diverged substantially from plants under WD or S separately. Our findings collectively highlight that the mechanisms behind acclimation reveal system-specific characteristics resulting from the interaction between its components, which emerge only when stresses are studied together and not when elements are analyzed separately (Vital et al., 2022).

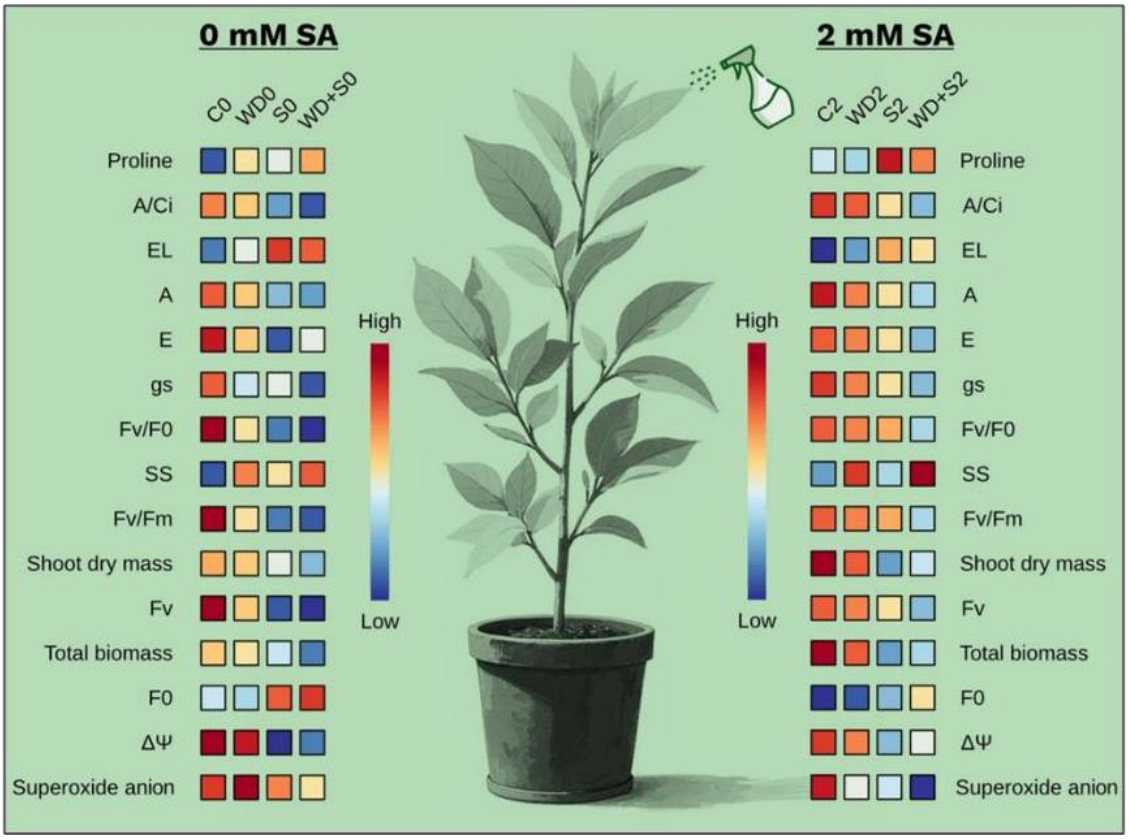

Figure 12. Major plant responses to each growing condition and 2 mM SA application.

6 CONCLUSIONS

The exposure of young citrus plants to water restriction and salt accumulation in the root zone soon after seedling transplanting negatively affected metabolism and inhibited subsequent plant growth and development. The reduction in cell turgor under stress conditions led to stomatal closure, limiting CO_2 assimilation. Moreover, the accumulation of reactive oxygen species increased electrolyte leakage, damaging photosynthetic pigments and compromising photosynthetic efficiency. Although the accumulation of soluble sugar, amino acid, and proline, concomitantly with the reduction in starch content, suggests an attempt at osmoregulation, this response was insufficient to maintain the relative water content in stressed plants. Furthermore, the antioxidant defense mechanism was shown to be ineffective in preventing oxidative damage. Our results suggest that the simultaneous stresses intensified the damaging effects of water deficit and salinity alone, causing more photosynthetic pigment degradation and reduced gas exchange and biomass production. Conversely, the application of 2 mM SA mitigated the harmful effects of stress by enhancing osmolyte accumulation and the antioxidant defense system, which benefited gas exchange and prevented ROS accumulation and oxidative damage.

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SUPPLEMENTARY MATERIAL

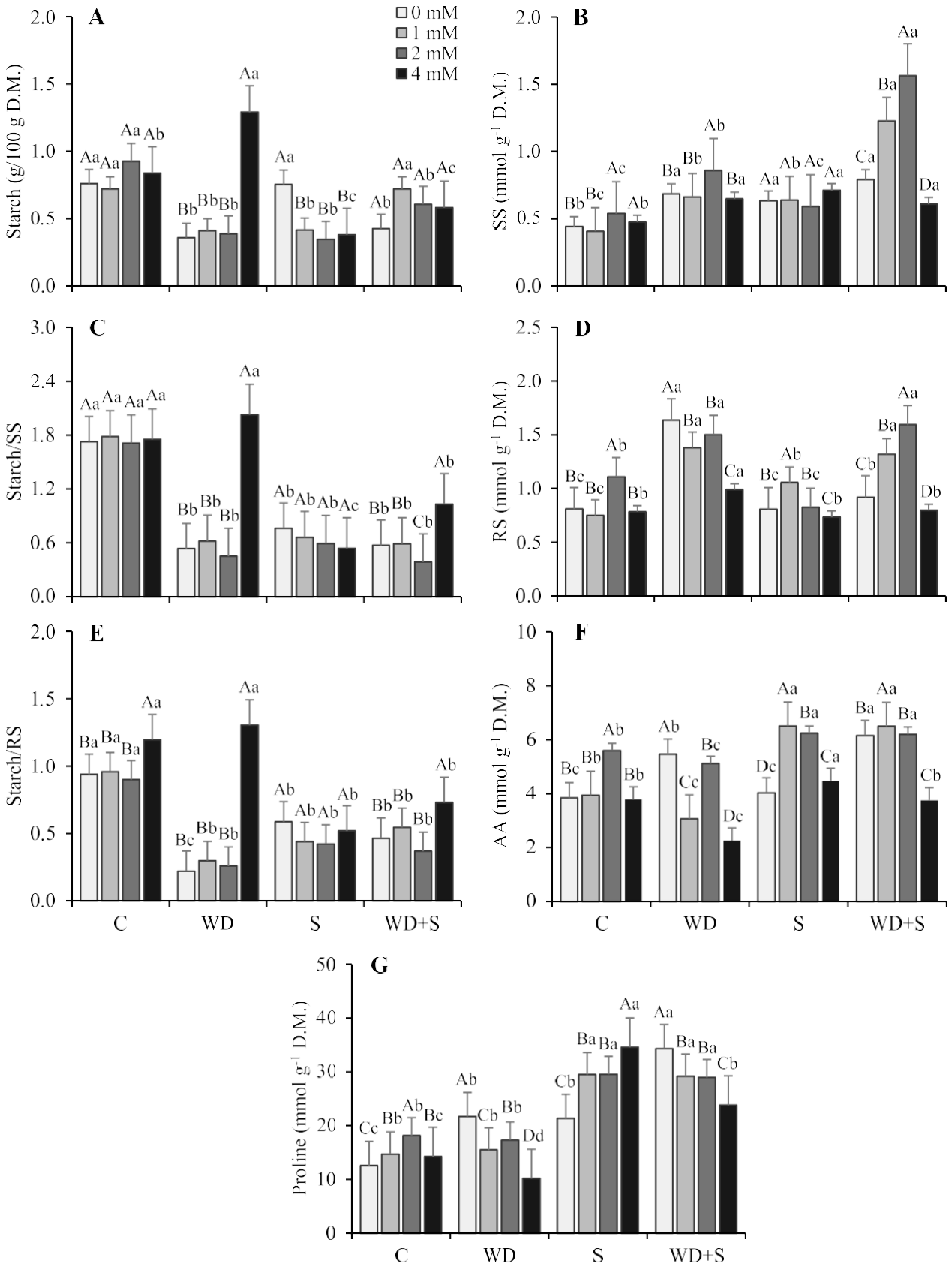

Figure S1. (**A**) Starch, (**B**) Soluble sugar (SS), (**C**) Starch/SS, (**D**) Reducing sugar (RS), (**E**) Starch/RS, (**F**) Amino acid (AA), and (**G**) Proline content of young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100

mM NaCl) conditions, and treated with different salicylic acid concentrations. Bars in each column indicate the standard error of means (n = 5). Uppercase letters compare salicylic acid concentrations within each growing condition, while lowercase letters compare the growing conditions within each salicylic acid concentration, by Scott-Knott's test (p<0.05).

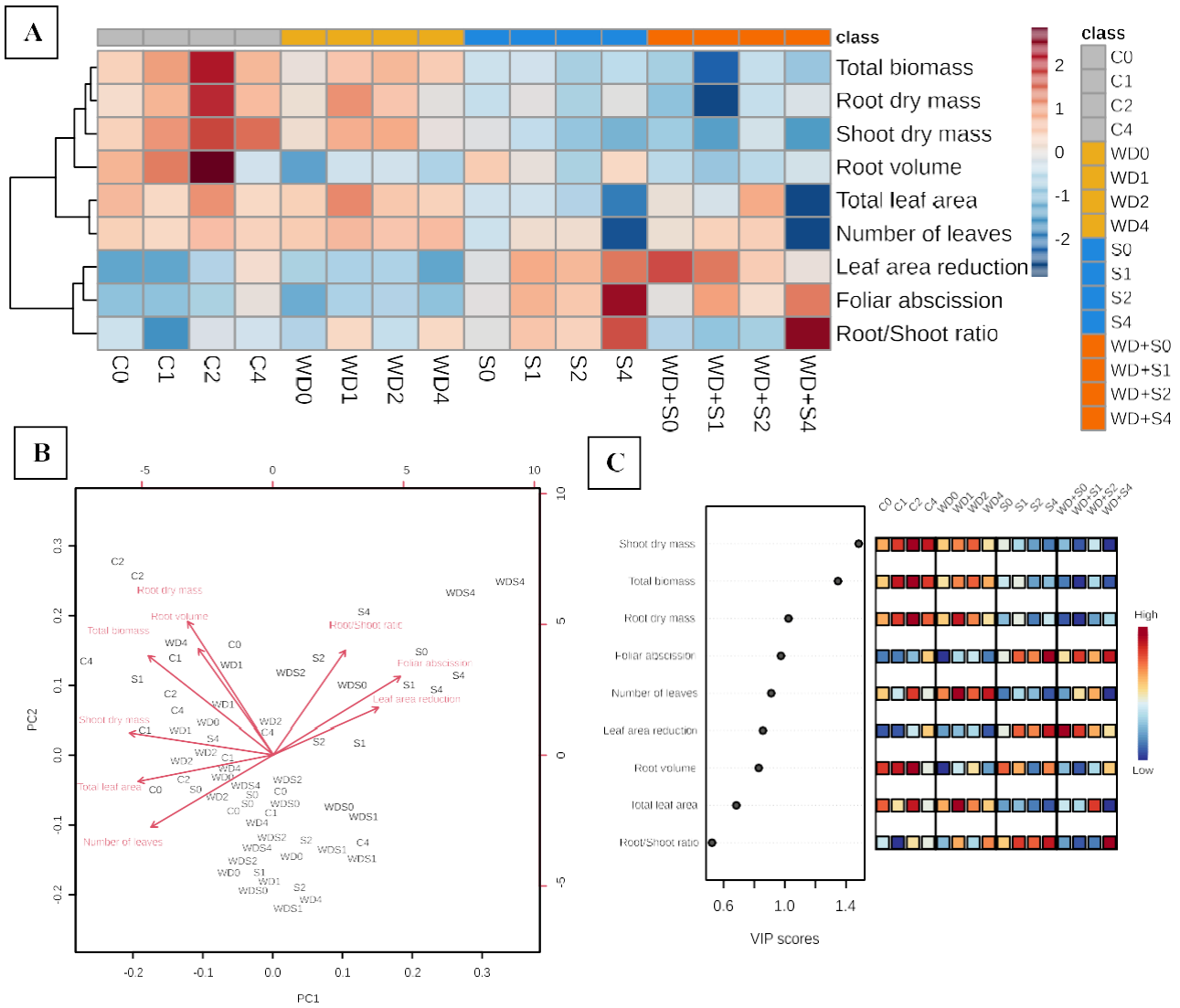

Figure S2. (**A**) Heatmap representation, (**B**) Principal Component Analysis Biplot (PCA Biplot), and (**C**) Variable Importance in Projection (VIP) scores of biometric attributes in young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with 0, 1, 2, and 4 mM salicylic acid concentrations. The data was normalized by using logarithmic and auto-scaling transformations in the MetaboAnalyst platform (n = 5).

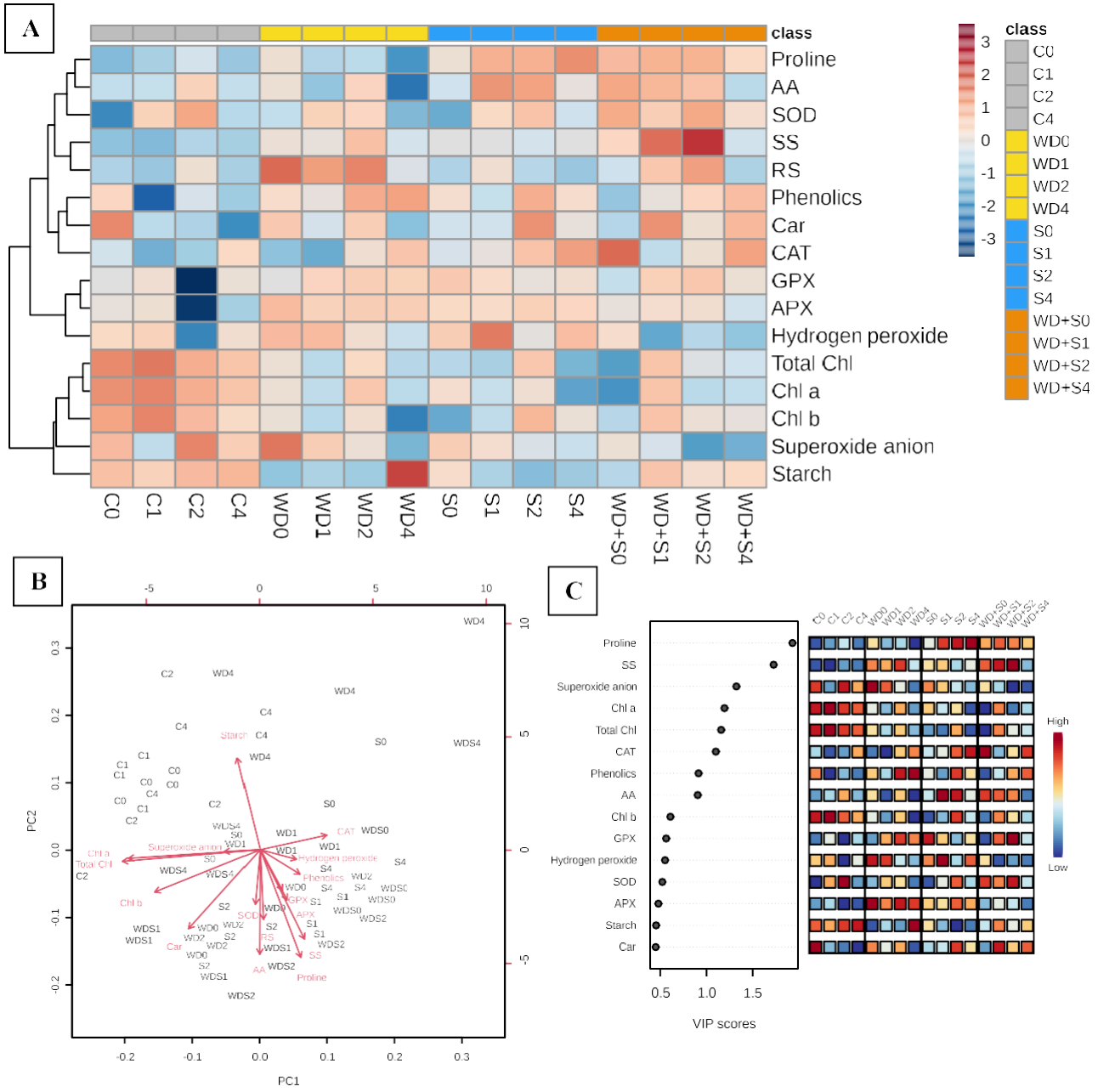

Figure S3. (**A**) Heatmap representation, (**B**) Principal Component Analysis Biplot (PCA Biplot), and (**C**) Variable Importance in Projection (VIP) scores of biochemical traits in young citrus plants under no stress (C, 90% pot capacity), water deficit (WD, 45% pot capacity), salinity (S, 90% pot capacity + 100 mM NaCl), and water deficit + salinity (WD+S, 45% pot capacity + 100 mM NaCl) conditions, and treated with 0, 1, 2, and 4 mM salicylic acid concentrations. The data was normalized by using logarithmic and auto-scaling transformations in the MetaboAnalyst platform (n = 5).

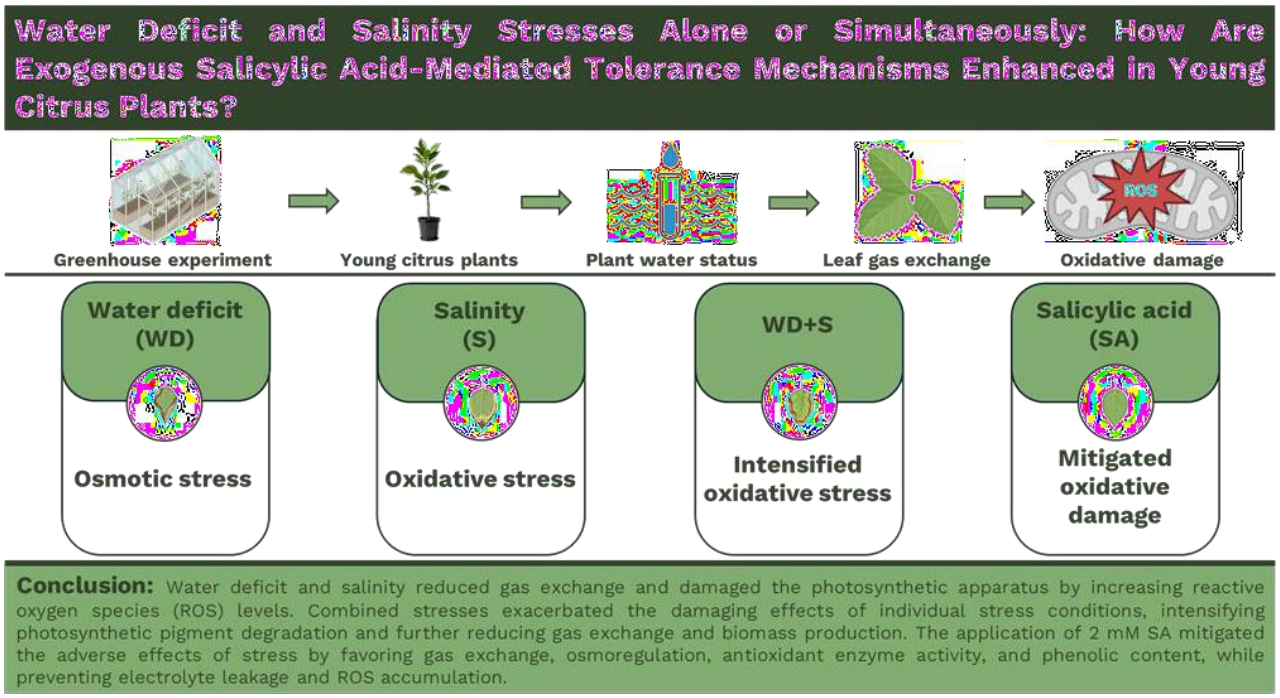

Figure S4. Graphical abstract.

Table S1. Summary of analysis of variance and coefficient of variation (CV) of CO_2 assimilation rate (*A*), transpiration rate (*E*), stomatal conductance (g_s), ratio of internal and external CO_2 concentration (C_i/C_a), water-use efficiency (WUE), intrinsic water-use efficiency (iWUE) and instantaneous carboxylation efficiency (A/C_i) in young citrus plants under different growing conditions (GC) and salicylic acid concentrations (SA).

Sources of variation	DF	Mean squares						
		A	*E*	g_s	C_i/C_a	WUE	iWUE	A/C_i
GC	3	172.51*	9.73*	0.0326*	0.0657*	2.88*	2951.87*	0.0396*
SA	3	15.39*	0.39*	0.0015*	0.0307^{ns}	4.34*	1328.57*	0.0039*
GC × SA	9	5.63*	0.36*	0.0009*	0.0373*	1.88*	466.45*	0.0012*
Residue	48	1.99	0.1	0.0003	0.0187	0.56	345.93	0.0008
CV (%)		27.59	27.69	27.44	24.59	17.23	20.62	20.61

ns not significant
*Significant ($p < 0.05$)

Table S2. Summary of analysis of variance and coefficient of variation (CV) of leaf water potential at pre-dawn (Ψ_w 5:00 a.m.), leaf water potential at midday (Ψ_w 12:00 p.m.), water potential difference between pre-dawn and midday ($\Delta\Psi$), and relative water content (RWC) in young citrus plants under different growing conditions (GC) and salicylic acid concentrations (SA).

Sources of variation	DF	Mean squares			
		Ψ_w 5:00 a.m.	Ψ_w 12:00 p.m.	$\Delta\Psi$	RWC
GC	3	2.45*	0.265*	1.48*	114.35*
SA	3	0.12*	0.008^{ns}	0.16*	21.67*
GC × SA	9	0.11*	0.006^{ns}	0.09*	13.85*
Residue	48	0.0302	0.0056	0.05	6.34
CV (%)		19.27	26.55	19.65	4.00

ns not significant
*Significant ($p < 0.05$)

Table S3. Summary of analysis of variance and coefficient of variation (CV) of starch, soluble sugar (SS), starch/SS, reducing sugar (RS), starch/RS, amino acid (AA), and proline content in young citrus plants under different growing conditions (GC) and salicylic acid concentrations (SA).

Sources of variation	DF	Mean squares						
		Starch	SS	Starch/SS	RS	Starch/RS	AA	Proline
GC	3	1.01*	1.48*	3.83*	0.1563*	0.9454*	10.27*	11.10*
SA	3	0.33*	0.29*	0.98*	0.0841*	0.6713*	13.56*	0.39*
GC × SA	9	0.55*	0.21*	0.50*	0.0233*	0.2056*	3.96*	1.07*
Residue	48	0.07	0.02	0.06	0.0007	0.0221	0.02	0.01
CV (%)		31.16	12.52	31.53	5.40	23.45	2.91	2.09

ns not significant
*Significant ($p < 0.05$)

Table S4. Summary of analysis of variance and coefficient of variation (CV) of minimal chlorophyll fluorescence (F_0), maximum chlorophyll fluorescence (F_m), variable chlorophyll fluorescence (F_v), electron transport capacity of photosystem II (F_v/F_0), and maximum quantum yield of photosystem II (F_v/F_m) in young citrus plants under different growing conditions (GC) and salicylic acid concentrations (SA).

Sources of variation	DF	Mean squares				
		F_0	F_m	F_v	F_v/F_0	F_v/F_m
GC	3	7672.65*	151170.24*	227534.93*	6.67*	0.0417*
SA	3	3222.57*	8345.38^{ns}	8194.54^{ns}	0.50*	0.0044*
GC × SA	9	794.25*	14783.00^{ns}	14654.67^{ns}	0.30*	0.0019*
Residue	48	524.76	14958.86	16502.55	0.28	0.0027
CV (%)		8.33	11.73	16.74	18.33	7.08

ns not significant
*Significant ($p < 0.05$)

Table S5. Summary of analysis of variance and coefficient of variation (CV) of total chlorophyll (Total Chl), chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), carotenoids (Car), and chlorophyll/carotenoids ratio (Chl/Car) in young citrus plants under different growing conditions (GC) and salicylic acid concentrations (SA).

Sources of variation	DF	Mean squares				
		Total Chl	Chl *a*	Chl *b*	Car	Chl/Car
GC	3	0.00223*	0.00125*	0.295*	0.000653^{ns}	116.81*
SA	3	0.00030^{ns}	0.00016^{ns}	0.068*	0.000555^{ns}	7.73*
GC × SA	9	0.00035*	0.00023^{ns}	0.063*	0.10*	5.08*
Residue	48	0.00018	0.00016	0.012	0.000338	2.50
CV (%)		18.49	19.95	24.73	10.25	12.36

ns not significant
*Significant ($p < 0.05$)

Table S6. Summary of analysis of variance and coefficient of variation (CV) of electrolyte leakage (EL) in young citrus plants under different growing conditions (GC) and salicylic acid concentrations (SA).

Sources of variation	DF	Mean squares
		EL
GC	3	3986.27*
SA	3	199.88*
GC × SA	9	147.51*
Residue	48	51.02
CV (%)		12.53

ns not significant
*Significant ($p < 0.05$)

Table S7. Summary of analysis of variance and coefficient of variation (CV) of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in young citrus plants under different growing conditions (GC) and salicylic acid concentrations (SA).

Sources of variation	DF	Mean squares	
		O_2^-	H_2O_2
GC	3	157.13*	0.111*
SA	3	92.24*	0.067*
GC × SA	9	30.16*	0.035*
Residue	48	10.92	0.001
CV (%)		2.50	2.94

ns not significant
*Significant ($p < 0.05$)

Table S8. Summary of analysis of variance and coefficient of variation (CV) of superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and total phenolics in young citrus plants under different growing conditions (GC) and salicylic acid concentrations (SA).

Sources of variation	DF	Mean squares			
		SOD	APX	GPX	Phenolics
GC	3	10.19*	16765.37*	0.0437*	4.16*
SA	3	4.08*	1803.12^{ns}	0.0352*	4.02*
GC × SA	9	3.49*	1985.99*	0.0494*	1.66*
Residue	48	1.85	1187.62	0.0031	0.01
CV (%)		26.12	17.95	16.19	1.44

ns not significant
*Significant ($p < 0.05$)