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# SCREENING ALGAE-BASED BIOSTIMULANTS FOR ACTIVATING DEFENSE MECHANISMS AND INCREASED PRODUCTIVITY OF SEMIARID-ADAPTED SOYBEAN UNDER WATER DEFICIT

**Bom Jesus – PI**
**2024**

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Dissertation presented to Postgraduate Program in Agricultural Sciences - PPGCA, Campus Professora Cinobelina Elvas - CPCE, at Federal University of Piauí - UFPI, in the concentration area of Plant Production, as a partial requirement for obtaining the title of Master in Agricultural Sciences.

Advisor: Prof. Dr. Rafael de Souza Miranda.

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*"When nothing seems to be going right, I go see the stonecutter hammering away at his rock, perhaps a hundred times without a single crack showing in it. Yet at the hundred and first blow, it splits in two, and I know it was not that blow that did it, but all that had gone before."*
*Jacob Riis*

## ABSTRACT

In the global scenario, climate change has been increasingly recurrent and accompanied by drought episodes, negatively affecting the agricultural yield. This constraint severely decreases soybean yield, even semiarid-adapted cultivars. The seaweed-based biostimulants has shown potential in enhancing the productive parameters and mitigating the water deficit damages in some plant species, including soybeans. However, evidence of how biostimulants alter plant metabolism in semiarid-typical soybean cultivars is still not fully elucidated, as discussed in Chapter I which presents the state of the art with results and investigations about the theme. Our experimental study was carried out to test the hypothesis that the application of seaweed-based biostimulants modulates the metabolism of semiarid soybean cultivars, promoting increases in productivity and tolerance to water deficit. The hypothesis was tested through the development of two greenhouse experiments. The first one aimed to select the dose and application timing capable of modulating the physiology and increasing soybean yield under well-irrigated conditions. At this stage, foliar application of the *Ascophyllum nodosum*-based biostimulant was carried out at doses of 0.5, 1.0, and 1.5 L $h^{-1}$, either as a single or split application (V4, V4+R1, R1, R1+R4), at different developmental stages of the Extrema soybean cultivar. The data, detailed in Chapter II, demonstrated that the *A. nodosum*-based biostimulant promoted better physiological conditioning and productive responses, depending on dose and application timing. The dose of 1.5 L $ha^{-1}$, applied in equal amounts at V4 and R1 stages, was characterized as the most effective, increasing the net photosynthesis, water status, and resulted in a 12% increase in soybean plant productivity. The second experiment, described in Chapter III, aimed to evaluate the efficiency of four commercial formulations in alleviating the drought effects on soybean performance during the vegetative stage, investigating growth and biochemical, physiological, and molecular pathways. The finding showed that drought drastically reduced photosynthetic pigments, stomatal conductance, transpiration, and increased non-photochemical energy dissipation, leading to severe restrictions in plant growth. The poor performance under drought was associated with greater membrane damage and hydrogen peroxide levels. The data also revealed that the plants activated defense pathways, such as maintaining water status and activating antioxidant enzymes; however, these mechanisms were not effective in neutralizing the stress damage. Under drought, the biostimulants stimulated the accumulation of metabolites involved in defense pathways (4-aminobutyric acid, benzoic acid, shikimic acid, erythronic acid, arabinose, phenylalanine, threonine, among others) and reprogrammed the antioxidant system, but they were ineffective in mitigating the stress effects on plant performance, at least during the studied period. In conclusion, biostimulants formulated with *A. nodosum* extracts are capable of enhancing the physiological and yield performance of soybean cultivars under well-irrigated conditions but do not mitigate the harmful effects of water deficit on the metabolism and growth of semiarid cultivars. Future research should focus on biostimulant application strategies under stressful conditions, integrating long-term responses with the investigation of productive parameters associated with defense mechanisms.



**Keywords:** *Ascophyllum nodosum*. Defense pathways. *Glycine max*. Plant metabolism. Water limitation. Yield.

## RESUMO

No cenário global, as alterações climáticas têm sido cada vez mais recorrentes e acompanhadas por episódios de seca, o que tem afetado negativamente a produtividade agrícola. Essa restrição não é diferente para a cultura da soja, mesmo que os produtores tenham acesso a cultivares geneticamente melhoradas para diferentes climas, como o sistema de sequeiro em regiões semiáridas. Os bioestimulantes à base de algas marinhas têm mostrado potencial para melhorar os parâmetros produtivos e mitigar os danos causados pelo déficit hídrico em algumas espécies de plantas, incluindo a soja. No entanto, as evidências de como os bioestimulantes alteram o metabolismo das cultivares de soja típicas de regiões semiáridas ainda não estão totalmente elucidadas, conforme discutido no Capítulo I, que apresenta o estado da arte com resultados e investigações sobre o tema. Nosso estudo experimental foi realizado para testar a hipótese de que a aplicação de bioestimulantes à base de algas marinhas modula o metabolismo das cultivares de soja cultivadas em regiões semiáridas, promovendo aumentos na produtividade e tolerância ao déficit hídrico. A hipótese foi testada por meio do desenvolvimento de dois experimentos em casa de vegetação. O primeiro teve como objetivo selecionar a dose e o momento de aplicação capazes de modular a fisiologia e aumentar a produtividade da soja em condições bem irrigadas. Nesta etapa, a aplicação foliar do bioestimulante à base de *Ascophyllum nodosum* foi realizada nas doses de 0,5, 1,0 e 1,5 L $ha^{-1}$, seja como aplicação única ou dividida (V4, V4+R1, R1, R1+R4), em diferentes estágios de desenvolvimento da cultivar de soja Extrema. Os dados, detalhados no Capítulo II, demonstraram que o bioestimulante à base de A. nodosum promoveu melhor condicionamento fisiológico e respostas produtivas, dependendo da dose e do momento de aplicação. A dose de 1,5 L $ha^{-1}$, aplicada em quantidades iguais nos estágios V4 e R1, foi caracterizada como a mais eficaz, aumentando a fotossíntese líquida, o estado hídrico e resultando em um aumento de 12% na produtividade das plantas de soja. O segundo experimento, descrito no Capítulo III, teve como objetivo avaliar a eficiência de quatro formulações comerciais na atenuação dos efeitos da seca no desempenho da soja durante o estágio vegetativo, investigando o crescimento e as vias bioquímicas, fisiológicas e moleculares. Os resultados mostraram que a seca reduziu drasticamente os pigmentos fotossintéticos, a condutância estomática, a transpiração e aumentou a dissipação de energia não fotoquímica, levando a severas restrições no crescimento das plantas. O fraco desempenho sob seca foi associado a maiores danos às membranas e níveis de peróxido de hidrogênio. Os dados também revelaram que as plantas ativaram vias de defesa, como a manutenção do estado hídrico e a ativação de enzimas antioxidantes; no entanto, esses mecanismos não foram eficazes em neutralizar os danos do estresse. Sob seca, os bioestimulantes estimularam o acúmulo de metabólitos envolvidos nas vias de defesa (ácido 4-aminobutírico, ácido benzóico, ácido chiquímico, ácido eritrônico, arabinose, fenilalanina, treonina, entre outros) e reprogramaram o sistema antioxidante, mas foram ineficazes em mitigar os efeitos do estresse no desempenho das plantas, pelo menos durante o período estudado. Em conclusão, os bioestimulantes formulados com extratos de *A. nodosum* são capazes de melhorar o desempenho fisiológico e produtivo de cultivares de soja em condições bem irrigadas, mas não mitigam os efeitos nocivos do déficit hídrico no metabolismo e no crescimento de cultivares de regiões semiáridas. Pesquisas futuras devem focar

em estratégias de aplicação de bioestimulantes sob condições de estresse, integrando respostas de longo prazo com a investigação de parâmetros produtivos associados a mecanismos de defesa.

**Palavras-chave**: *Ascophyllum nodosum*. *Glycine max*. Limitação hídrica. Metabolismo. Produção. Rotas de defesa.

## LIST OF FIGURES

**Figure 1 -** Location of the experimental area and dynamics of mean, maximum and minimum temperature, and air relative humidity during the trials with soybean plants, from June to September 2022…….. **39**

**Figure 2 -** Summary of *Ascophyllum nodosum*-based biostimulant treatments on different stages of soybean crop under greenhouse conditions…… **40**

**Figure 3 -** Net photosynthesis, transpiration, stomatal conductance, relative water content, instantaneous and intrinsic water use efficiency rates of soybean plants at V4 stage, after application of biostimulants..... **44**

**Figure 4 -** Number of flowers, plant height and pollen viability of soybean plants at R2 stage, after application of biostimulants…..…….. **45**

**Figure 5 -** Net photosynthesis, transpiration, stomatal conductance, relative water content, instantaneous and intrinsic water use efficiency rates of soybean plants at R4 stage, after application of biostimulants..... **46**

**Figure 6 -** Net photosynthesis, transpiration, stomatal conductance, relative water content, instantaneous and intrinsic water use efficiency rates of soybean plants at R6 stage, after application of biostimulants..... **48**

**Figure 7 -** Number of grains per pod, weight of a thousand seeds and yield of soybean plants treated with biostimulants……..**49**

**Figure 8 -** Principal component analysis of the mean parameters of soybean plants, Extrema genotype, evaluated at different stages (V4, R2, R4 and R6) after application of the biostimulants……. **50**

**Figure 9 -** Summary of biostimulant treatments based on A*scophyllum nodosum* e *Lithothamnium* in trials under greenhouse with soybean plants subjected to well-irrigated and drought conditions..…… **64**

**Figure 10 -** Irrigation depth applied and total consumed by soybean plants treated with different biostimulant formulations, under well-irrigated and drought conditions..…….. **65**

**Figure 11 -** Dynamics of environmental conditions during the experimental period in the greenhouse with soybean plants exposed to different water regimes……. **66**

**Figure 12 -** Phenological aspects of representative soybean plants, at the end of the experiment, carried out in a greenhouse, growing under well-irrigated and water deficit conditions, in absence and presence of different algae-based biostimulants……..……..... **74**

**Figure 13 -** Stem diameter, plant height, number of trefoils, root length and root volume of soybean plants, with full irrigation (control) and under water deficit, in absence and presence of different algae-based biostimulants……..……..... **75**

**Figure 14 -** Dry mass of shoot, root and total, leaf area and relative drought tolerance of soybean plants exposed to water regimes and treated with different algae-based biostimulants ……**77**

**Figure 15 -** Relative water content, osmotic potential, leaf succulence, leaf temperature, instantaneous and intrinsic water use efficiency of soybean plants exposed to water regimes and treated with different algae-based biostimulants……. **78**

**Figure 16 -** Thermography of a representative soybean plant, cultivar Extrema, at 53 days of cultivation (25 days after water deficit exposition), and treated with different algae-based biostimulants……. **80**

**Figure 17 -** Net photosynthesis, transpiration, stomatal conductance and Rubisco carboxylation efficiency of soybean plants exposed to water regimes and treated with different algae-based biostimulants……**81**

**Figure 18 -** Maximum (Fv/Fm) and effective quantum efficiency of photosystem II (ΦPSII), photochemical (qP) and non-photochemical (NPQ) quenching dissipation, and electron transport rate (ETR) of soybean plants exposed to water regimes and treated with different algae-based biostimulants……... **82**

**Figure 19 -** Contents of chlorophyll a, chlorophyll b, chlorophyll total and carotenoids of soybean plants exposed to water regimes and treated with different algae-based biostimulants……. **83**

**Figure 20 -** Activity of ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (G-POD) and superoxide dismutase (SOD) of soybean plants exposed to water regimes and treated with different algae-based biostimulants……..… **84**

**Figure 21 -** Malondialdehyde/lipid peroxidation (MDA) and hydrogen peroxide ($H_2O_2$) contents in leaves and roots of soybean plants exposed to water regimes and treated with different algae-based biostimulants…… **85**

**Figure 22 -** Contents of free amino acids, soluble carbohydrates and soluble proteins in soybean plants exposed to water regimes and treated with different algae-based biostimulants…….. **87**

**Figure 23 -** Principal component analysis (PCA) of the metabolic profile in leaves of soybean plants growing under well-irrigated and drought conditions, and subjected to different treatments with biostimulants……..**88**

**Figure 24 -** Principal component analysis (PCA) of the metabolic profile in leaves of soybean plants as affected by water regimes, and volcano plot with different metabolites responsive to water deficit.....**89**

**Figure 25 -** Principal component analysis (PCA) of the metabolic profile with score plot, and hierarchical cluster analysis of metabolite variations with heat map and correlation network, showing the relative abundance of different significant metabolites in leaves of soybean plants exposed to drought, and subjected to different biostimulant treatments……. **90**

## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| *A* | Net photosynthesis |
| *A*/Ci | Carboxylation efficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) |
| *A*/*E* | Instantaneous water use efficiency |
| $A/g_s$ | Intrinsic efficiency of water use |
| ABA | Abscisic acid |
| ANE | *Ascophyllum nodosum* extracts |
| APX | Ascorbate peroxidase |
| Bio 01 | Biostimulant 01 |
| Bio 02 | Biostimulant 02 |
| Bio 03 | Biostimulant 03 |
| Bio 04 | Biostimulant 04 |
| C3 | Photosynthetic metabolism |
| $C_3H_8O_2$ | Propilenoglicol |
| $CaCO_3$ | Calcium carbonate |
| CAT | Catalase |
| Ci | Internal dioxide carbon concentration |
| $CO_2$ | Carbon dioxide |
| DMSO | Dimethyl sulfoxide |
| DW | Dry weight |
| E | Transpiration |
| EDTA | Ethylenediaminetetraacetic acid |
| ETR | Apparent electron transport rate |
| FC | Field capacity |
| Fm | Maximum fluorescence |
| Fo | Initial fluorescence |
| Fo’ | Illuminated plant tissue |
| Fv | Variable fluorescence |
| Fv’/Fm’ | Potential quantum yield of PSII |
| FW | Fresh weight |

| | |
|---|---|
| G-POD | Guaiacol peroxidase |
| GABA | Gamma-aminobutyric acid |
| GC-MS | Gas chromatography coupled with mass spectrometer |
| GMD | Golm Metabolome Database |
| GR | Glutathione reductase |
| gs | Stomatal conductance |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2SO_4$ | Sulfuric acid |
| IRGA | Infrared gas analyser |
| LA | Leaf area |
| LS | Leaf succulence |
| MDA | Malondialdehyde |
| MSTFA | N-methyl-N-(trimethylsilyl) trifluoroacetamide |
| $N_2$ | Liquid nitrogen |
| NBT | Nitrotetrazolium blue chloride |
| NGP | Number of grains per plant |
| NGV | Number of grains per pod |
| NPP | Number of pods per plant |
| NPQ | Non-photochemical quenching |
| PCA | Principal component analysis |
| PMG | 1000-grain weight |
| POD | Peroxidase |
| PPFD | Photosynthetic photon flux density |
| PSII | Photosystem II |
| qP | Photochemical quenching |
| R2 | Reproductive stage 2 |
| R4 | Reproductive stage 4 |
| R6 | Reproductive stage 6 |
| R8 | Reproductive stage 8 |
| ROS | Oxigen reactive species |
| RWC | Relative water content |
| SOD | Superoxide dismutase |
| TBA | Thiobarbituric acid |

| | |
|---|---|
| TCA | Trichloroacetic acid |
| TW | Turgid weight |
| UA | Unit of enzymatic activity |
| V4 | Vegetative stage 4 |
| VP | Pollen viability |
| W. BIO | Without biostimulant |
| WTG | Weight of one thousand grains |
| WUE | Water use efficiency |
| Y | Yield |
| Y(NPQ) | Regulated energy dissipation |
| ϕPSII | Effective quantum yields of linear electron flow through PSII |

**SUMMARY**


| | | |
|---|---|---|
| **1** | **GENERAL INTRODUCTION.....** | **20** |
| **2** | **EXPERIMENTAL STRATEGY.....** | **22** |
| **3** | **CHAPTER I: THEORETICAL FRAMEWORK……** | **23** |
| **3.1** | **Water stress in plants……...** | **23** |
| **3.2** | **Response mechanisms against water deficit……** | **25** |
| **3.3** | **Use of biostimulants as mitigators of water stress……..** | **26** |
| **3.4** | **Soybean crop……..** | **28** |
| | **REFERENCES……..** | **30** |
| **4** | **CHAPTER II: *Ascophyllum nodosum*-DERIVED BIOSTIMULANT PROMOTES PHYSIOLOGICAL CONDITIONING TO INCREASE SOYBEAN YIELD IN A SEMIARID CLIMATE……...** | **36** |
| | **ABSTRACT……...** | **36** |
| **4.1** | **Introduction……** | **37** |
| **4.2** | **Material and methods……** | **38** |
| 4.2.1 | Study site, experimental conditions and treatments……..… | 38 |
| 4.2.2 | Photosynthetic parameters…… | 41 |
| 4.2.3 | Hydric status. ……... | 41 |
| 4.2.4 | Biometric measurements and yield……... | 41 |
| 4.2.5 | Pollen viability……... | 42 |
| 4.2.6 | Statistical analyses…….. | 42 |
| **4.3** | **Results.....** | **43** |
| 4.3.1 | Physiological responses to treatments during V4 stage…… | 43 |
| 4.3.2 | Reproductive and biometric responses at R2 stage……... | 44 |
| 4.3.3 | Physiological performance and number of pods at R4 and R6 stage.. | 45 |
| 4.3.4 | Soybean yield…… | 48 |
| 4.3.5 | Principal component analysis (PCA) …… | 49 |
| **4.4** | **Discussion.....** | **51** |
| **4.5** | **Conclusion.....** | **53** |

**REFERENCES..... 55**

**5 CHAPTER III: SEAWEED-BASED BIOSTIMULANTS MODULATE METABOLIC PROFILE AND ANTIOXIDANT SYSTEM BUT DO NOT ALTER PHYSIOLOGICAL PERFORMANCE AND DROUGHT TOLERANCE IN SEMIARID-ADAPTED SOYBEAN……. 60**

**ABSTRACT..... 60**

**5.1 Introduction..... 61**

**5.2 Material and methods..... 63**

5.2.1 Study site, experimental conditions, treatments and irrigation management……. 63

5.2.2 Growth parameters…….. 66

5.2.3 Hydric status……. 67

5.2.4 Gas exchanges……. 66

5.2.5 Chlorophyll a fluorescence…… 68

5.2.6 Photosynthetic pigments……. 68

5.2.7 Biochemical analyzes…….. 69

*5.2.7.1 Soluble carbohydrates*……. 69

*5.2.7.2 Free amino acids*…….. 69

*5.2.7.3 Antioxidative enzymes*……. 70

*5.2.7.4 Lipid peroxidation*…….. 71

*5.2.7.5 Hydrogen peroxide ($H_2O_2$)* ……. 72

5.2.8 Metabolic profile…… 72

5.2.9 Statistical analyses……... 73

**5.3 Results..... 74**

5.3.1 Growth performance……. 74

5.3.2 Hydric status…….. 77

5.3.3 Efficiency of the photosynthetic machinery…….. 80

5.3.4 Biochemical regulation, oxidative damage and antioxidant system…. 83

5.3.5 Modulation of the metabolic profile…… 88

**5.4 Discussion..... 91**

5.4.1 Water deficit limits growth and biomass accumulation, and biostimulant formulations do not confer stress tolerance……... 91

5.4.2 The biostimulant formulations do not influence the water status and efficiency of the photosynthetic machinery of soybean plants under water deficit…… 92

5.4.3 Biostimulants promote relevant responses in the plant metabolome and activation of the antioxidant system under stress…… 93

**5.5 Conclusion..... 95**

**REFERENCES..... 96**

**6 FINAL CONSIDERATIONS..... 103**

**APPENDICES..... 105**

# 1 GENERAL INTRODUCTION

Abiotic stresses and their interactions are responsible for hindering or limiting agricultural production in large portions of the globe's arable land (Chaudhry; Sidhu, 2021). Water deficit conditions are one of these major constraints, as plants lose a significant amount of absorbed water in the transpiration process (Kapoor *et al*., 2020). Therefore, one of the key challenges in productivity is to have water conditions that favor a good balance between transpiration and photosynthesis rates, based on the available soil water (Gleason *et al*., 2021). Thus, under water limitation conditions, it is important to identify which strategies plants activate to cope with water scarcity with the lowest possible energy cost (Seleiman *et al*., 2021).

Water stress may also occur in conditions of excess water, which is less common in dry and semiarid regions, and when the water supply in the soil is less than the amount spent in the transpiration process (Fang *et al*., 2019). In the latter case, the initial impact is on the turgor state, which directly affects cell elongation and division, ceasing growth, followed by several other biochemical and physiological changes (Steiner *et al*., 2022).

Stomatal closure constitutes the first plant response to drought, in order to minimize water loss; however, this also implies a decrease in $CO_2$ absorption, causing sharp reductions in assimilatory rate (Inoue *et al*., 2021). As a consequence, the lower $CO_2$ assimilation rate leads to a metabolic imbalance characterized by an increase in reactive oxygen species production (Chandrasekaran; Paramasivan, 2022). Thus, the plant needs to activate mechanisms to cope with water limitation and also increase the synthesis of important antioxidant compounds to maintain redox homeostasis (El-beltagi; Mohamed; Sofy, 2020). Additionally, under field conditions and in semiarid climates, along with water restriction, high temperatures, high radiation, and nutritional imbalance linked to absorption inefficiency can also exacerbate the effects of water scarcity.

In recent years, the search for signaling molecules that can mitigate the negative effects of stress has been intense (Hasanuzzaman *et al*., 2018; Hidangmayum *et al*., 2019; Ghosh *et al*., 2021; Khan *et al*., 2021; Raza *et al*., 2021; Tiwari *et al*., 2021). These studies report that the application of signaling molecules, in addition to contributing to the maintenance of physiological processes and assisting in

the restoration of water status, may enable the development of new cultivation techniques. In this regard, biostimulants emerge as powerful tools, which are compounds formed by a mixture of plant growth regulators containing one or more chemical components such as amino acids, nutrients and vitamins (Shahrajabian *et al*., 2021; Gupta *et al*., 2022).

Biostimulants may optimize gene expression, stimulate root growth, and contribute to hormonal balance, providing an alternative to assist plants in water stress conditions by acting on stomatal conductance, plant transpiration, and stimulating the defense system (Shahrajabian *et al*., 2021). Additionally, they promote an increase in photosynthetic rate by inducing the dissipation of excess energy, enhancing the activity of antioxidant enzymes, regulating post-stress metabolic activities, leaf water potential, and water use efficiency (Rosa *et al*., 2021).

Soybean (*Glycine max*) has been cultivated in Brazil for almost 140 years and is one of the most important products for the Brazilian economy, possibly one of the crops that have shown the most significant growth in cultivated areas and also in the agro-industrial segment. The crop is of great economic importance for the country and the world (AGROSTAT, 2024), it is being widely explored in MATOPIBA (region composed of states of Maranhão, Tocantins, Piauí e Bahia), considered the last agricultural frontier of the Brazil. However, although plant breeding generates genetic material to cultivate in semiarid, water availability is still a challenge, considering that precipitation is an uncontrollable factor and periods of drought occur, even during the rainy season.

Despite all the breeding efforts aimed at adapted cultivars, soybean is a crop that suffers considerably from changes in water availability. This limitation causes changes in the plant's metabolism and physiology, affecting vegetative and reproductive stages and reducing the quality of the final product (Felisberto *et al*., 2022). Therefore, considering the importance of drought episodes in soybean cultivation and the potential use of biostimulants algae-based as a drought mitigator, we expect to identify physiological, biochemical, and morphological mechanisms activated by biostimulants in plants responses to drought.

## 2 EXPERIMENTAL STRATEGY

The study was developed in two dependent and consecutive stages. In the first one, an experiment was set up in a greenhouse to assess the performance of different soybean genotypes in response to the application of biostimulants based on *Ascophyllum nodosum* in three different doses, at different time points, under full irrigation conditions. Here, we aimed to select the time and dosage of a promisor *A. nodosum*-extract biostimulant capable of promoting the best physiological and productive responses. The parameters net $CO_2$ assimilation rate, stomatal conductance, transpiration rate, water use efficiency, pollen viability, number of grains per pod, 1000-grain weight, and soybean productivity in kilograms per hectare were investigated. The data are detailed in Chapter II.

Once the cultivar and biostimulant dosage was defined, the second stage was set up. Trials were again conducted in a greenhouse, and plants were exposed to two water regimes (well-irrigated and drought) and subjected to five treatments with biostimulants (four commercial formulations + negative control (spraying with distilled water)). In this stage, biometric assays (plant height, stem diameter, number of trifoliate, leaf area, root system volume and length, root and shoot dry mass); physiological and water-related parameters (gas exchanges, osmotic potential, chlorophyll *a* fluorescence, photosynthetic pigments, relative water content, leaf succulence, stress tolerance, and leaf temperature); and biochemical analyses (antioxidant enzymes, soluble carbohydrates, free amino acids, reactive oxygen species, and metabolic profile) were investigated after 25 days of drought imposition. The detailed data are described in Chapter III.

# 3 CHAPTER I

# THEORETICAL FRAMEWORK

## 3.1 Water stress in plants

Abiotic stresses and their interactions are responsible for preventing or limiting agricultural production over large areas of arable land worldwide, with water scarcity being one of the major limiting factors (Chaudhry; Sidhu, 2021). In plants, water plays a crucial role in several cellular processes. Therefore, the water absence or restriction affects all fundamental processes (Kapoor *et al*., 2020), since plants lose most of the water absorbed through transpiration. Thus, one of agriculture's major challenges is to favor a good balance between transpiration and photosynthesis rates based on the available soil water (Gleason *et al*., 2021).

Stress occurs in drought situations when water content is below critical thresholds for the crop due to insufficient precipitation or irrigation (Veimrober Júnior *et al*., 2022). In this scenario, temperature and air humidity will also have a significant impact, considering the dynamics of water throughout the soil-plant-atmosphere system and how this relationship influences water availability for the plant. These effects are more intense in arid and/or semiarid climates, where high temperatures and low water availability result in annual evaporation rates exceeding rainfall replenishment (Hatfield; Dold, 2019).

When soil water is less than the plant's water requirement, turgor loss occurs, directly affecting growth, as water intake is responsible for cell elongation and expansion. Indirectly, without water, there is no photosynthesis (Hemati *et al*., 2022). Thus, the metabolic imbalance induced by water stress will compromise cell division, also affecting leaf gas exchange, phloem transport, and the rigidity and mechanical stability of tissues (Wang *et al*., 2019).

According to Ihuoma and Madramootoo (2017), drought-stressed plants activate response mechanisms, comprising physiological, biochemical, metabolic, and/or molecular pathways. Together, the routes induce multifaceted influence on the

plant's entire functional machinery, which may be reversible or irreversible depending on the degree of impairment of important structures. In this context, stomatal closure to prevent water loss is one of the first strategies that alter the water use mechanism and increase water use efficiency (Hatfield; Dold, 2019; Ilyas *et al*., 2020). However, stomatal closure directly impacts the reduction of $CO_2$ absorption, which will limit photosynthesis, reducing the production of photoassimilates and metabolites necessary for plant growth and development (Chen *et al*., 2017; Escalera *et al*., 2019). In field conditions, high radiation and temperatures exacerbate the stress condition caused by drought, resulting in the plant redirecting its energy expenditure towards root expansion to seek water and absorb nutrients.

Conditions of prolonged water restriction led to productivity losses, considering the essentiality of water from germination to stand stabilization (Zuffo *et al*., 2020). During germination process, the seed needs to imbibe a large quantity of water to initiate the degradation of reserve tissues and activation of metabolism (Bradford, 1995). Also, once gene expression is environment-dependent (Park *et al*., 2018), under water restriction conditions may interfer in vigor, consequently affecting stand uniformity (Zuffo *et al*., 2020).

In the reproductive stage, the water restriction reduces the anthesis period, which is the flower opening period (Chowdhury *et al*., 2021). For species that depend on cross-pollination and pollinators, water limitation can promote an absence of insects according to their cycle. Additionally, the energy produced is limited, and photoassimilates do not reach the flower, resulting in a much higher abortion percentage due to the high flower energy requirement (Lamin-Samu *et al*., 2021), which subsequently shortens the cycle. During grain filling, the direction of photoassimilates is also reduced, impairing the respiratory process and consequently leading to a decrease in production (Farooq *et al*., 2016).

The main reason for exacerbated drought damage is the reduced photosynthetic capacity, which directly or indirectly affects numerous other plant processes. Therefore, Kramer and Boyer (1995) already reported that the effects of drought will vary according to a series of factors, such as intensity and duration, including abrupt restriction, dry spells, or prolonged drought, as well as the growth stage at the time of stress exposure. All these variation factors should be considered in studies with water stress.

### 3.2 Response mechanisms against water deficit

Along with evolution, plants have developed mechanisms to counteract drought damage as a strategy to acclimate and/or adapt to water stress conditions, whether through drought escape or increased tolerance (Lamaoui *et al*., 2018). As morphological mechanisms, plants seek to avoid drought by reducing the energy expenditure of the reproductive cycle through flower abortion and by escaping drought through stomatal closure (Molinari *et al*., 2021), as well as by developing roots to seek and store water in cells and tissues. Expanding the root system is crucial for obtaining water from the environment, directing energy towards its expansion in size, density and proliferation (Kunert *et al*., 2016).

As a physiological mechanism, in addition to enhancing membrane stability, antioxidants production and growth regulation, plants may compartmentalize protective solutes and osmolytes, which are organic molecules that act to protect and stabilize membranes and other cellular structures. These molecules also act as osmoregulators, optimizing water absorption and retention, thereby maintaining turgor pressure and reducing damage and injuries caused by water restriction (Anjum *et al*., 2017). Additionally, among the molecular mechanisms, studies report an increase in the number of aquaporins (Andrade *et al*., 2016), the synthesis of stress proteins, and the production of signaling molecules (Chi *et al*., 2019).

The accumulation of proline is a frequent physiological response observed in various species under various stressful conditions (Siddique; Kandpal; Kumar, 2018). Pioneering studies by Smirnoff and Cumbes (1989) showed that proline can act as a defense molecule by adjusting the osmotic potential as compatible osmolytes, as well as scavenging reactive oxygen species (ROS) and stabilizing protein structures.

Similarly, to any other stress that affects plants, one inevitable consequence of drought is oxidative stress, because any imbalance in cellular structures and function promotes an increase in ROS, which can lead to oxidative damage in plants (Ashraf *et al*., 2019). Depending on stress intensity, the resulting damage can compromise basic organelle functions. Failure in ROS scavenging through efficient antioxidant machinery can impair energy and electron fluxes and prevent redox-regulated signaling processes (Noctor; Foyer, 2016).

Plants naturally possess antioxidant defense mechanisms that eliminate reactive oxygen species to prevent damage to cellular components (Noctor; Foyer, 2016). These mechanisms can be enzymatic and non-enzymatic pathways. Enzymatic mechanisms involve enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX). Non-enzymatic mechanisms include the activity and synthesis of carotenoids, glutathione, and ascorbate, which also aid in the elimination of reactive species (Sarker; Oba, 2018).

Therefore, understanding signaling molecules, assessing enzymatic and non-enzymatic activities, key synthesis points for stress response, and the transcription factors involved in activating antioxidant and protein mechanisms to cope with drought are crucial. Thus, discovering management techniques and cultivation strategies can be determinant for agricultural production in many productive areas facing water scarcity.

### 3.3 Use of biostimulants as mitigators of water stress

In the last few years, biostimulants have emerged as an important growth regulator for plants around the world (Bulgari; Franzoni; Ferrante, 2019). They are metabolic compounds not classified as fertilizers, employed to enhance crop performance and productivity, aiding in mitigating the effects of various stresses, including water deficit (Shukla *et al*., 2018). The biostimulants are considered eco-sustainable, with environmental benefits by not leaving residues in the soil, addressing current challenges for agricultural productivity (Campobenedetto *et al*., 2021).

These compounds consist of a complex mixture of bioactive molecules with various functions depending on the extraction method and application mode (Shukla *et al*., 2018). The biostimulants are grouped according to their composition, including those based on amino acids and protein hydrolysates (vegetal or animal), formulations based on humic substances, seaweed extracts, and microbiological-based products (Shahrajabian *et al*., 2021). Among these formulations, seaweed extract-based biostimulants are widely used in agriculture (Campobenedetto *et al*., 2021), applicable via soil, hydroponic systems, or foliar spray in order to optimize crop growth, mitigating

abiotic and biotic stresses, and enhancing grain or fruit quality (Stirk; Van Staden, 2020).

Due to complex composition, understanding the mechanisms by which biostimulants positively regulate plant physiological processes, leading to increased abiotic stress tolerance, poses a significant challenge (Yakhin *et al*., 2017). Physiologically, biostimulants act on nitrogen metabolism, antioxidant defense systems, and water relations (Goñi *et al*., 2016); biochemically, they affect hydrolysis, biosynthesis of molecules, and accumulation of reserves (Sunmonu *et al*., 2016); and genetically, they regulate genes involved in ROS production pathways of antioxidant mechanisms and hormonal regulation (Ertani *et al*., 2019). Moreover, they also impact plant epigenetics by inducing changes through chromatin modulation (Gupta *et al*., 2022).

Omidbakhshfard *et al*. (2020), studying how biostimulants alleviate oxidative stress in Arabidopsis plants, observed transcription factors and genes linked to stress adaptation mechanisms, including oxidative stress control by reducing reactive oxygen species production and autophagy. The authors also reported positive effects on enhancing photosynthesis, signaling, transcription of growth-related genes, and regulation of metabolites such as maltose and raffinose, contributing to defense under stress conditions. The beneficial outcomes of seaweed extract-based applications may be attributed to the synergy of various compounds like hormones, betaines, polyamines, alginates, carrageenans, fucans, and phlorotannins (Omidbakhshfard *et al*., 2020).

Even at low application rates, seaweed-based biostimulants have demonstrated potential in numerous studies to stimulate plant growth, improve productive characteristics, expand root systems, and enhance plant tolerance to salt excess, water deficiency, and heat stress (Battacharyya *et al*., 2015). Rosa *et al*. (2021), studying increased soybean tolerance to water deficiency using biostimulants based on fulvic acids and *A. nodosum* (L.) seaweed extract, found that all treated plants produced more than stressed-nontreated plants, showing higher photosynthetic rates, more efficient mechanisms to dissipate excess energy, and increased antioxidant enzyme activities. Testing the responses of this compound at various stages of crop development, combined with different dosages and already adapted genotypes, is a great advantage.

Shukla *et al*. (2018) observed that soybean plants treated with *A. nodosum* (L.)

seaweed extract biostimulant exhibited reduced wilting degree, as well as 50% higher water content and 46% higher stomatal conductance compared to controls. Furthermore, the authors noted that treated plants captured more ROS and had faster rehydration rates, while also modulating ABA biosynthesis and increasing gene expression related to water stress tolerance.

In this context, numerous studies are being conducted to improve the development and productivity of crops under drought conditions mediated by biostimulants. Thus, understanding the biostimulant actions in plant physiology, biochemistry, and morphology become essential to elucidate the defense mechanisms in different plant species. Particularly, evidence of how biostimulants can counteract drought damage and improve the productivity of soybean cultivars adapted to semiarid regions remains to be elucidated.

### 3.4 Soybean crop

Soybean (*Glycine max*) is an oilseed native to Asia that has been cultivated in Brazil for over a century. The crop presents significant investment in research throughout its production chain, based on the edaphoclimatic conditions in various regions of the country (Lima *et al*., 2019). The soybean holds great economic importance for Brazil and the world, driving billions of dollars in revenue (AGROSTAT, 2024). In Brazil, the production is approximately 147 million tons over an area of 45 million hectares, with a yield of 3,259 kg/ha (CONAB, 2024). This amount represents a 7.7% decrease in productivity compared to the previous season, due to the El Niño climate event, which contributed to increased global temperatures and extreme weather conditions in several producing regions. In Piauí, despite exceptional climatic events, soybean-cultivated areas are progressively increasing, accomplished with satisfactory yields.

Adequate water availability is determinant to the success of this oilseed crop (He *et al*., 2017), particularly with the progresses through the development stages, where water demand increase reaching the maximum peak during the reproductive stage (flowering R1-R2, fruiting R3-R4, and grain filling R5-R6). These stages are the most sensitive to water restriction and have the greatest negative impact on

productivity (Oya *et al*., 2004; Montoya *et al*., 2017).

Water scarcity during reproductive phase may result in fewer and smaller pods and grains, lower seed weight, leaf senescence, and a shortened plant life cycle, which can impair grain filling (Farooq *et al*., 2016). According to He *et al*. (2017), drought stress can significantly reduce soybean grain yield while increasing water use efficiency (WUE). This efficiency is an important parameter for indicating plant resistance under water stress (Hatfield; Dold, 2019).

The development of new soybean genotypes for cultivation in semiarid regions, by plant breeders, usually includes several requirements, like i.) resistance to water scarcity and high temperatures (Yang *et al*., 2020); ii.) short cycle to avoid the most critical period of drought (Saleem *et al*., 2022); iii.) elevated efficiency in water use (Feng *et al*., 2022); iv.) and resistance to pests and diseases (Tripathi *et al*., 2022). In regions containing abundance of water availability, cultivars are selected mainly for their high yield and longer development cycle, taking advantage of the continuous availability of water (Umburanas *et al*., 2022).

In the last few years, numerous biological compounds have been available for cultivating agricultural species. However, for effective use, we need to understand how soybeans physiologically respond in water-limited environments to improve productivity guides future breeding goals (Koester *et al*., 2014). Given its national, regional, and local economic importance, studies on stress-attenuating products are extremely important, as periods of drought can significantly compromise productive outcomes.

**REFERENCES**

AGROSTAT - Estatística de Comércio Exterior do Agronegócio Brasileiro. **Indicadores da agricultura**. 2024. Disponível em: <https://indicadores.agricultura.gov.br/agrostat/index.htm_>. Acessed at: Jun 08, 2024.

ANDRADE, L. M.; NOBILE, P. M.; RIBEIRO, R. V.; OLIVEIRA, J. F. N. C. DE; FIGUEIRA, A. V. O.; FRIGEL, L. T. M.; NUNES. D.; PERECIN, D.; BRITO, M. S.; PIRES, R. C. M. Characterization of PIP2 aquaporins in Saccharum hybrids. **Plant Gene**, v. 5, p. 31-37, 2016.

ANJUM, S.; ASHRAF, U.; TANVEER, M.; KHAN, I.; HUSSAIN, S.; SHAHZAD, B.; ZOHAIB, A.; ABBAS, F.; SALEEM, M.F.; ALI, I.; WANG, L.C. drought induced changes in growth, osmolyte accumulation and antioxidant metabolism of three maize hybrids. **Frontiers in Plant Science**, v. 8, p. 69, 2017.

ASHRAF, M. A.; RIAZ, M.; ARIF, S. M.; RASHEED, R.; IQBAL, M.; HUSSAIN, I.; MUBARIK, M. S. The role of non-enzymatic antioxidants in improving abiotic stress tolerance in plants. *In:* HASANUZZAMAN, M.; FUJITA, M.; OKU, H.; ISLAM, M. T. **Plant Tolerance to Environmental Stress**. 1. ed. Serbia: CRC Press, 2019, p. 129 - 144.

BATTACHARYYA, D.; BABGOHARI, M. Z.; RATHOR, P.; PRITHIVIRAJ, B. Seaweed extracts as biostimulants in horticulture. **Scientia Horticulturae**, v. 196, p. 39-48, 2015.

BRADFORD, K. J. Water Relations in Seed Germination. In: KIGEL, J.; GALILI, G. **Seed Development and Germination**. 1. ed. California: Routledge, 1995, p. 351-396.

BULGARI, R.; FRANZONI, G.; FERRANTE, A. Biostimulants application in horticultural crops under abiotic stress conditions. **Agronomy**, v. 9, n. 6, p. 306, 2019.

CAMPOBENEDETTO, C.; AGLIASSA, C.; MANNINO, G.; VIGLIANTE, I.; CONTARTESE, V.; SECCHI, F.; BERTEA, C. M. A. Biostimulant based on seaweed (*Ascophyllum nodosum* and *Laminaria digitata*) and yeast extracts mitigates water stress effects on tomato (*Solanum lycopersicum* L.). **Agriculture**, v. 11, n. 6, p. 557, 2021.

CHANDRASEKARAN, M.; PARAMASIVAN, M. Arbuscular mycorrhizal fungi and antioxidant enzymes in ameliorating drought stress: a meta-analysis. **Plant and Soil**, p. 1-9, 2022.

CHAUDHRY, S.; SIDHU, G. P. S. Climate change regulated abiotic stress mechanisms in plants: a comprehensive review. **Plant Cell Reports**, v. 41, n. 1, p. 1-31, 2021.

CHEN, D.; CHAI, S.; MCINTYRE, C. L.; XUE, G. Overexpression of a predominantly root-expressed NAC transcription factor in wheat roots enhances root length, biomass and drought tolerance. **Plant Cell Reports**, v. 37, n. 2, p. 225-237, 2017.

CHI, Y. H.; HUN, P. J.; BAE, S. B.; KIM, M. G.; KOO, S. S.; PHAN, K. A. T; PAENG, S. K.; KIM, A.; OH, H. T.; WI, S. D.; CHAE, H. B.; YUN, D. J.; LEE, E. S.; KANG, C. H.; LEE, S. Y. The physiological functions of universal stress proteins and their molecular mechanism to protect plants from environmental stresses. **Frontiers in Plant Science**, v. 10, p. 750, 2019.

CHOWDHURY, M. K.; IQBAL, M. A.; ELSANAFAWY, N. E.; ISLAM, M. S.; MA, H.; JAVED, T.; ANWAR, S.; BAHADUR, M. M.; RAZA, A.; ALAMRI, S.; ISLAM, M. R.; SHABBIR, R.; SABAGH, A. E.; HAKIM, M. A.; SOROUR, S. Evaluation of drought tolerance of some wheat (*Triticum aestivum* L.) genotypes through phenology, growth, and physiological indices. **Agronomy**, v. 11, n. 9, p. 1792, 2021.

CONAB - Companhia Nacional de Abastecimento. **Acompanhamento da Safra Brasileira de Grãos**: décimo levantamento. Brasília. 2024.

EL-BELTAGI, H. S.; MOHAMED, H. I.; SOFY, M. R. Role of ascorbic acid, glutathione and proline applied as singly or in sequence combination in improving chickpea plant through physiological change and antioxidant defense under different levels of irrigation intervals. **Molecules**, v. 25, n. 7, p. 1702, 2020.

ERTANI, A.; CORTES, S. S.; NARDI, S.; FOGGIA, M. D.; FRANCIOSO, O.; SCHIAVON, M. Effects of two protei n hydrolysates obtained from chickpea (*Cicer arietinum* L.) and *Spirulina platensis* on *Zea mays* (L.) plants. **Frontiers in Plant Science**, v. 10, p. 954, 2019.

ESCALERA, R. A. V.; PIMENTEL, J. R.; TROYJACK, C.; CARVALHO, I. R.; SZARESKI, V. J.; PETER, M.; FACHI, S. M.; SILVA, F. L.; FONSECA, L. L.; JAQUES, L. B. A. Physiological quality of rice and soybean seeds produced under hydric stress in greenhouse. **Journal of Agricultural Science**, v. 11, n. 12, p. 22, 2019.

FANG, W.; HUANG, S.; HUANG, Q.; HUANG, G.; WANG, H.; LENG, G.; WANG, L.; GUO, Y. Probabilistic assessment of remote sensing-based terrestrial vegetation vulnerability to drought stress of the Loess Plateau in China. **Remote Sensing of Environment**, v. 232, p. 111290, 2019.

FAROOQ, M.; GOGOL, N.; BARTHAKUR, S.; BAROOWA, B.; BHARADWAJ, N.; ALGHAMDI, S. S.; SIDDIQUE, K. H. M. Drought stress in grain legumes during reproduction and grain filling. **Journal of Agronomy and Crop Science**, v. 203, n. 2, p. 81-102, 2016.

FELISBERTO, G.; SCHWERZ, F.; UMBURANAS, F.S.; DOURADO-NETO, D.; REICHARDT, K. Physiological and productive responses of soybeans under water deficit. **Journal of Crop Science and Biotechnology**, v. 12, p. 809692, 2022.

FENG, Y.; RICHARDS, R. A.; JIN, Y.; SIDDIQUE, K. H. M.; LI, F.; HE, J. Yield and water-use related traits in landrace and new soybean cultivars in arid and semi-arid areas of China. **Field Crops Research**, v. 283, p. 108559, 2022.

GHOSH, U. K.; ISLAM, M. N.; SIDDIQUI, M. N.; CAO, X.; KHAN, M. A. R. Proline, a multifaceted signalling molecule in plant responses to abiotic stress: understanding the physiological mechanisms. **Plant Biology**, v. 24, n. 2, p. 227-239, 2021.

GLEASON, S. M.; NALEZNY, L.; HUNTER, C.; BENSEN, R.; CHINTAMANANI, S.; COMAS, L. H. Growth and grain yield of eight maize hybrids are aligned with water transport, stomatal conductance, and photosynthesis in a semi-arid irrigated system. **Physiologia Plantarum**, v. 172, n. 4, p. 1941-1949, 2021.

GOÑI, O.; ANTOINE, F.; QUILLE, P.; MCKEOWN, P. C.; SPILLANE, C.; O'CONNELL, S. Comparative transcriptome analysis of two *Ascophyllum nodosum* extract biostimulants: same seaweed but different. **Journal of Agricultural and Food Chemistry**, v. 64, n. 14, p. 2980-2989, 2016.

GUPTA, S.; DOLEŽAL, K.; KULKARNI, M. G.; BALÁZS, E.; VAN STADEN, J. Role of non-microbial biostimulants in regulation of seed germination and seedling establishment. **Plant Growth Regulation**, v. 97, n. 2, p. 271-313, 2022.

HASANUZZAMAN, M.; NAHAR, K.; ANEE, T. I.; KHAN, M. I.; FUJITA, M. Silicon-mediated regulation of antioxidant defense and glyoxalase systems confers drought stress tolerance in *Brassica napus* L. **South African Journal of Botany**, v. 115, p. 50-57, 2018.

HATFIELD, J. L; DOLD, C. Water-use efficiency: advances and challenges in a changing climate. **Frontiers in Plant Science**, v. 10, p. 103, 2019.

HE, J.; DU, Y.; WANG, T.; TURNER, N. C.; YANG, R. P.; JIN, Y.; XI, Y.; ZHANG, C.; CUI, T.; FANG, X. W. Conserved water use improves the yield performance of soybean (*Glycine max* (L.) Merr.) under drought. **Agricultural Water Management**, v. 179, p. 236-245, 2017.

HEMATI, A.; MOGHISEH, E.; AMIRIFAR, A.; MOFIDI-CHELAN, M.; LAJAYER, B. A. Effects of drought stress in plants. **Plant Stress Mitigators**, p. 113-124, 2022.

HIDANGMAYUM, A.; DWIVEDI, P.; KATIYAR, D.; HEMANTARANJAN A. Application of chitosan on plant responses with special reference to abiotic stress. **Physiology and Molecular Biology of Plants**, v. 25, n. 2, p. 313-326, 2019.

IHUOMA, S.; MADRAMOOTO, C. A. Recent advances in crop water stress detection. **Computers and Electronics in Agriculture**, v. 141, p. 267-275, 2017.

ILYAS, M.; NISAR, M.; KHAN, N.; HAZRAT, A.; KHAN, A. H.; HAYAT, K.; FAHAD, S.; KHAN, A.; ULLAH, A. Drought tolerance strategies in plants: a mechanistic approach. **Journal of Plant Growth Regulation**, v. 40, n. 3, p. 926-944, 2020.

INOUE, T.; MATSUSHIMA, Y.; SUNAGA, M.; SAKODA, K.; ITO, M.; YAMORI, W.; YUNHEN, Q. Minimizing VPD fluctuations maintains higher stomatal conductance and photosynthesis, resulting in improvement of plant growth in lettuce. **Frontiers in Plant Science**, v. 12, p. 646144, 2021.

KAPOOR, D.; BHARDWAJ, S.; LANDI, M.; SHARMA, A.; RAMAKRISHNAN, M.; SHARMA, A. The impact of drought in plant metabolism: how to exploit tolerance mechanisms to increase crop production. **Applied Sciences**, v. 10, n. 16, p. 5692, 2020.

KHAN, M. I. R.; ASHFAQUE, F.; CHHILLAR, H.; IRFAN, M.; KHAN, N. A. The intricacy of silicon, plant growth regulators and other signaling molecules for abiotic stress tolerance: an entrancing crosstalk between stress alleviators. **Plant Physiology and Biochemistry**, v. 162, p. 36-47, 2021.

KOESTER, R. P.; SKONECZKA, J. A.; CARY, T. R.; DIERS, B. W.; AINSWORTH, E. A. Historical gains in soybean (*Glycine max* Merr.) seed yield are driven by linear increases in light interception, energy conversion, and partitioning efficiencies. **Journal of Experimental Botany**, v. 65, n. 12, p. 3311-3321, 2014.

KRAMER, P. J; BOYER, J. S. **Water relations of plants and soils**. San Diego: Academic Press. 1995, 512 p.

KUNERT, K. J; VORSTER, B. J.; FENTA, B. A.; KIBIDO, T.; DIONISIO, G.; FOYER, C. H. Drought stress responses in soybean roots and nodules. **Frontiers in Plant Science**, v. 7, p. 1015, 2016.

LAMAOUI, M.; JEMO, M.; DATLA, R.; BEKKAOUI, F. Heat and drought stresses in crops and approaches for their mitigation. **Frontiers in Chemistry**, v. 6, p. 26, 2018.

LAMIN-SAMU, A. T.; FARGHA, L. M.; ALI, M.; LU, G. Morpho-physiological and transcriptome changes in tomato anthers of different developmental stages under drought stress. **Cells**, v. 10, n. 7, p. 1809, 2021.

LIMA, M.; SILVA JUNIOR, C. A.; RAUSCH, L.; GIBBS, H. K.; JOHANN, J. A. Demystifying sustainable soy in Brazil. **Land Use Policy**, v. 82, p. 349-352, 2019.

MOLINARI, M. D. C.; FUGANTI-PAGLIARINI, R.; MARCOLINO-GOMES, J.; BARBOSA, D. A.; MARIN, S. R. R.; MERTZ-HENNING, L. M.; NEPOMUCENO, A. L. sensitivity to drought. **Journal of Plant Interactions**, v. 16, n. 1, p. 187-200, 2021.

NOCTOR, G.; FOYER, C. H. Intracellular redox compartmentation and ROS-related communication in regulation and signaling. **Plant Physiology**, v. 171, n. 3, p. 1581-1592, 2016.

OMIDBAKHSHFARD, M. A.; SUJEETH, N.; GUPTA, S.; OMRANIAN, N.; GUINAN, K. J.; BROTMAN, Y.; NIKOLOSKI, Z.; FERNIE, A. R.; MUELLER-ROEBER, B.; GECHEV, T. S. A Biostimulant obtained from the seaweed *Ascophyllum nodosum* protects *Arabidopsis thaliana* from severe oxidative stress. **International Journal of Molecular Sciences**, v. 21, n. 2, p. 474, 2020.

OYA, T.; NEPOMUCENO, A. L.; NEUMAIER, N.; FARIAS, J. R. B.; TOBITA, S.; ITO, O. Drought tolerance characteristics of brazilian soybean cultivars— evaluation and characterization of drought tolerance of various brazilian soybean cultivars in the field. **Plant Production Science**, v. 7, n. 2, p. 129-137, 2004.

PARK, S. J.; SANGGEUN, C.; YANG, G. S.; KIM, P. M.; YOON, S.; KIM, J. H.; SUNG, J. The chemical fluctuation theorem governing gene expression. **Nature Communications**, v. 9, n. 1, p. 1-12, 2018.

RAZA, A.; TABASSUM, J.; MUBARIK, M. S.; ANWAR, S.; ZAHRA, N.; SHARIF, Y.; HAFEEZ, M. B.; ZHANG, C.; CORPAS, F. J.; CHEN, H. Hydrogen sulfide: an emerging component against abiotic stress in plants. **Plant Biology**, v. 24, n. 4, p. 540-558, 2021.

ROSA, V. R.; SANTOS, A. L. F.; SILVA, A. A.; SAB, M. P. V.; GERMINO, G. H.; CARDOSO, F. B.; SILVA, M. A. Increased soybean tolerance to water deficiency through biostimulant based on fulvic acids and *Ascophyllum nodosum* (L.) seaweed extract. **Plant Physiology and Biochemistry**, v. 158, p. 228-243, 2021.

SALEEM, A.; APER, J.; MUYLLE, H.; BORRA-SERRANO, I.; QUATAERT, P.; LOOTENS, P.; SWAEF, T.; ROLDÁN-RUIZ, I. Response of a diverse European soybean collection to “short duration” and “long duration” drought stress. **Frontiers in Plant Science**, v. 13, p. 818766, 2022.

SARKER, U.; OBA, S.; Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of Amaranthus tricolor. **Scientific Reports**, v. 8, n. 1, p. 1-12, 2018.

SELEIMAN, M. F.; AL-SUHAIBANI, N.; ALI, N.; AKMAL, M.; ALOTAIBI M.; REFAY, Y.; DINDAROGLU, T.; ABDUL-WAJID, H. H.; BATTAGLIA, M. L. Drought stress impacts on plants and different approaches to alleviate its adverse effects. **Plants**, v. 10, n. 2, p. 259, 2021.

SHAHRAJABIAN, M. H.; CHASKI, C.; POLYZOS, N.; PETROPOULOS, S. A. Biostimulants application: a low input cropping management tool for sustainable farming of vegetables. **Biomolecules**, v. 11, n. 5, p. 698, 2021.

SHUKLA, P. S.; SHOTTON, K.; NORMAN, E.; NEILY, W.; CRITCHLEY, A. T.; PRITHIVIRAJ, B. Seaweed extract improve drought tolerance of soybean by regulating stress-response genes. **AoB Plant**s, v. 10, n. 1, p. plx051, 2018.

SIDDIQUE, A.; KANDPAL, G.; KUMAR P. Proline accumulation and its defensive role under diverse stress condition in plants: an overview. **Journal of Pure and Applied Microbiology**, v. 12, n. 3, p. 1655-1659, 2018.

SMIRNOFF, N.; CUMBES, Q. J. Hydroxyl radical scavenging activity of compatible solutes. **Phytochemistry**, v. 28, n. 4, p. 1057-1060, 1989.

STEINER, F.; ZUFFO, A. M.; OLIVEIRA, C. E. S.; ARDON, H. J. V.; SOUSA, T. O.; AGUILERA, J. G. Can potassium fertilization alleviate the adverse effects of drought stress on soybean plants? **Revista em Agronegócio e Meio Ambiente**, v. 15, n. 1, p. 1-17, 2022.

STIRK, W. A.; VAN STADEN, J. Potential of phytohormones as a strategy to improve microalgae productivity for biotechnological applications. **Biotechnology Advances**, v. 44, p. 107612, 2020.

SUNMONU, T. O.; KULKARNI, M. G.; VAN STADEN, J.; SMOKE-WATER. karrikinolide and gibberellic acid stimulate growth in bean and maize seedlings by efficient starch mobilization and suppression of oxidative stress. **South African Journal of Botany**, v. 102, p. 4-11, 2016.

TIWARI, R. K.; LAL, M. K.; KUMAR, R.; CHOURASIA, K. N.; NAGA, K. C.; KUMAR, D.; DAS, S. K.; ZINTA G. Mechanistic insights on melatonin-mediated drought stress mitigation in plants. **Physiologia Plantarum**, v. 172, n. 2, p. 1212-1226, 2021.

TRIPATHI, N.; TRIPATHI, M. K.; TIWARI, S.; PAYASI, D. K. Molecular breeding to overcome biotic stresses in soybean: update. **Plants**, v. 11, n. 15, p. 1967, 2022.

UMBURANAS, R. C.; KAWAKAMI, J.; AINSWORTH, E. A.; FAVARIN, J. L.; ANDERLE, L. Z.; DOURADO-NETO, D.; REICHARDT, K. Changes in soybean cultivars released over the past 50 years in southern Brazil. **Scientific Reports**, v. 12, n. 1, p. 508, 2022.

VEIMROBER JÚNIOR, L. A. A.; SILVA, A. J. P.; GHEYI, H. R.; NASCIMENTO, F. A. L.; SILVA, M. G.; VELLAME, L. M. Water productivity of passion fruit under different forms of propagation and soil-based irrigation management criteria. **Irrigation Science**, v. 40, n. 3, p. 423-433, 2022.

WANG, B.; LIU, C.; ZHANG, D.; HE, C.; ZHANG, J.; LI, Z. Effects of maize organ-specific drought stress response on yields from transcriptome analysis. **BMC plant biology**, v. 19, n. 1, p. 1-19, 2019.

YAKHIN, O. I.; LUBYANOV, A. A.; BROUN, P. H.; YAKHIN, I. A. Biostimulants in plant science: a global perspective. **Frontiers in Plant Science**, v. 7, p. 2049, 2017.

YANG, M.; JAHUFER, M. Z. Z.; HE, J.; DONG, R.; HOFMANN, R.; SIDDIQUE, K. H. M.; LI, FM. Effect of traditional soybean breeding on water use strategy in arid and semi-arid areas. **European Journal of Agronomy**, v. 120, p. 126128, 2020.

ZUFFO, A. M.; STEINER, F.; AGUILERA, J. G.; TEODORO, P. E.; TEODORO, L. P. R.; BUSCH, A. Multi-trait stability index: a tool for simultaneous selection of soya bean genotypes in drought and saline stress. **Journal of Agronomy and Crop Science**, v. 206, n. 6, p. 815-822, 2020.

# 4 CHAPTER II

## *Ascophyllum nodosum*-DERIVED BIOSTIMULANT PROMOTES PHYSIOLOGICAL CONDITIONING TO INCREASE SOYBEAN YIELD IN A SEMIARID CLIMATE


## ABSTRACT

The use of algae-based biostimulants as promoters for the growth, flowering, and fruiting phases has been intensified in recent years, despite a lack of information about the ideal doses for maximum efficiency by the plant. This study aimed to select the concentration and application timing of *Ascophyllum nodosum*-derived biostimulant capable of modulating the physiology and increasing the yield of soybean plants from semiarid regions. The trials were carried out in a greenhouse by applying leaf spraying of biostimulant at 0, 0.5, 1.0 and 1.5 L $ha^{-1}$, in a single or divided dosage, in different developmental stages (V4, V4+R1, R1, R1+R4) of Extrema soybean cultivar. Growth, water content, pollen viability, gas exchanges and yield were investigated. The biostimulant promoted better physiological conditioning and productive responses, depending on the dose and application timing. *A. nodosum*-based biostimulant at 1.5 L $ha^{-1}$, applied 0.75 L $ha^{-1}$ at V4 + 0.75 L $ha^{-1}$ at R1 (T12), was found to be the most significant treatment, improving the net photosynthesis, water status and plant growth, which resulted in a 12% increase in yield. In conclusion, the dosage and timing of application play pivotal roles in eliciting physiological and productive responses through biostimulants. Seaweed-based biostimulants emerge as essential components to optimizing the cultivation and yield of soybean plants, serving as efficient and sustainable biological regulators.


**Keywords:** *Glycine max.* Growth regulators. Semiarid region. Seaweed algae extracts. Plant performance.

## 4.1 Introduction

Soybean is an important leguminous crop broadly explored in countries like Brazil, United States, Argentina and China, corresponding to the largest producers responsible for over 86% of global production in the 2022/23 season (AGROSTAT, 2022; USDA, 2023). Brazil stands out as the world's largest soybean producer, with a planted area exceeding 43 million hectares (CONAB, 2023; USDA, 2023). The crop is widely cultivated in semiarid regions and presents relative tolerance to water stress.

In the last decades, climate change has led to significant alterations in environmental conditions worldwide, mostly in temperature and precipitation patterns (Malhi; Kaur; Kaushik, 2021). Environmental stresses such as drought and high temperatures have affected arable areas and decreased the yield of numerous plant species (Shahzad *et al*., 2021; Leite-Filho *et al*., 2021). The situation is challenging for the soybean crop due to severe damage to morphological, physiological, and biochemical pathways, impacting all stages of plant development and yield, and resulting in economic losses (Chaudhry; Sidhu, 2021; Silva *et al*., 2021; CONAB, 2023; USDA, 2023).

The increasing demand for techniques to mitigate the damages caused by climate variations has forced producers to select cultivars adapted to adverse environments, even if they present lower productivity capacity (Hassan *et al*., 2021). The biostimulants can constitute an alternative strategy to overcome environmental limitations, given their composition of active principles capable of activating plant metabolic processes (Hasanuzzaman *et al*., 2018; Hidangmayum *et al*., 2019; Khan *et al*., 2021; Raza *et al*., 2021; Tiwari *et al*., 2021). These compounds consist of a mixture of plant growth regulators containing numerous chemical compounds, such as amino acids and nutrients, which not only contribute to crop yield but can also alleviate the deleterious effects of specific abiotic stresses (Shahrajabian *et al*., 2021; Cocetta *et al*., 2022; Gupta *et al*., 2022).

Numerous studies have evidenced the contributions of biostimulants based on seaweed *A. nodosum* (ANE) to the performance of species under stressful conditions (Shukla; Prithiviraj, 2021; Shahzad *et al*., 2023). In tomato plants, the biostimulant protected against membrane damage and reactive oxygen species (ROS), resulting in reduced oxidative stress and improved photochemical performance under stress

(Hernández-Herrera *et al*., 2022; Villa *et al*., 2023). In stressed-watermelon plants, the biostimulants supported the root system, leaf biomass, and increased leaf number (Bantis; Koukounaras, 2022). In soybeans, ANE-biostimulants were able to improve the photosynthetic efficiency by dissipating energy excess and increasing the enzymatic antioxidant system under drought (Rosa *et al*., 2021). In addition, ANE-treated stressed plants displayed faster rehydration associated with elevated water content and stomatal conductance and enhanced ROS scavenging (Shukla *et al*., 2018).

Nevertheless, there is no evidence of ANE-biostimulants improving the performance of soybean cultivars from semiarid regions, particularly regarding the timing of application and sampling procedures, which are crucial factors in eliciting positive responses in plants (Andreotti *et al*., 2022; Ali *et al*., 2023). These biostimulant aspects become critical in production of crops from semiarid regions that utilize cultivars with diverse levels of resilience and productive potential.

Our working hypothesis is that the *A. nodosum*-based biostimulant at specific concentrations and application time improves the physiological and productive performance of soybean plants. This hypothesis was tested by cultivating a semiarid-adapted soybean cultivar (Extrema) using three ANE-biostimulant dosage levels applied at three distinct development stages. The photosynthetic performance, water status, growth and yield trials were assessed in different stages of plant development.

## 4.2 Material and Methods

### 4.2.1 Study site, experimental conditions and treatments

The experiment was carried out in a greenhouse at the Federal University of Piauí (UFPI), Campus Professora Cinobelina Elvas, Bom Jesus, Piauí, Brazil (located at 9°05'02.6"S, 44°19'32.8"W, and 277 m above sea level), between June and September 2022. During the trials, temperature and relative humidity data inside the greenhouse were monitored using a digital thermo-hygrometer. Figure 1 shows the

environmental data along the study, encompassing the highest temperature peaks and low air humidity, characteristic of semiarid regions.

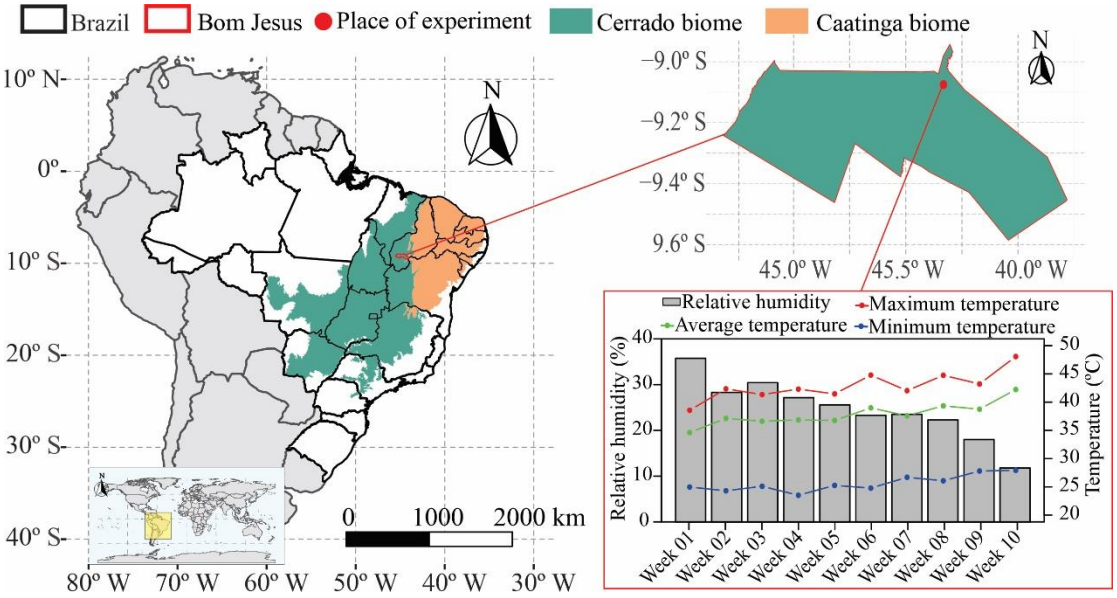



**Figure 1** - Location of the experimental area and dynamics of mean, maximum and minimum temperature, and air relative humidity during the trials with soybean plants, Extrema cultivar, from June to September 2022.

During the trials, soil samples of typical dystrophic Yellow Latosol from the UFPI experimental area were collected from the 0-0.20 layer and subjected to chemical analysis (Appendix A). The soil was corrected and fertilized according to the recommendations for the cerrado soil fertilization (Sousa; Lobato, 2004), and thereafter used to fill plastic pots of 11 dm³. Sowing was done by adding five seeds from Extrema cultivar per pot. Thinning procedures were carried out at 7 and 14 days after sowing to maintain only one plant per pot. The Extrema cultivar is characterized mainly by its indeterminate growth cycle, moderate fertility requirements, high yield potential and stature, and suitability for semiarid regions.

Irrigation management was conducted daily using the pot weighing method (Veihmeyer; Hendrickson, 1931), with water replacement performed according to the amount required to reach 60% of the field capacity (FC) during the vegetative stage and 80% FC during the reproductive stage (Miranda *et al.*, 2023). The seaweed-based biostimulant provided by Brandon Bioscience (Tralee, Ireland) contains 48% extract (480 g/L), is a fluid solution by physical nature, composed of 100% *A. nodosum*, and is initially recommended at a rate of 1.0 $ha^{-1}$ for cereals in general. In all cases, 2.0 mL of the biostimulant, appropriately diluted, were sprayed in the leaves considering the

crop stage (Vegetative 4 – V4, Reproductive 1 – R1, Reproductive 4 – R4), application timing and dose (L $ha^{-1}$) considering a population of 220 thousand plants $ha^{-1}$ in the field (Figure 2).

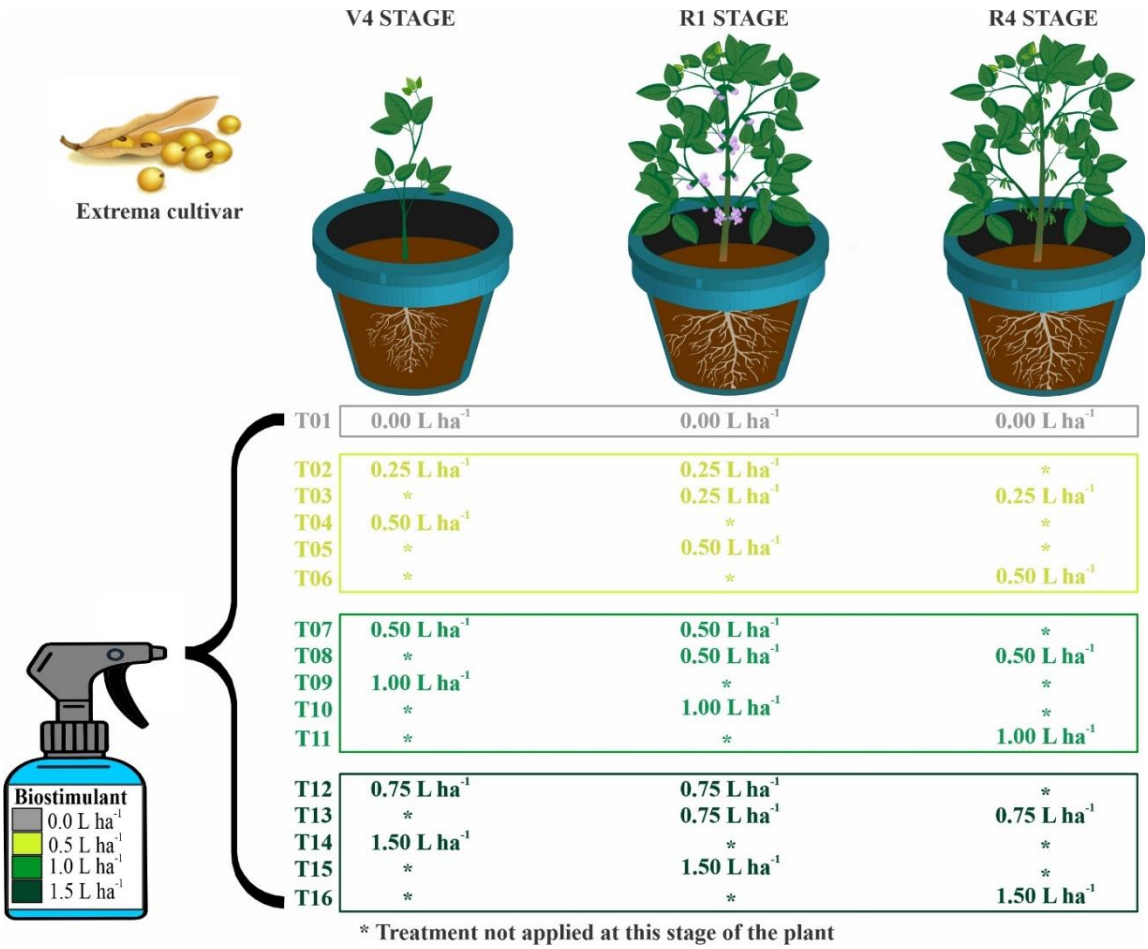



**Figure 2 –** Summary of *Ascophyllum nodosum*-based biostimulant treatments on different stages of soybean crop, Extrema cultivar, under greenhouse conditions. The plants from T01 were sprayed with distilled water, constituting the negative control. The biostimulant was applied at doses 0, 0.5, 1.0 and 1.5 L $ha^{-1}$.

The design was completely randomized, with sixteen treatments and four replications. The treatments consisted of a combination of the biostimulant dose (0.5, 1.0 e 1.5 L $ha^{-1}$) and five application timings, which occurred in a single application or two applications depending on the crop development stage (V4, V4-R1, R1, R1-R4, and R4) (Figure 2). A group of plants was sprayed with 2.0 mL of distilled water, constituting the control treatment (T1). Registers of the entire experiment are documented by photos (Appendix B).

### 4.2.2 Photosynthetic parameters

Gas exchange measurements were conducted on the first fully expanded leaf using an infrared gas analyzer (IRGA, Model GFS3000 - Walz) between 8:30 and 10:30 a.m. on completely sunny days at the V4, R4, and R6 stages, seven days after biostimulant applications according to Figure 2. The assays of net photosynthesis (*A*), stomatal conductance ($g_s$), and transpiration (*E*) were performed with a photosynthetic photon flux density (PPFD) of 1,000 μmol $m^{-2}$ $s^{-1}$ and an internal $CO_2$ concentration of 400 ppm.

### 4.2.3 Hydric status

To estimate the relative water content (RWC), 10 discs with a diameter of 1.0 cm were extracted from fully expanded leaves of each treatment. Initially, the fresh weight (FW) of the discs was measured, and subsequently, the samples were immersed in distilled water for 24 hours to determine the turgid weight (TW). The material was then placed in an oven at 60 °C for 72 h to obtain the dry weight (DW). Finally, the RWC was calculated following the methodology of Čatský (1960), using the following formula:

$$RWC = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{TW} - \mathrm{DW})} \times 100$$

### 4.2.4 Biometric measurements and yield

The plant height was measured in the reproductive stage (R2 and R4), post-application of the biostimulant, from the first visible internode above the ground to the last node of the apex, using a measuring tape. The number of flowers and pods was counted manually.

Soybean yield was estimated at the end of the experiment (R8), 94 days after planting, in kilograms per hectare (kg $ha^{-1}$), according to the formula:

$$\boldsymbol{Yield} = \frac{220.000 \times NPP \times NGP \times WTG}{1000}$$

Where NPP is the number of pods per plant, NGP is the number of grains per plant, and WTG is the weight of one thousand grains.

### 4.2.5 Pollen viability

To verify the influence of the biostimulant on plant fertility, four floral buds at R2 stage were collected during the pre-anthesis stage, between 9:00 and 10:00 a.m., in each replication. The material was used to prepare slides using the anther crushing technique (Guerra; Souza, 2002). The identification of viable pollen was performed using the Alexander reactive stain (Alexander, 1980). In each slide, 200 pollen grains were counted using an optical microscope with a 40x objective. Pollen grains with purple coloring were classified as viable, while those without internal coloring were considered non-viable. The percentage of viable pollen (VP) was determined by the equation:

$$\boldsymbol{VP}\ (\%) = \frac{\text{Nº of viable pollen}}{200} \times 100$$

### 4.2.6 Statistical analyses

Attending the normality and homogeneity, the data were subjected to analysis of variance, followed by mean comparison using the Scott-Knott test ($p \leq 0.05$) (Borges; Ferreira 2003). To provide a comprehensive overview, the data were initially standardized (Cao *et al*., 1999), and then a principal component analysis (PCA) was performed to characterize the variables that most discriminated the structural

characteristics in each treatment (Hair Junior *et al*., 2009). Parametric tests, figures and PCA were performed using the statistical software R (R Core Team, 2023).

## 4.3 Results

### 4.3.1 Physiological responses to treatments during V4 stage

The net photosynthesis of soybean plants remained unaltered by biostimulant treatment in a fractionated manner during stages V4-R1 (T02, T09, and T14); however, it was reduced in treatments with a single dose (T04, T07, and T12) (Figure 3a). It's noteworthy that, at the evaluation time (stage V4), only 50% of the dose had been applied for treatments T02, T09, and T14 (equivalent to 0.25, 0.5, and 0.75 L $ha^{-1}$, respectively) (Figure 2a).

The biostimulant at rates of 1.0 and 1.5 L $ha^{-1}$ (T09 and T14, respectively) significantly increased the transpiration rate of plants compared to the other treatments (Figure 3b). In contrast, the application of 0.5 to 0.75 L $ha^{-1}$ at V4 (T04, 50% of the dose in T07 and T12) significantly reduced the stomatal conductance ($g_s$) of soybean plants compared to the control, while the application of 1.5 L $ha^{-1}$ (T14) resulted in an increased $g_s$ at the V4 stage (Figure 3c).

Biostimulant at 0.5 (T02 and T04) and 1.0 L $ha^{-1}$ (T07 and T09) reduced the relative water content (RWC) compared to control plants, while the dose of 1.5 L $ha^{-1}$ (T12 and T14) did not significantly alter the RWC of plants at vegetative stage 4 (Figure 3d). The intrinsic water use efficiency (*A/gs*) was higher in T04 and T12, surpassing all other treatments and the control. Meanwhile, the instantaneous water use efficiency (*A/E*) did not exhibit statistical differences (Figure 3e, f).

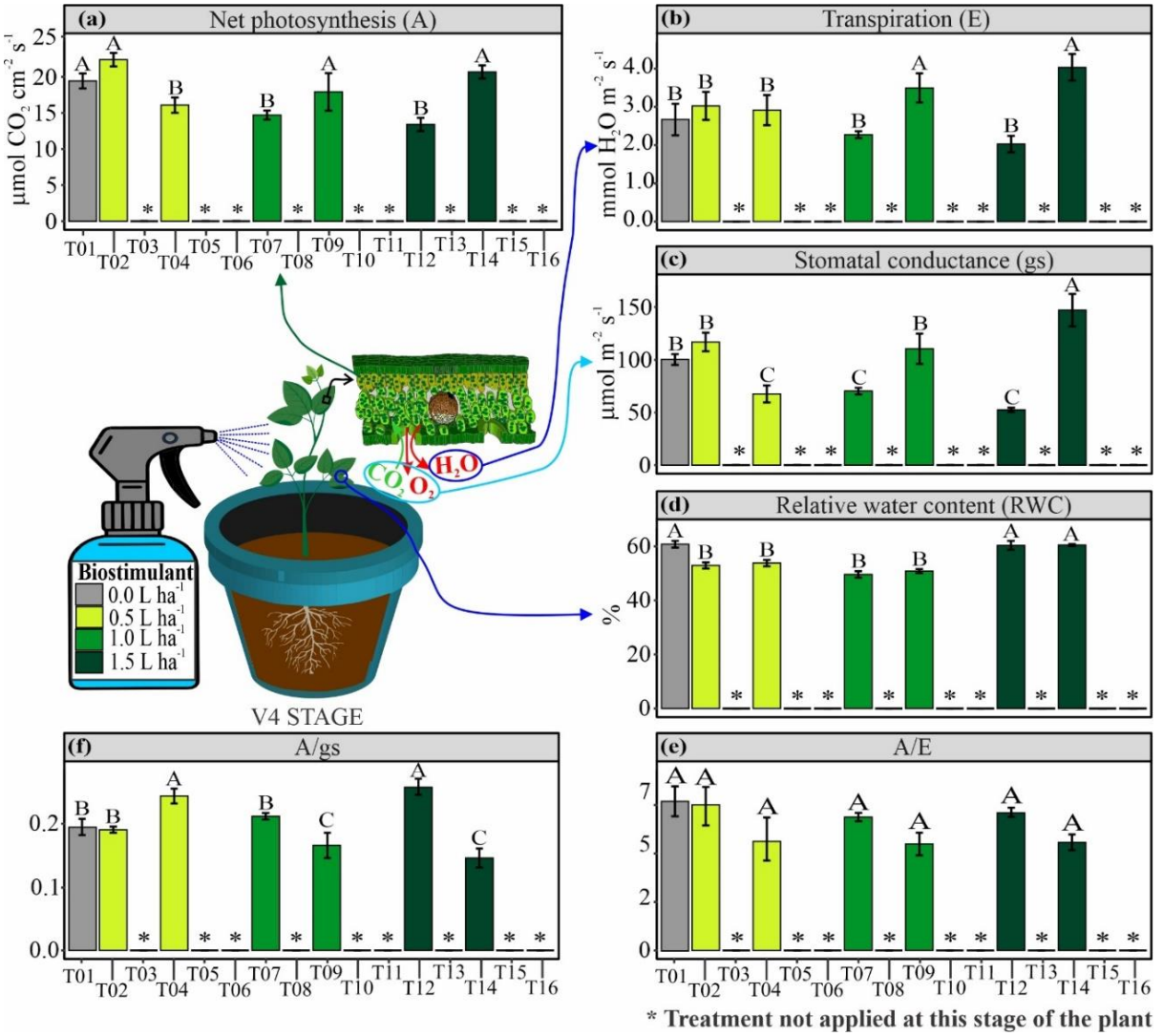



**Figure 3** - Net photosynthesis (*A*, **a**), transpiration (*E*, **b**), stomatal conductance ($g_s$, **c**), relative water content (RWC, **d**), instantaneous water use efficiency (*A/E*, **e**) and intrinsic water use efficiency ($A/g_s$, **f**) rate of soybean plants at V4 stage, Extrema cultivar, after application of biostimulant at 0.5, 1.0 and 1.5 L ha$^{-1}$, as detailed in figure 02. Different uppercase letters represent significant differences due to biostimulant treatments according to the Scott–Knott test ($p<0.05$). *treatment was not initiated in the V4-stage time-point according to Figure 2.

### 4.3.2 Reproductive and biometric responses at R2 stage

At R2 stage, the biostimulant significantly decreased the number of flowers, with the more prominent effects in a single sprayment at 0.5 (T04) and 1.5 L ha$^{-1}$ (T15) treatments, except for T13 plants as compared to the control (Figure 4a).

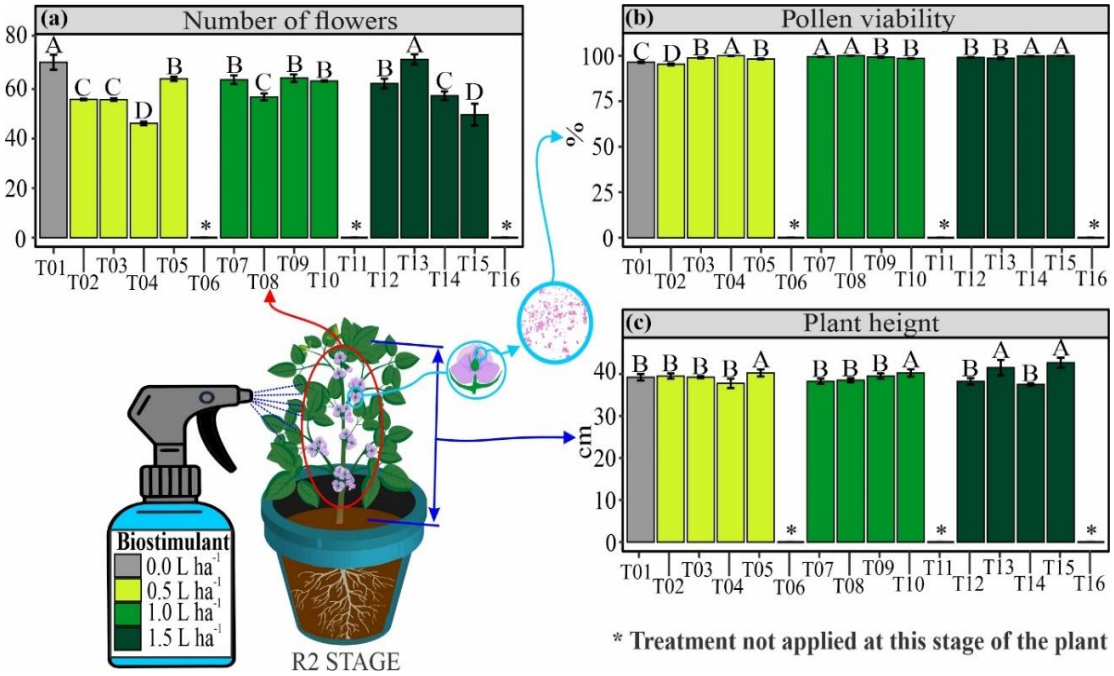



**Figure 4 -** Number of flowers (**a**), plant height (**b**) and pollen viability (**c**) of soybean plants at R2 stage, Extrema genotype, after application of biostimulant at 0.5, 1.0 and 1.5 L $ha^{-1}$, as detailed in figure 02. Different uppercase letters represent significant differences due to biostimulant treatments according to Scott–Knott test ($p<0.05$). *treatment was not initiated in the R2-stage time-point.

In the present study, although the biostimulant application promoted negative effects in the number of flowers (T04 and T15), in most cases it promoted higher pollen viability to the remaining flowers compared to the control, reaching values equal to or higher than 99% (T04, T07, T08, T14, and T15). Thus, except for T02, the application of the biostimulant significantly increased pollen viability in soybean plants, regardless of the dose and application timing, as compared to the control (Figure 4b). In general, soybean plant height at the R2 stage was slightly stimulated by treatments T05, T10, T13, and T15, while the remaining treatments did not result in any significant alteration compared to the control (Figure 4c).

### 4.3.3 Physiological performance and number of pods at R4 and R6 stage

At R4 stage, the net photosynthesis was not changed by biostimulant application; on the other hand, there was a reduction in plants from treatments T05, T06, T07, T08, T09, T10, and T16 compared to the control T01 (Figure 5a). In contrast,

transpiration rate was increased in plants treated with biostimulant at T03, T04, T07, T08, T09, T10, and T15 compared to the control (Figure 5b), but no significant changes were observed in stomatal conductance (Figure 5c).

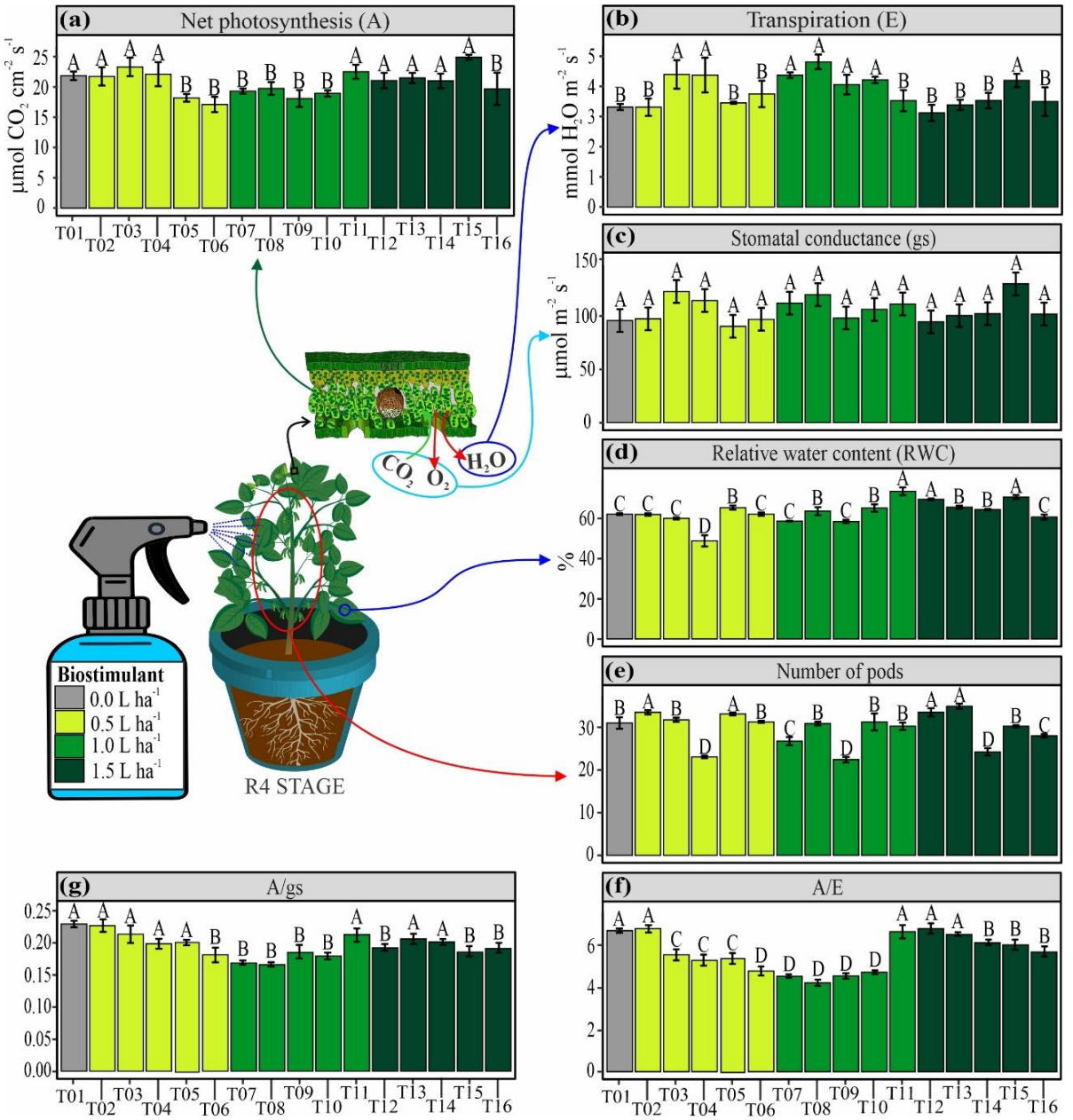



**Figure 5** – Net photosynthesis (*A*, **a**), transpiration (*E*, **b**), stomatal conductance ($g_s$, **c**), relative water content (RWC, **d**), number of pod (**e**) instantaneous water use efficiency (*A/E*, **f**) and intrinsic water use efficiency (*A/gs*, **g**) of soybean plants, Extrema cultivar, at R4 stage, after treatments with biostimulant as detailed in figure 02. Different uppercase letters represent significant differences due to biostimulant treatments according to the Scott–Knott test ($p<0.05$).

Soybean plants at the R4 stage exhibited a higher relative water content under biostimulant treatment at 1.0 (T11) and 1.5 L $ha^{-1}$ (T12 and T15) in the R4 and R1

stages, respectively, as compared to the control (Figure 5d). On the other hand, soybean plants exhibited a higher number of pods when the biostimulant was applied at 1.5 L $ha^{-1}$ in two applications at V4-R1 (T12) or R1-R4 (T13), not differing from T02 and T05 but surpassing the control and other treatments (Figure 5e).

The intrinsic and instantaneous water use efficiencies of biostimulant-treated plants were found to be similar or little reduced as compared to the control treatments. The decreases were particularly perceptible in plants from 1.0 L $ha^{-1}$ dose, except for T11 (Figure 5g, f).

At the R6 stage, soybean plants showed elevated rates of net photosynthesis when the biostimulant was applied as T04, T09, T10, and T14, as compared to control and other treatments (Figure 6a). In contrast, transpiration and stomatal conductance rates were stimulated in plants from treatments T04, T05, T06, T07, T09, T10, T14, and T16 compared to the control, except for stomatal conductance in T06 (Figure 6b, c).

In general, except for 1.5 L $ha^{-1}$ dose, the biostimulant significantly increased the relative water content of soybean plants compared to the control (T01), with particular emphasis on treatment T07 (Figure 6d). In contrast, the number of pods in soybean plants either remained the same or was reduced compared to the control treatment when the biostimulant was applied at 1.0 (V4-R1) or 1.5 L $ha^{-1}$ (R1-R4 or R1) (Figure 6e). Intrinsic and instantaneous water use efficiency did not exhibit significant changes at this stage (Figure 6f, g).

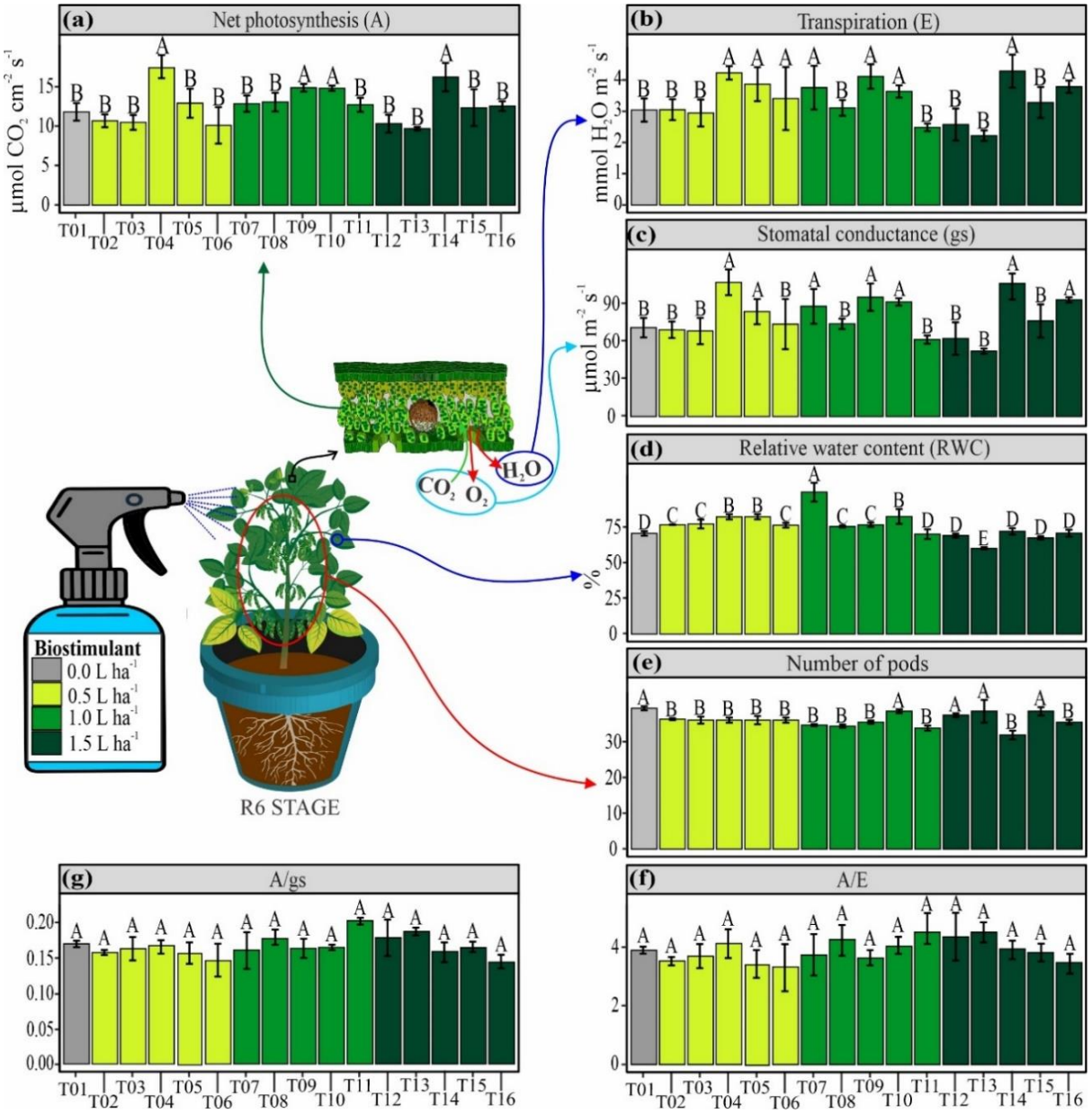



**Figure 6** – Net photosynthesis (*A*, **a**), transpiration (*E*, **b**), stomatal conductance ($g_s$, **c**), relative water content (RWC, **d**), number of pod (**e**) instantaneous water use efficiency (A/E, **f**) and intrinsic water use efficiency (A/gs, **g**) of soybean plants, Extrema cultivar, at R6 stage, after treatments with biostimulant as detailed in figure 02. Different uppercase letters represent significant differences due to biostimulant treatments according to the Scott–Knott test ($p<0.05$).

### 4.3.4 Soybean yield

The biostimulant application at 0.5 (T02, T03, T04), 1.0 (T07, T08, T11), and 1.5 L ha$^{-1}$ (T12, T13, T14, T15) promoted a significant increase in the number of grains

per pod of soybean plants, while T06 resulted in a significant decrease (Figure 7a). A similar pattern was observed for grain weight, with plants treated with the biostimulant at 0.5 (T02, T03, T06), 1.0 (T07, T09), and 1.5 L ha$^{-1}$ (T12, T13, T14) showing a significant gain compared to the control (Figure 7b).

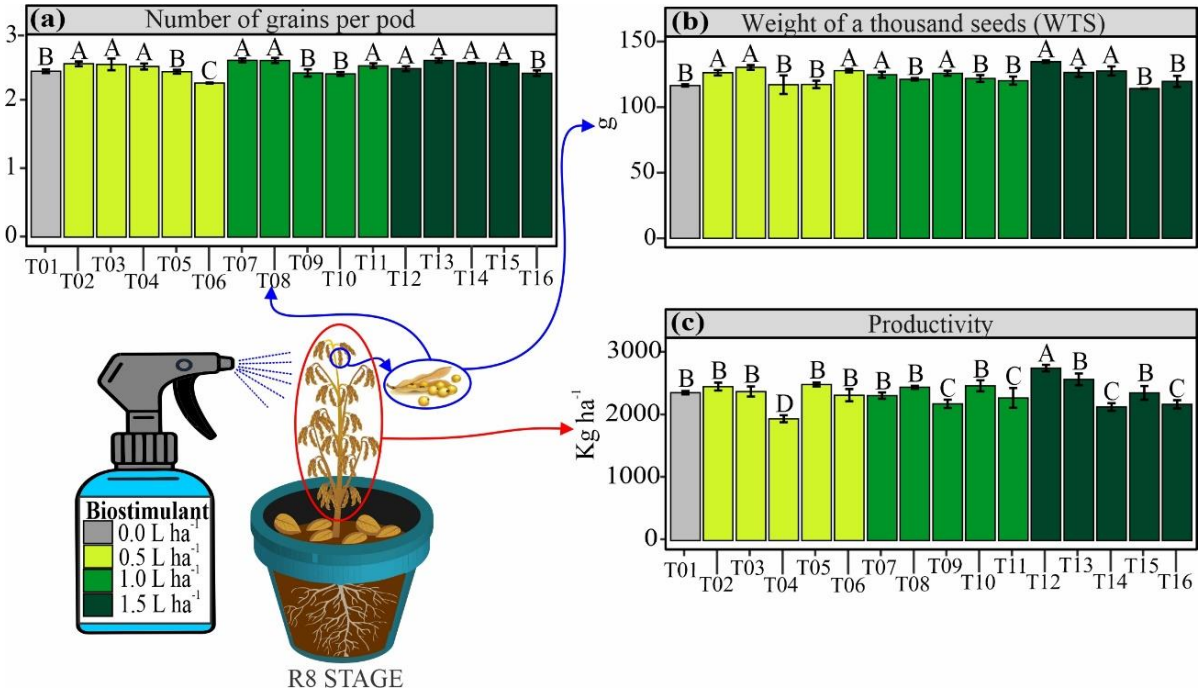



**Figure 7** – Number of grains per pod (**a**), weight of a thousand seeds (**b**) and yield (**c**) of soybean plants, Extrema genotype, after treatment with biostimulant at 0.5, 1.0 and 1.5 L ha$^{-1}$ in the V4, R1 and R4 stages, as detailed in figure 02. Different uppercase letters represent significant differences due to biostimulant treatments according to the Scott–Knott test ($p<0.05$).

The soybean yield gain as affected by biostimulant treatment was dependent on the dose and application stage (Figure 7c). The highest yield data were observed only when the biostimulant was applied at 1.5 L ha$^{-1}$, divided between the V4-R1 stages (T12), resulting in a value 12% higher than the control.

### 4.3.5 Principal Component Analysis (PCA)

The data revealed that Principal Component Analysis (PCA) accounted for 72.38% of the total variation, with 45.01% attributed to component 1 and 27.3% to component 2 (Figure 8). The results indicated distinct clustering patterns among

soybean plant stages, with overlaps observed between application timing and biostimulant dosage.

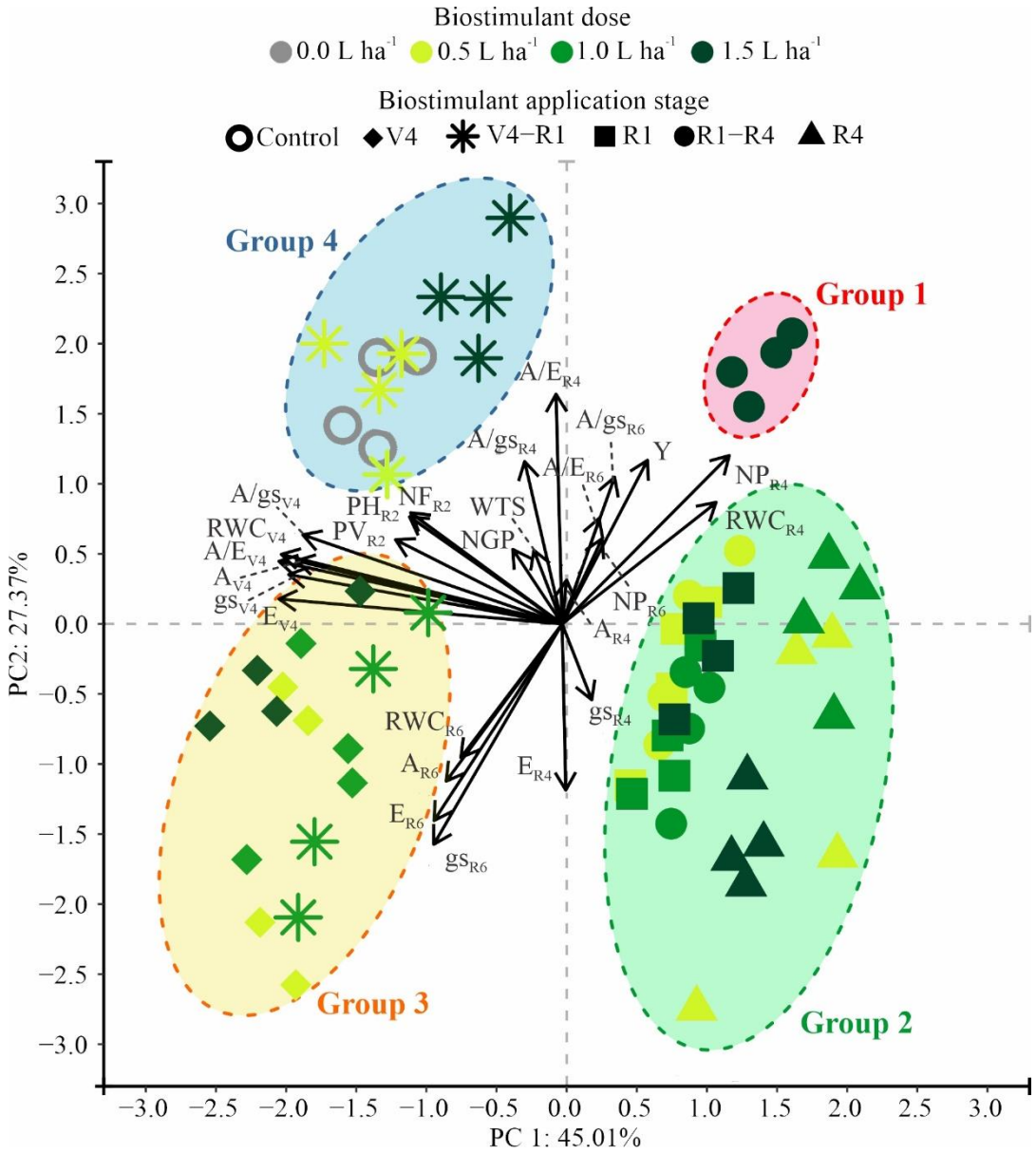



**Figure 8** – Principal component analysis of the mean parameters of soybean plants, Extrema genotype, evaluated at different stages (V4, R2, R4 and R6) after application of the biostimulant, as detailed in figure 02. Net photosynthesis (*A*), transpiration (*E*), stomatal conductance ($g_s$), relative water content (RWC), instantaneous water use efficiency (*A/E*) and intrinsic water use efficiency (*A/gs*), plant height (H), number of flowers (NF), pollen viability (VP), number of pod (NP), number of grains per pod (NGP), weight of a thousand seeds (WTS) and yield (Y). Groups were formed by dose and application timing.

Regarding PC2, an overlap was noted between group 4 and group 1, where the application of 1.5 L $ha^{-1}$ at V4-R1 or R1-R4, and 0.5 L $ha^{-1}$ (at V4-R1), exhibited stronger correlations with productivity factors and instantaneous water use efficiency

across all assessed stages. A higher correlation was observed between these variables and their respective treatments. However, in terms of PC1, these two groups were distinctly separated, displaying variations in the grouped variables.

In group 2, treatments applied solely during the reproductive period (either in a single or divided dose) clustered together and showed stronger associations with stomatal conductance. Conversely, in group 3, applying all three doses at the V4 stage, along with a 1 L $ha^{-1}$ dose at V4-R1, exhibited weaker correlations with productivity, photosynthetic factors, and variables related to flowering. Nonetheless, photosynthetic parameters evaluated at R6 (A, E, RWC, and gs) showed significant influence following application at the V4 stage.

## 4.4 Discussion

Biostimulants have been considered as an emerging solution for enhancing plant resilience to environmental conditions and increasing crop yield (Andreotti *et al*., 2022; Franzoni *et al*., 2022). Although *A. nodosum*-based biostimulants are widely marketed globally, their effectiveness may vary depending on the application method, dosage and adopted crop (Appendix C). Thus, numerous studies are underway to optimize the use of biostimulants, searching for optimum dosage and the most effective application stage (Deolu-Ajayi *et al*., 2022).

In a previous report, Repke *et al*. (2022) reported that the optimal performance of the 95R95-IPRO soybean cultivar was observed when the *A. nodosum*-based biostimulant was applied at 1.0 L $ha^{-1}$, a response associated with higher rates of net photosynthesis and stomatal conductance, even in plants under stress. In agreement, soybean plants from Extrema cultivar also displayed improved physiological conditioning when treated with *A. nodosum*-based biostimulant at 1.0 and 1.5 L $ha^{-1}$, but the productive performance was improved only by 1.5 L $ha^{-1}$ treatment (Figures 3, 5, 6, 7, 8).

At the vegetative stage V4, T14-treated plants exhibited stomatal conductance higher than those from the control treatment, which was associated with an elevated transpiration rate (Figure 3). However, although a higher instantaneous water use efficiency was not observed, treatments T12 and 14 showed unchanged relative water

content, suggesting a restricted control of thermal balance, as transpiration process is determinant for heating dissipation and cooling the leaf (Ranawana *et al*., 2023). These findings may arise from the reduction of the leaf waxy layer immediately after the application of the biostimulant, which consequently decreased the intrinsic resistance of the water boundary layer near the stomata (Petrík *et al*., 2023), and also led to improvements in water use efficiency (Figure 5f and g). Thus, the application at V4 may be linked to optimal growth, highlighting a fine adjustment in water consumption at vegetative stage, an important response for plant performance (Liao *et al*., 2022; Graziani *et al*., 2022; Jiménez-Arias *et al*., 2022; Sujata *et al*., 2022; Rahimi *et al*., 2022).

The data also indicate that the biostimulant at 1.5 L $ha^{-1}$ elicits significant responses in the physiological conditioning of soybean plants, as it significantly increased the relative water content (RWC) at the V4 (T12 and T14) and R4 (T11, T12, T15) stages (Figures 3d, 5d). The results suggest a better balance of water potential, involving tight control of stomatal conductance, which directly influences transpiration and leaf temperature regulation (Hassan *et al*., 2020).

The growth morphology has a direct relationship with flowering and grain yield in soybean cultivation, particularly due to increased pod formation in the internodes of the plants (Xue *et al*., 2022). Nevertheless, our data suggest that the relationship between vegetative and reproductive stages can be modulated by biostimulant application. Soybean plants exhibited increased height when treated with the biostimulant at 0.5 (T05), 1.0 (T10), and 1.5 (T13 and T15) L $ha^{-1}$ (Figure 4c). The height increase was associated with a higher number of flowers in T13 at the R2 stage (Figure 4a) and a higher number of pods in T13 at R4 (Figures 5e, 6e). Treatment T12 also stimulated the number of pods at the R4 and R6 stages (Figures 5e, 6e), but there was no direct relationship with the height of the plants. This phenomenon likely resulted from the application method of the biostimulant dose, which was split in T12 (0.75 + 0.75 L $ha^{-1}$ at V4 and R1, respectively), as compared to the single dose in T13. In several plant species, this response was attributed to fluctuations in gibberellin levels (Mosa *et al*., 2022; Zhao *et al*., 2022).

The results also suggest that the induction of the photosynthetic machinery at the R6 stage by the *A. nodosum*-based biostimulant was crucial for plant production. It supplied seeds with assimilates and increased fertilization rates, resulting in a higher number of grains per pod (Figures 4, 5, 6, 7, 8). This metabolic adjustment was

essential for reserve accumulation in the seeds, leading to an increase in thousand-seed weight (Figure 7b) and higher plant productivity (Figure 7c). These findings align with previous studies that emphasized the biostimulant's role in maintaining photosynthetic metabolism at the R6 stage, thereby enhancing plant performance, as observed by Repke *et al*. (2022).

Previous studies have demonstrated that biostimulants play a role in the hydrolysis and biosynthesis of molecules, as well as in the accumulation of reserves during seed formation (Sunmonu *et al*., 2016), contributing to pod production and plant yield. In this study, soybean plants exhibited a higher number of pods when exposed to 0.5 (T02 and T05) and 1.5 (T12 and T13) L $ha^{-1}$ at the R4 stage (Figure 5e). This increase in pod number was not directly associated with the modulation of the number of flowers, which was even lower than the control (Figure 4a). These findings suggest that, in the application configuration of these treatments, the biostimulant increased fertilization rates likely due to the rise in viable pollen (Figure 4b), resulting in a higher number of pods (Figure 5e), as previously demonstrated by Sun *et al*. (2022).

The data reinforce recent reports that algae-based biostimulants modulate soybean yield depending on dosage and application timing (Repke *et al*., 2022). Here, a typical semiarid cultivar displayed the highest yield treated in a split manner at different stages, at a rate of 1.5 L $ha^{-1}$ (Figure 7c), specifically in the form of T12 (0.75 L $ha^{-1}$ at V4 + 0.75 L $ha^{-1}$ at R1), with productivity values 12% higher than the control (Figure 7c). These results are consistent with those found by Repke *et al*. (2022), who reported the influence of the application of ANE on the productive responses of soybean plants.

## 4.5 Conclusion

Our data evidence that the *A. nodosum*-derived biostimulant through foliar spray promotes better physiological performance in soybean plants under greenhouse conditions in a timing and dose-dependent manner. The superior performance of biostimulant-treated plants was achieved in a split application at 1.5 L $ha^{-1}$, divided into 0.75 L $ha^{-1}$ at V4 + 0.75 L $ha^{-1}$ at R1 (T12), which reflected in net photosynthesis, improved number of pods, number of grains per pod, weight of a thousand seeds and

yield. Further studies are necessary to investigate the role of biostimulants at molecular and biochemical levels in cultivated crops, employing them as a strategy to activate plant responses against environmental stresses. The findings provide crucial information to support soybean cultivation in semiarid regions.

**REFERENCES**

AGROSTAT – Estatística de Comércio Exterior do Agronegócio Brasileiro, 2022. **Indicadores da agricultura**. Disponível em: <https://indicadores.agricultura.gov.br/agrostat/index.htm_>. Accessed at: Nov 28, 2023.

ALEXANDER, M. P. A versatile stain for pollen fungi, yeast and bacteria. **Stain technology**, v. 55, p. 13-18,1980.

ALI, A.; HUSSAIN, T.; TANTASHUTIKUN, N.; HUSSAIN, N.; COCETTA, G. Application of smart techniques, internet of things and data mining for resource use efficient and sustainable crop production. **Agriculture**, v. 13, p. 397, 2023.

ANDREOTTI, C.; ROUPHAEL, Y.; COLLA, G.; BASILE, B. Rate and timing of application of biostimulant substances to enhance fruit tree tolerance toward environmental stresses and fruit quality. **Agronomy**, v. 12, p. 603, 2022.

BANTIS, F.; KOUKOUNARAS, A. *Ascophyllum nodosum* and silicon-based biostimulants differentially affect the physiology and growth of watermelon transplants under abiotic stress factors: the case of drought. **Horticulturae**, v. 12, p. 1177, 2022.

BORGES, L. C.; FERREIRA, D. F. Poder e taxas de erro tipo I dos testes Scott-Knott, Tukey e Student-Newman-Keuls sob distribuições normal e não normais dos resíduos. **Revista de Matemática e Estatística**, v. 21, n. 1, p. 67-83, 2003.

CAO, Y.; WILLIAMS, D. D.; WILLIAMS, N. E. Data transformation and standardization in the multivariate analysis of river water quality. **Ecological Applications**, v. 9, p. 669-677, 1999.

ČATSKÝ, J. Determination of water deficit in disks cut out from leaf blades. **Biologia Plantarum**, v. 2, p. 76-78,1960.

CHAUDHRY, S.; SIDHU G. P. S. Climate change regulated abiotic stress mechanisms in plants: a comprehensive review. **Plant Cell Reports**, v. 41, p.1-31, 2021.

COCETTA, G.; LANDONI, M.; PILU, R.; REPISO, C.; NOLASCO, J.; ALAJARIN, M.; UGENA, L.; LEVY, C. C. B.; SCATOLINO, G.; VILLA, D. Priming treatments with biostimulants to cope the short-term heat stress response: a transcriptomic profile evaluation. **Plants**, v. 11, n. 9, p. 1130, 2022.

CONAB - Companhia Nacional de Abastecimento. **Acompanhamento da Safra Brasileira de Grãos**: quarto levantamento, Brasília. 2023.

DEOLU-AJAYI, A. O.; MEER, I. M. D.; WERF, A. V. D.; KARLOVA R. The power of seaweeds as plant biostimulants to boost crop production under abiotic stress. **Plant, Cell e Environment**, v. 45, p. 2537-2553, 2022.

FRANZONI, G.; COCETTA, G.; PRINSI, B.; FERRANTE, A.; ESPEN, L. Biostimulants on crops: their impact under abiotic stress conditions. **Horticulturae**, v. 8, p. 189, 2022.

GRAZIANI, G.; CIRILLO, A.; GIANNINI, P.; CONTI, S.; EL-NAKHEL, C.; ROUPHAEL, Y.; RITIENI, A.; VAIO, C. D. Biostimulants improve plant growth and bioactive compounds of young olive trees under abiotic stress conditions. **Agriculture**, v. 12, n. 2, p. 227, 2022.

GHOSH, U. K.; ISLAM, M. N.; SIDDIQUI, M. N.; CAO, X.; KHAN, M. Proline, a multifaceted signalling molecule in plant responses to abiotic stress: understanding the physiological mechanisms. **Plant Biology**, v. 24, p. 227-239, 2021.

GUERRA, M.; SOUZA, M. J. **Como observar cromossomos: um guia de técnicas em citogenética vegetal, animal e humana**: Ribeirão Preto, Funpec, 2002. 131 p.

GUPTA, S.; DOLEŽAL, K.; KULKARNI, M. G.; BALÁZS, E.; VAN STADEN J. Role of non-microbial biostimulants in regulation of seed germination and seedling establishment. **Plant Growth Regulation**, v. 97, p. 271-313, 2022.

HAIR JR, J. F.; BLACK, W. C.; BABIN, B. J.; ANDERSON, R. E.; TATHAM, R. L. **Análise multivariada de dados**. Porto Alegre: Bookman, 2009, 688 p.

HASANUZZAMAN, M.; NAHAR, K.; ANEE, T. I.; KHAN, M. I.; FUJITA, M. Silicon-mediated regulation of antioxidant defense and glyoxalase systems confers drought stress tolerance in *Brassica napus L.* **South African Journal of Botany**, v.115, p. 50-57, 2018.

HASSAN, M. U.; RASOOL, T.; IQBAL, C.; ARSHAD A. Linking plants functioning to adaptive responses under heat stress conditions: a mechanistic review. **Journal of Plant Growth Regulation**, v. 41, p. 2596-2613, 2021.

HASSAN, M. U.; AAMER, M.; CHATTHA, M. U.; The critical role of zinc in plants facing the drought stress. **Agriculture**, v.10, p. 396, 2020.

HERNÁNDEZ-HERRERA, R. M.; SÁNCHEZ-HERNÁNDEZ, C. V.; PALMEROS-SUÁREZ, P. A.; OCAMPO-ALVAREZ, H.; SANTACRUZ-RUVALCABA, F.; MEZA-CANALES, I. D.; BECERRIL-ESPINOSA, A. Seaweed extract improves growth and productivity of tomato plants under salinity stress. **Agronomy**, v.12, p. 2495, 2022.

HIDANGMAYUM, A.; DWIVEDI, P.; KATIYAR, D.; HEMANTARANJAN, A. Application of chitosan on plant responses with special reference to abiotic stress. **Physiology and Molecular Biology of Plants**, v. 25, p. 313-326, 2019.

KHAN, M.; ASHFAQUE, F.; CHHILLAR, H.; IRFAN, M.; KHAN, N. A. The intricacy of silicon, plant growth regulators and other signaling molecules for abiotic stress

tolerance: an entrancing crosstalk between stress alleviators. **Plant Physiology and Biochemistry**, v. 162, p. 36-47, 2021.

JIMÉNEZ-ARIAS, D.; HERNÁNDIZ, A. E.; MORALES-SIERRA, S.; GARCÍA-GARCÍA, A. L.; GARCÍA-MACHADO, F. J.; LUIS, J. C.; BORGES, A. A. Applying biostimulants to combat water deficit in crop plants: research and debate. **Agronomy**, v. 12, p. 571, 2022.

LEITE-FILHO, A. T.; SOARES-FILHO, B. S.; DAVIS, J. L.; ABRAHÃO, G. M.; BÖRNER, J. Deforestation reduces rainfall and agricultural revenues in the Brazilian Amazon. **Nature communications**, v.12, p. 1-7, 2021.

LIAO, Z.; ZENG, H.; FAN, J.; LAI, Z.; ZHANG, C.; ZHANG, F.; WANG, H.; CHENG, M.; GUO, J.; LI, Z. Effects of plant density, nitrogen rate and al irrigation on photosynthesis, root growth, seed yield and water-nitrogen use efficiency of soybean under ridge-furrow plastic mulching. **Agricultural Water Management**, v. 268, p. 107688, 2022.

MALHI, G. S.; KAUR, M.; KAUSHIK, P. Impact of climate change on agriculture and its mitigation strategies: a review. **Sustainability**, v. 13, p. 1318, 2021.

MIRANDA, R. S.; FONSECA, B. S. F.; PINHO, D. S.; BATISTA, J. Y.N.; BRITO, R. R.; SILVA, E. M.; FERREIRA, W. S.; COSTA, J. H.; LOPES, M. S.; SOUSA, R. H. B. Selection of soybean and cowpea cultivars with superior performance under drought using growth and biochemical aspects. **Plants**, v. 12, n. 17, p. 3134, 2023.

MOSA, W. F. A.; SAS-PASZT, L.; GŁUSZEK, S.; GÓRNIK, K.; ANJUM, M. A.; SALEH, A. A.; ABADA, H. S.; AWAD, R. M. Effect of some biostimulants on the vegetative growth, yield, fruit quality attributes and nutritional status of apple. **Horticulturae**, v.9, p. 32, 2022.

MZIBRA, A.; AASFAR, A.; KHOULOUD, M.; FARRIE, Y.; BOULIF, R.; KADMIRI, I.M.; BAMOUH, A.; DOUIRA, A. Improving growth, yield, and quality of tomato plants (*Solanum lycopersicum* L) by the application of moroccan seaweed-based biostimulants under greenhouse conditions. **Agronomy**, v.11, p. 1373, 2021.

PATANÈ, C.; COSENTINO, S. L.; ROMANO, D.; TOSCANO, S. Relative water content, proline, and antioxidant enzymes in leaves of long shelf-life tomatoes under drought stress and rewatering. **Plants**, v.11, p. 3045, 2022.

PETRÍK, P.; PETEK-PETRIK, A.; MUKARRAM, M.; SCHULDT, B.; LAMARQUE, L.; J. Leaf physiological and morphological constraints of water-use efficiency in C3 plants. **AoB Plants**, v. 15, p. plad047, 2023.

R Core Team. **A Language and Environment for Statistical Computing**: RFoundation for Statistical Computing, Vienna, Austria, 2023.

RAHIMI, A.; MOHAMMADI, M. M.; MOGHADDAM, S. S.; HEYDARZADEH, S.; GITARI, H. Effects of stress modifier biostimulants on vegetative growth, nutrients,

and antioxidants contents of garden thyme (*Thymus vulgaris* L.) under water deficit conditions. **Journal of Plant Growth Regulation**, v. 41, p. 2059-2072, 2022.

RANAWANA, S. R. W. M. C. J. K.; BRAMLEY, H.; PALTA, J. A.; SIDDIQUE, K. H. M. Role of transpiration in regulating leaf temperature and its application in physiological breeding. *In:* MAMRUTHA, H. M.; KRISHNAPPA, G.; KHOBRA, R.; SINGH, G.; SINGH, G. P. (ed.). **Translating physiological tools to augment crop breeding**. Singapure: Springer, 2023, p. 91-119.

RAZA, A.; TABASSUM, J.; MUBARIK, M. S.; Blume, Y. B. Hydrogen sulfide: an emerging component against abiotic stress in plants. **Plant Biology**, v. 24, p. 540-558. 2021.

REPKE, R. A.; SILVA, D. M. R.; SANTOS, J. C. C.; SILVA, M. A. Increased soybean tolerance to high-temperature through biostimulant based on *Ascophyllum nodosum* (L.) seaweed extract. **Journal of Applied Phycology**, v. 34, p. 3205-3218, 2022.

ROSA, V. R.; SANTOS, A. L. F.; SILVA, A. A.; SAB, M. P. V.; GERMINO, G. H.; CARDOSO, F. B.; SILVA, M. A. Increased soybean tolerance to water deficiency through biostimulant based on fulvic acids and *Ascophyllum nodosum* (L.) seaweed extract. **Plant Physiology and Biochemistry**, v. 158, p. 228-243, 2021.

SHAHRAJABIAN, M. H.; CHASKI, C.; POLYZOS, N.; PETROPOULOS, S. A. Biostimulants application: a low input cropping management tool for sustainable farming of vegetables. **Biomolecules**, v. 11, n. 5, p. 698, 2021.

SHAHZAD, A.; ULLAH, S.; DAR, A. A.; SARDAR, M. F.; MEHMOOD, T.; TUFAIL, M. A.; SHAKOOR, A.; HARIS M. Nexus on climate change: agriculture and possible solution to cope future climate change stresses. **Environmental Science and Pollution Research**, v. 28, p. 14211-14232, 2021.

SHAHZAD, R.; HARLINA, P. W.; GALLEGO, P. P.; FLEXAS, J.; EWAS, M.; LEIWEN, X.; KARUNIAWAN, A. The seaweed *Ascophyllum nodosum*-based biostimulant enhances salt stress tolerance in rice (*Oryza sativa* L.) by remodeling physiological, biochemical, and metabolic responses. **Journal of Plant Interactions**, v. 18, p. 2266514, 2023.

SHUKLA, P. S.; PRITHIVIRAJ, B. *Ascophyllum nodosum* biostimulant improves the growth of *Zea mays* grown under phosphorus impoverished conditions. **Frontiers in Plant Science**, v. 11, p. 601843, 2021.

SHUKLA, P. S.; SHOTTON, K.; NORMAN, E.; NEILY, W.; CRITCHLEY, A. T.; PRITHIVIRAJ, B. Seaweed extract improve drought tolerance of soybean by regulating stress-response genes. **AoB Plant**s, v. 10, n. 1, p. plx051, 2018.

SILVA, E. H. F.; ANTOLIN, L. A. S.; ZANON, A. L.; ANDRADE-JUNIOR, A. S.; SOUZA, H. A.; CARVALHO, K. S.; VIEIRA-JUNIOR, N. A.; MARIN, F. R. Impact assessment of soybean yield and water productivity in Brazil due to climate change. **European Journal of Agronomy**, v. 129, p. 126329, 2021.

SOUSA, D. M. G.; LOBATO, E. (ed.). **Cerrado: correção do solo e adubação.** 2. ed. Planaltina: Embrapa Informação Tecnológica, 2004. 416 p.

STAYKOV, N. S.; ANGELOV, M.; PETROV, V.; MINKOV, P.; KANOJIA, A.; GUINAN, K. J.; ALSEEKH, S.; FERNIE, A. R.; SUJEETH, N.; GECHEV, T. S. An *Ascophyllum nodosum*-derived biostimulant protects model and crop plants from oxidative stress. **Metabolites**, v. 11, n. 1, p. 24, 2020.

SUJATA; G. V.; BALIYAN, V.; AVTAR, R.; MEHROTRA, S. Alleviating drought stress in *Brassica juncea* (L.) by foliar application of biostimulants-orthosilicic acid and seaweed extract. **Applied Biochemistry and Biotechnology**, v. 195, n. 1, p. 693-721, 2022.

SUN, X.; SUN, X.; PAN, X.; ZHANG, H.; WANG, Y.; REN, H.; WANG, F. Identification and fine mapping of a quantitative trait locus controlling the total flower and pod numbers in soybean. **Agronomy**, v. 12, p. 790, 2022.

SUNMONU, T. O.; KULKARNI, M. G.; VAN STADEN, J. Smoke-water, karrikinolide and gibberellic acid stimulate growth in bean and maize seedlings by efficient starch mobilization and suppression of oxidative stress. **South African Journal of Botany**, v. 102, p. 4-11, 2016.

TIWARI, R. K.; LAL, M. K.; KUMAR, R.; CHOURASIA, K. N.; NAGA, K. C.; KUMAR, D.; DAS, S. K.; ZINTA, G. Mechanistic insights on melatonin-mediated drought stress mitigation in plants. **Physiologia Plantarum**, v. 172, p. 1212-1226, 2021.

USDA - United States Department Agriculture. **Soybean Area, Yield, and Production**. 2023. Available at <https://apps.fas.usda.gov/psdonline/reporthandler.ashx?reportId=906&templateId=1&format=html&fileName=Table%2011%20Soybean%20Area,%20Yield,%20and%20Production>. Acessed at: Nov 28, 2023.

VEIHMEYER, F. J.; HENDRICKSON, A. H. The moisture equivalent as a measure of the field capacity of soils. **Soil Science**, v. 32, p. 181-194, 1931.

VILA, V. V.; MARQUES, P. A. A.; REZENDE, R. Deficit irrigation with *Ascophyllum nodosum* extract application as a strategy to increase tomato yield and quality. **Agronomy**, v. 13, p. 1853, 2023.

XUE, Y.; ZHANG, Y.; SHAN, J.; JI, Y.; ZHANG, X.; LI, W.; LI, D.; ZHAO, L. Growth repressor *GmRAV* binds to the *GmGA3ox* promoter to negatively regulate plant height development in soybean. **International Journal of Molecular Sciences** v. 23, p.1721,2022.

ZHAO, C.; MA, J.; ZHANG, Y.; YANG, S.; FENG, X.; YAN, J. The miR166 mediated regulatory module controls plant height by regulating gibberellic acid biosynthesis and catabolism in soybean. **Journal of Integrative Plant Biology**, v. 64, p. 995-1006, 2022.

# 5 CHAPTER III

## SEAWEED-BASED BIOSTIMULANTS MODULATE METABOLIC PROFILE AND ANTIOXIDANT SYSTEM BUT NOT ALTER PHYSIOLOGICAL PERFORMANCE AND DROUGHT TOLERANCE IN SEMIARID-ADAPTED SOYBEAN

## ABSTRACT

## ABSTRACT

In recent decades, water stress has become one of the most limiting environmental factors for the growth and productivity of crops, such as soybean. The use of seaweed-based biostimulants has emerged as an alternative to mitigate the damage caused by abiotic stresses. In this study, we aimed to evaluate the efficiency of different commercial biostimulant formulations on the performance of soybean cultivars typical of semiarid regions under water deficit, investigating growth and biochemical, physiological, and molecular pathways. The trial was conducted in a greenhouse using the Extrema cultivar, in a completely randomized experimental design, factorial 5 × 2, corresponding to five treatments with biostimulants (without biostimulant - negative control, and four commercial biostimulants - Bio 01, Bio 02, Bio 03, and Bio 04) and two irrigation levels (well-irrigated control and water deficit). The soybean plants were subjected to water stress 30 days after sowing, and the harvest was carried out after 25 days of stress imposition. Bio 01 was applied based on the results of the previous experiment (dose and timing of application). The other biostimulants were applied according to the manufacturers' recommendations. The results showed that water deficit significantly reduces the levels of photosynthetic pigments, stomatal conductance, and transpiration, and increases the non-photochemical quenching of energy dissipation, leading to severe restrictions in plant growth. The poor performance under stress was associated with increased membrane damage and hydrogen peroxide contents. The data also revealed that the soybean plants activated pathways for osmotic adjustment, maintaining water status and antioxidant enzymes, which were not effective in neutralizing oxidative damage. Under drought, the

biostimulants promoted significant responses in the accumulation of metabolites involved in defense pathways (such as 4-aminobutyric acid, benzoic acid, shikimic acid, erythronic acid, arabinose, phenylalanine, threonine, among others) and on reprogramming of the antioxidant system, but they were not effective in mitigating the effect of stress on the physiology and growth of plants, at least during the studied period. In conclusion, the biostimulants do not improve the performance of soybean cultivars typical of semiarid regions under water restriction. Future research should focus on strategies for applying biostimulants under stressful conditions, integrating long-term responses with the investigation of productive parameters associated with defense mechanisms.

**Keywords:** *Ascophyllum nodosum*. *Glycine max*. *Lithothamnium*. Metabolic profile. Plant defense. Water restriction.

## 5.1 Introduction

Abiotic stresses are the major limiters of agricultural production worldwide (Yasir *et al*., 2023). Episodes of flooding and drought, high and low temperatures combined with high light intensity, salinity, and heavy metals compromise the growth and profitability of crops (Yadav *et al*., 2020). These negative effects have been exacerbated by constant climate changes (Zandalinas *et al*., 2022). Among environmental stresses, water stress is a major restraint of plant growth (Rai *et al*., 2023), as water plays a fundamental role in all plant metabolic processes (Gul; Paul; Chehri, 2023). This situation is more pronounced in arid and/or semiarid regions, where high temperatures and low water availability cause annual evaporation rates to exceed rainfall replenishment (Asif *et al*., 2023). The climatic characteristics of these regions amplify the cumulative and multidimensional effects of stress, affecting various structures and functions of plants, which will need to activate defense pathways to overcome and cope with the stressful condition (Alori *et al*., 2020).

During the imposition of stress, the reduction in water absorption negatively impacts the plant's physiological processes, such as stomatal opening dynamics and transpiration. At the same time, severe changes occur in chlorophyll levels and

Rubisco activity, leading to a reduction in photosynthesis, halting or reducing cell elongation and expansion (Yang *et al*., 2021). And, since water is a vehicle for nutrient absorption, its restriction in the root zone also causes nutritional deficiencies (Nawaz *et al*., 2020). Secondarily, water stress can cause imbalances in many metabolic pathways, particularly resulting in the excessive accumulation of reactive oxygen species (ROS) (Dumanović *et al*., 2021; Yang *et al*., 2021).

Plant defense pathways against water deficit include complex mechanisms, ranging from morphoanatomical, physiological, and biochemical changes to metabolic and molecular modulations. These mechanisms differ according to the level of stress and the species (Seleiman *et al*., 2021). Among the main defense pathways, those related to osmotic adjustment (Ozturk *et al*., 2020; Zia *et al*., 2021) and the activation of the enzymatic (Sachdev *et al*., 2021; Jomova *et al*., 2024) and non-enzymatic (Mansoor *et al*., 2022) antioxidant defense systems can be mentioned.

To deal with water stress, decreasing as much damage as possible, the plant needs to invest a high degree of energy, which often becomes a limiting factor. Thus, the energy for biosynthesis, typically used for growth and yield, is redirected to activate defense mechanisms (Seleiman *et al*., 2021). In this context, several researchers have employed various strategies to assist plants in reducing damage caused by stress, aiming to maintain productive performance.

Biostimulants are products composed of a combination of hormones, humic substances, amino acids, seaweed extract, and micronutrients. Under normal conditions, these bioproducts have been widely used in agriculture to assist plants in increasing production. The exogenous application of biostimulants, via soil or foliar spraying, has also been shown to be beneficial for soybean, maize, rice, watermelon, and tomatoes under stressful conditions (Rosa *et al*., 2021; Shukla *et al*., 2021; Bantis *et al*., 2022; Cocetta *et al*., 2022; Repke *et al*., 2022; Engel *et al*., 2023; Shahzad *et al*., 2023; Vila *et al*., 2023). Those derived from the alga *Ascophyllum nodosum* have proven effective in activating plant responses under drought conditions, delaying leaf senescence, improving nutrient absorption and photosynthetic performance, leaf temperature, and activating defense mechanisms for ROS elimination (Omidbakhshfard *et al*., 2020; Mandal *et al*., 2023). Similarly, those derived from *Lithothamnium* have also shown efficiency in improving productive parameters of cultivated plants (Amatussi *et al*., 2020; Mógor *et al*., 2021; Amatussi *et al*., 2022). All

these factors highlight that this active ingredient is of great interest for use in crops of significant economic importance and susceptible to environmental stresses.

Soybean is a C3 photosynthetic metabolism species that is among the most widely cultivated and economically valuable crops. Considered one of the main commodities in the agricultural sector, it displays reduced productive potential during dry spells, leading to significant drops in yield and grain quality (Farooq *et al*., 2016; He *et al*., 2017; Mertz-Henning *et al*., 2018; Staniak; Szpunar-Krok; Kocira, 2023). Despite numerous studies, the role of biostimulants in the metabolism and tolerance of typical soybean cultivars in semiarid regions remains unknown.

Our working hypothesis was that seaweed-based biostimulants activate metabolic defense responses for improved performance of semiarid-adapted soybean plants under water deficit. The hypothesis was tested through the evaluation of growth, physiological, biochemical, and molecular indicators in stressed plants treated with different commercial formulations of biostimulants.

## 5.2 Material and methods

### 5.2.1 Study site, experimental conditions, treatments and irrigation management

The experiment was carried out in a greenhouse of the Federal University of Piauí, Campus Professora Cinobelina Elvas, located in the municipality of Bom Jesus-PI, at 9° 04' 46" South latitude, 44° 19' 38" West longitude. The experimental design was completely randomized (CRD), in a 2 × 5 factorial scheme, with two water deficit treatments (control and water deficit) and five biostimulant treatments (four formulations of commercial biostimulants and one control treatment - spray with distilled water), totaling 10 treatments, with 5 replications.

To install the experiment, 11 L pots were filled with soil from the 0-20 cm layer of the UFPI experimental area. The soil was analyzed for chemical composition, and correction was done according to the Cerrado soil fertilization manual (Sousa; Lobato, 2004). Sowing began with the addition of five seeds per pot. Thinning was then carried out at 7 and 14 days after sowing, leaving only one plant per pot.

The biostimulants were purchased from the commercial market, all derived from formulations based on *A. nodosum*, except for Bio 03, which is based on the seaweed *Lithothamnium*. Applications were made via foliar spray at doses recommended by the manufacturers (Appendix D). In all cases, 2.0 mL of appropriately diluted biostimulant was applied via foliar spray at vegetative stage 4 (V4) and reproductive stage 1 (R1), considering a population of 220,000 plants $ha^{-1}$ in the field. A group of plants was sprayed with 2.0 mL of distilled water, constituting the control treatment (Figure 9).

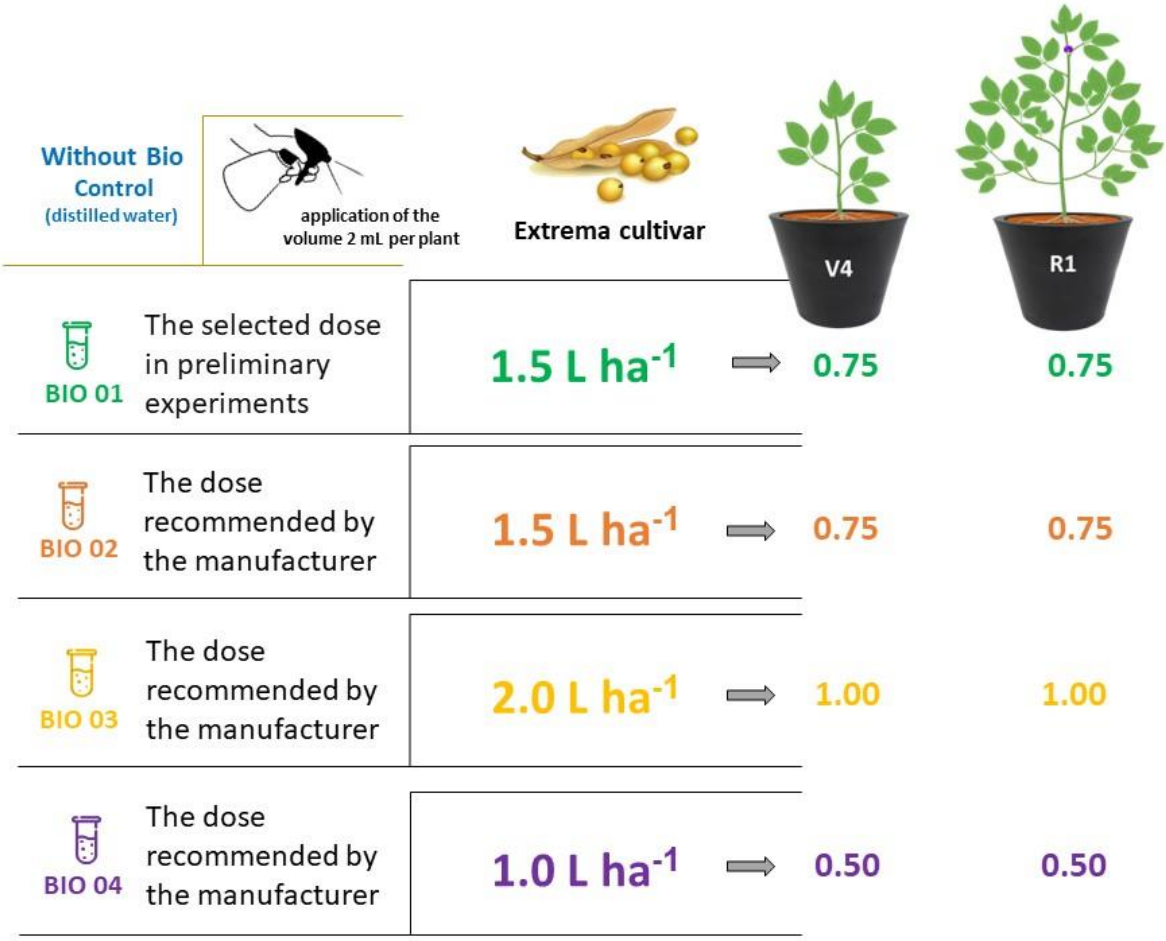



**Figure 9 -** Summary of biostimulant treatments based on *Ascophyllum nodosum* e *Lithothamnium* in soybean cultivation, cultivar Extrema, in a greenhouse. Plants without biostimulant were sprayed with distilled water, constituting the negative control. The biostimulant application was defined as manufacture recommendation.

The irrigation management was carried out daily by checking the soil water content using the pot weighing method (Veihmeyer; Hendrickson, 1931). Throughout the first 24 days after sowing, soil moisture was maintained at 75% of field capacity (FC), after which the water treatments were differentiated. During the stress imposition, the replenishment of water was applied to reach 60% FC for the well-irrigated treatment

and 45% FC for the water deficit treatment (Miranda *et al*., 2023) (Figure 10). Harvest was conducted 25 days after the application of the water deficit treatments, at stage R2 of the plants.

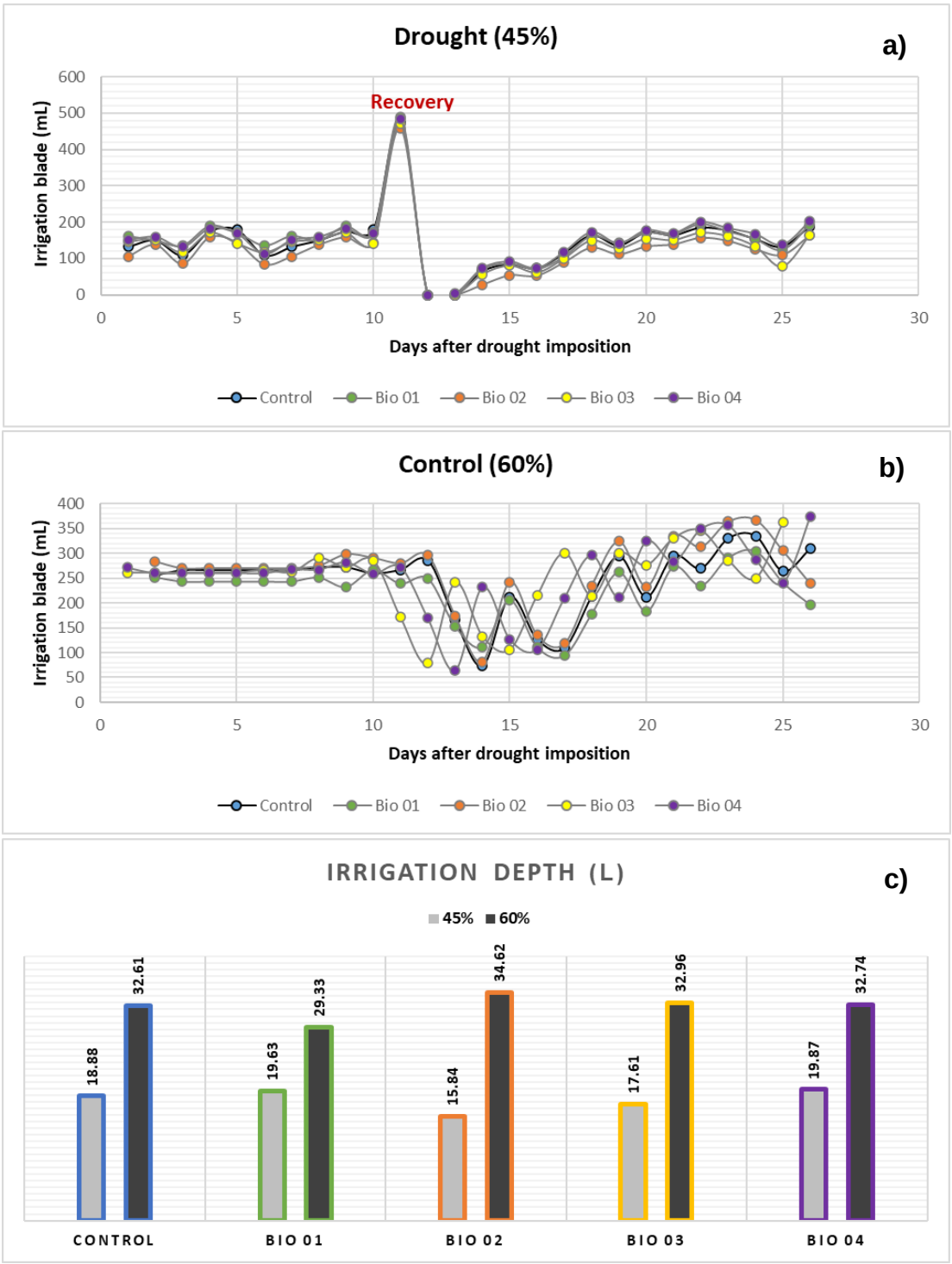



**Figure 10 -** Irrigation depth applied (a, b) and total consumed (c) by soybean plants treated with different biostimulant formulations, under well-irrigated (60% field capacity) and drought (45% field capacity) conditions. A full irrigation (recovery) was applied on the tenth day of drought, corresponding to irrigation depth similar to that applied in control treatment.

Throughout the experiment, the environmental conditions of average temperature during the day and night, as well as the data of relative air humidity, were recorded using a digital thermo-hygrometer (Figure 11).

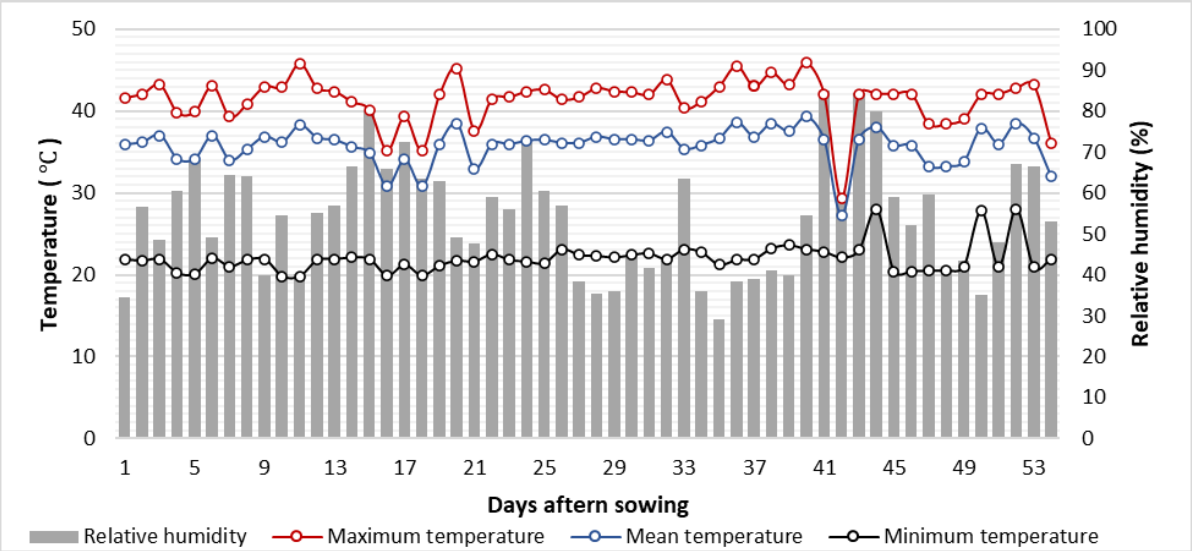



**Figure 11-** Dynamics of average, maximum, minimum temperature and relative air humidity in the greenhouse during the experimental period with soybean plants.

5.2.2 Growth parameters

The height of the plants and stem diameter were estimated using a tape measure and a digital caliper, respectively. The number of trifoliates was recorded and used to estimate the average number of trifoliates per plant. Leaf area was determined using an area meter (model LI-3100C, LI-COR, Inc. Lincoln, Nebraska, USA). Root volume was measured by the displacement of water caused by the introduction of roots into a graduated cylinder (Basso, 1999), and root length was obtained using a tape measure. During the harvest, to obtain the dry mass of the root and aerial parts (leaves + stem), the plants were cut at the internode between the first node from the base and the soil mark, followed by drying in a forced-air circulation oven at 72°C until reaching constant dry mass, with weighing performed on a precision scale.

The relative drought tolerance index was calculated considering the total dry mass of stressed plants compared to the dry mass of control plants, following adaptations by Miranda *et al*. (2021) and Silva *et al*. (2023).

### 5.2.3 Hydric status

To estimate the relative water content (RWC), one leaf from the plants of each treatment was excised and used to extract ten leaf disks with a diameter of 1.0 cm, which were weighed to obtain the fresh mass (FM). They were then immersed in distilled water for 24 hours to determine the turgid mass (TM). Subsequently, the material was placed in an oven at 60°C for 72 h for drying, to obtain the dry mass (DM). Finally, the RWC was calculated according to the methodology of Čatský (1960), using the following formula:

$$RWC = \frac{(\mathrm{FM} - \mathrm{DM})}{(\mathrm{TM} - \mathrm{DM})} \times 100$$

To determine the osmotic potential (Ψs), leaves were pressed to extract the leaf sap into microtubes and centrifuged at 3,000 × g for 15 min. Osmolarity was determined by readings of the respective extracts in a micro-osmometer (Vapor Pressure Osmometer, model 5600, Wescor, Utah, USA).

Leaf succulence (LS) was determined using a methodology adapted from Mantovani (2011), based on the water content in the leaves relative to the leaf area, using the following formula:

$$LS_{\,(g\,H_2O\,cm^{-2})} = \frac{(\mathrm{FLM} - \mathrm{DLM})}{(\mathrm{LA})}$$

The leaf temperature was measured on the first fully expanded leaf with the aid of a thermal camera (model FLIR TG165), between 11:30 and 12:00h on fully sunny days.

### 5.2.4 Gas exchanges

The net assimilation rate of $CO_2$ ($A$), stomatal conductance ($g_s$), transpiration rate ($E$), and internal carbon concentration (Ci) were measured on the first fully expanded leaf of all soybean plants, using an infrared gas analyzer (IRGA, Model

GFS3000 - Walz), between 08:30 and 10:30 a.m. on fully sunny days, the time of maximum photosynthesis for soybean crop. The instantaneous and intrinsic water use efficiency (WUE) were calculated as the ratios *A*/*E* and *A*/$g_s$, respectively, and the Rubisco carboxylation efficiency as the ratio *A*/Ci.

### 5.2.5 Chlorophyll a fluorescence

The chlorophyll fluorescence parameters were obtained in mature and fully expanded leaves, after 30 min of dark adaptation, using a fluorometer coupled to the IRGA. The variables analyzed were initial fluorescence (Fo), maximum fluorescence (Fm), variable fluorescence (Fv), and potential quantum yield of PSII (Fv'/Fm') (Genty *et al*., 1989). The variables of the slow phase of fluorescence induction, fluorescence in a light-adapted sample before the saturation pulse (F), and maximum fluorescence in a light-adapted sample (Fm') were obtained sequentially with the application of a saturating actinic light pulse (>3000 µmol photons $m^{-2}$ $s^{-1}$).

The parameters of minimum fluorescence of illuminated plant tissue (Fo') (Oxborough; Baker, 1997) and the estimation of open reaction centres of PSII (qP) (Kramer *et al*., 2004) were calculated from F and Fm'. The effective quantum yields of linear electron flow through PSII (ϕPSII), regulated energy dissipation (Y(NPQ)), and ϕPSII were also used to estimate the apparent electron transport rate (ETR). The photochemical extinction coefficient was calculated as qP = (Fm' Fs) / (Fm' - Fo'), and the non-photochemical extinction coefficient as NPQ = (Fm - Fm') / Fm'.

### 5.2.6 Photosynthetic pigments

The determination of photosynthetic pigments (chlorophylls a, b, total, and carotenoids) was based on the methodology described by Wellburn (1994). Leaf discs of 1.0 cm diameter were incubated in a solution containing dimethyl sulfoxide (DMSO) saturated with calcium carbonate, shielded from light for 48 h at room temperature. After this time, the extracts were incubated in a water bath at 65°C for 1.0 h. Once at

room temperature, the mixture was read in a spectrophotometer at 480, 649, and 665 nm, and the contents of chlorophylls a, b, total, and carotenoids were determined using the equations described by Wellburn (1994).

### 5.2.7 Biochemical analyzes

#### *5.2.7.1 Soluble carbohydrates*

The extraction of total soluble carbohydrates followed the methodology of Rinne *et al*. (2012), where 50 mg of lyophilized plant material (leaf and root samples) were transferred to test tubes containing 5.0 mL of 80% ethanol, and incubated in a water bath at 85°C for 30 min. Subsequently, the cooled material at room temperature was centrifuged at 10,000 x g for 10 min and the supernatant was collected.

After extraction, the total soluble carbohydrates were quantified according to the methodology of Dubois *et al*. (1956). Aliquots of 200 µL of the extract (supernatant) were homogenized with 200 µL of 5% (v/v) phenol and 1.0 mL of concentrated sulfuric acid ($H_2SO_4$). The mixture was then shaken and allowed to stand for about 10 min for cooling. Subsequently, absorbance readings were taken at 490 nm using a microplate reader (Agilent, BioTek Synergy MX, USA), and the soluble carbohydrate content was established based on a standard curve previously constructed with increasing concentrations of D (+)-anhydrous glucose solutions.

#### *5.2.7.2 Free amino acids*

For the determination of free amino acids, the same alcoholic extract prepared for soluble carbohydrates was used. In test tubes, 500 µL of the extract, 250 µL of 0.2 M citrate buffer (pH 5.0), 500 µL of 0.2 mM potassium cyanide in 100% methylcellosolve ($C_3H_8O_2$), and 100 µL of 5% ninhydrin in 100% methylcellosolve were added. Subsequently, the tubes were closed, vigorously shaken, and placed in a water

bath at 100°C for 20 min. The reaction was stopped in an ice bath, and then 650 µL of 60% ethanol were added. The quantification of soluble N-amino was performed based on absorbance readings at 570 nm using a microplate reader. The levels of total soluble amino acids were obtained based on the standard glycine curve with increasing concentrations, following the methodology of Yemm and Cocking (1955).

*5.2.7.3 Antioxidative enzymes*

The assays to measure the activities of the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and guaiacol peroxidase (G-POD) were conducted to verify the activation of antioxidant mechanisms in response to the treatments studied. Crude extracts were prepared by homogenizing 300 mg of fresh leaves in 200 mM potassium phosphate buffer (pH 7.8), 10 mM ethylenediaminetetraacetic acid (EDTA), 200 mM ascorbic acid, and ultrapure Milli-Q® water. After homogenization, the mixture was centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant was collected and used as the enzyme extract. Protein content was determined in each extract by reaction with Coomassie Brilliant Blue G-250, through absorbance readings at 595 nm, using bovine serum albumin as the standard (Bradford, 1976).

The activity of SOD was measured by adding an aliquot of the enzyme extract to a reaction medium containing 100 mM phosphate buffer (pH 7.8), 10 µM disodium EDTA, 13 µM methionine, 75 µM NBT, 50 mM $Na_2CO_3$, 0.025% (m/v) Triton X-100, and 2.0 µM riboflavin. Absorbance readings were taken at 560 nm using a microplate reader, and the results were expressed in UA $mg^{-1}$ protein. Each UA (unit of enzymatic activity) was defined as the amount of enzyme capable of causing 50% inhibition of the NBT photoreduction, according to the methodology of Beyer and Fridovich (1987).

The activity of APX was determined based on the methodology of Nakano and Asada (1981). Aliquots of the enzyme extract were mixed with 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM sodium ascorbate, 0.1 mM $H_2O_2$, and 0.1 mM disodium EDTA. After homogenization, the reaction kinetics were recorded using a microplate reader, and APX activity was quantified based on the decrease in absorbance at 290 nm over 5 min with readings taken at 30-second intervals. For the

calculations, the molar extinction coefficient (ε = 2.8 mM-1 cm-1) of ascorbate was considered, and the stoichiometry of the reaction was two moles of ascorbate for each mole of hydrogen peroxide ($H_2O_2$) formed. The results were expressed in μmol $H_2O_2$ $min^{-1}$ $mg^{-1}$ protein.

The activity of CAT was quantified according to the methodology of Havir and McHale (1987) and Anderson *et al*. (1995). The reaction medium was composed of aliquots of the extract, 200 mM potassium phosphate buffer (pH 7.0), Milli-Q water, and 250 mM $H_2O_2$. Enzyme kinetics were estimated by absorbance readings at 240 nm using a microplate reader. Based on the readings and the molar extinction coefficient (ε = 36 M-1 cm-1), the results were expressed in μmol $H_2O_2$ $min^{-1}$ $mg^{-1}$ protein.

The activity of GPX was measured following the methodology of Kar and Mishra (1976). The reaction medium was composed of the enzyme extract, 100 mM potassium phosphate buffer (pH 7.0), 0.02 M guaiacol, and 60 mM $H_2O_2$. GPX activity was determined by absorbance readings at 420 nm, considering the formation of tetraguaiacol. For calculate GPX activity, the molar extinction coefficient of tetraguaiacol (ε = 26.6 $mM^{-1}$ $cm^{-1}$) and the reaction stoichiometry, where four moles of $H_2O_2$ are consumed for each mole of tetraguaiacol formed, were considered. The results were expressed in μmol $H_2O_2$ $min^{-1}$ $mg^{-1}$ protein.

*5.2.7.4 Lipid peroxidation*

Lipid peroxidation was measured levels following the methodology suggested by Heath and Packer (1968). Extraction and quantification procedures were carried out using fresh leaves with the use of trichloroacetic acid (TCA) and thiobarbituric acid (TBA). MDA levels were determined by spectrophotometric readings at 532 nm and 600 nm, based on the molar extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$ for MDA (Heath; Packer, 1968).

*5.2.7.5 Hydrogen peroxide ($H_2O_2$)*

To estimate $H_2O_2$ levels, samples of fresh leaf tissue were macerated in liquid $N_2$ and homogenized with 5.0% TCA at 4.0 °C. Subsequently, the homogenate was centrifuged at 12,000 × g for 15 min, and the supernatant was collected. $H_2O_2$ levels were determined by absorbance readings at 390 nm after the reaction of $H_2O_2$ with potassium iodide, based on a standard curve obtained from solutions with increasing concentrations of $H_2O_2$ (Alexieva *et al*., 2001).

5.2.8 Metabolic profile

For metabolome analysis, samples of plant material were collected and immediately frozen in liquid $N_2$, and metabolites were extracted using an extraction solution containing water, methanol, and chloroform (1:2.5:1 v/v/v) for 30 min at 4 °C. During this process, ribitol was added as an internal standard. Subsequently, the homogenate was centrifuged for 10 min at 14,000 × g, and 150 µL aliquots of the polar phase were collected and then dried in a Speedvac overnight. The dried samples were then derivatized with methoximation reagent - methoxylamine (20 mg $mL^{-1}$ in pyridine) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), followed by incubation at 37 °C for 2 h in a dry bath with agitation (thermomixer).

The identification and quantification of metabolites were performed using a Gas Chromatography system coupled with a Mass Spectrometer (GCMS), based on the methodology of Roessner *et al*. (2001). During the process, 1.0 µL of the derivatized sample was injected in split mode (1:10), at a flow rate of 1.2 mL $min^{-1}$. Helium gas was used as the carrier gas, and the separation of metabolites was carried out using an RTX-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The temperature program was set to an initial temperature of 80 °C for 2.0 min, with increments of 10 °C $min^{-1}$ until reaching a final temperature of 315 °C, which was maintained for 8.0 min. The injection, interface, and ion source temperatures were set at 230, 250, and 200 °C, respectively. The mass spectrometer operated at 70 eV (EI), with a mass fragment detection range of 40-700 (m/z), after a 3-minute cutoff time. Chromatograms and

mass spectra were analyzed using the XcaliburTM 2.1 software (Thermo Fisher Scientific, Waltham, MA, USA). Metabolite identification was based on retention time and fragmentation spectrum, using an internal library built from a mixture of standards and fragmentation spectra recorded in the Golm Metabolome Database (GMD). Results were expressed as the relative concentration of each metabolite by determining and normalizing the peak area to the internal standard Ribitol and the mass of the sample used.

### 5.2.9 Statistical analysis

For all evaluated parameters, the data were subjected to analysis of variance (ANOVA), and significances were observed using the F-test at a 5% probability level. Means were compared using the Tukey test at a 5% significance level, using R software (R Development Core Team, 2016) for data analysis and SigmaPlot Version 15.0 software (SYSTAT SOFTWARE, 2006) for graph generation.

The metabolome data were shown in relative content according to the internal standard (ribitol) and the respective dry mass of each sample. For multivariant analysis, data was submitted to MetaboAnalyst 6.0 software and pre-processed using square root transformation and auto-scaling. Principal Component Analysis (PCA) was run to obtain an overview of metabolic profile variation among biostimulant treatments. In addition, a univariate analysis (ANOVA one-way followed by Tukey test) and heatmap analysis were performed to identify the significant metabolites that contribute to variance among the treatments.

## 5.3 Results

### 5.3.1 Growth performance

In the present study, the phenological aspect of soybean plants showed differential behavior in response to the treatments, with variations dependent on both water regimes and biostimulants (Figure 12).

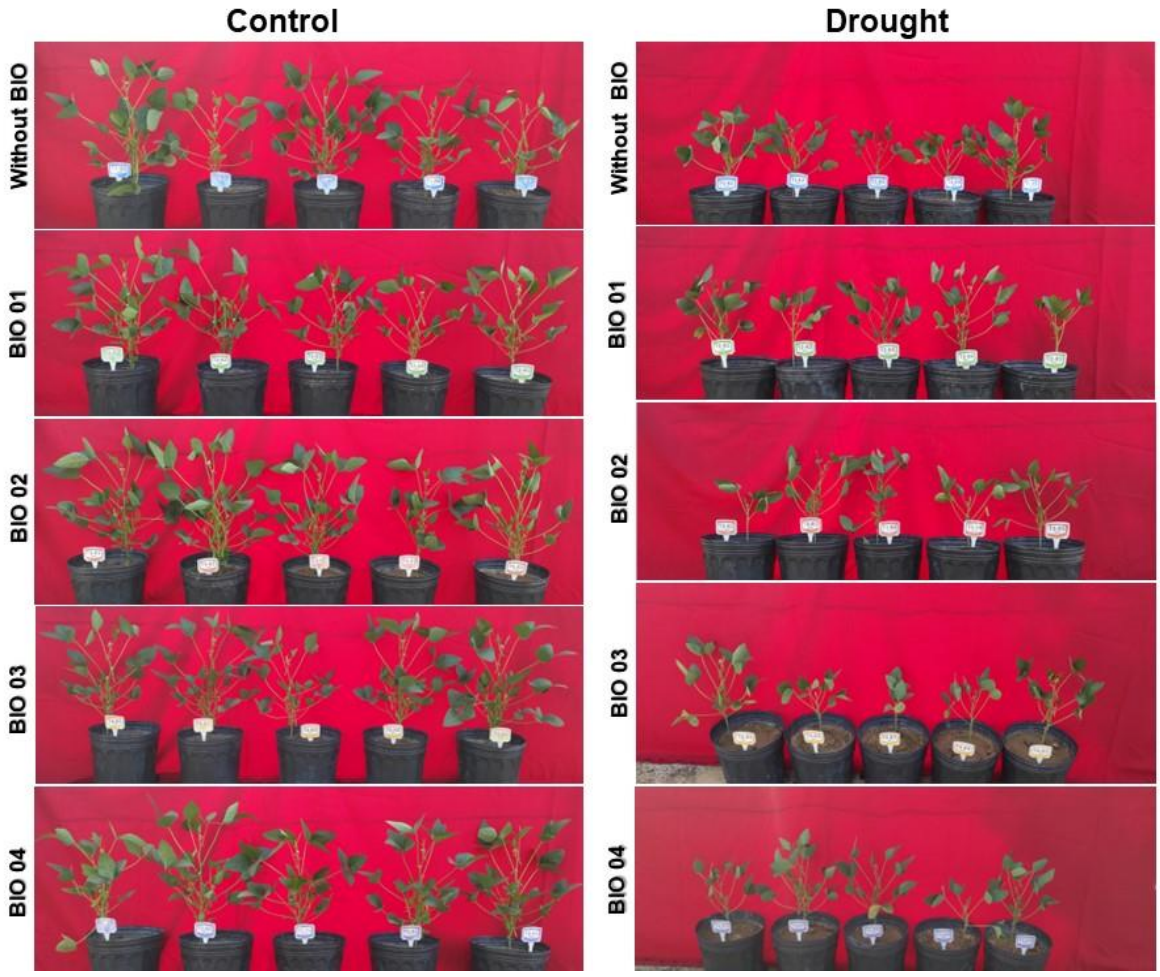



**Figure 12 -** Phenological aspects of representative soybean plants/plots at the end of the experiment carried out in a greenhouse after 53 days growing under well-irrigated conditions (control) and 25 days of water deficit, in absence and presence (Bio 01, Bio 02, Bio 03, Bio 04) of biostimulants.

The biometric data is presented in Figure 13. Overall, the water deficit significantly reduced all studied biometric variables, except for root growth (Figure 13d). Under well-irrigated conditions, changes due to the formulations were only

recorded for stem diameter, where plants from Bio 02 and Bio 03 treatments showed higher values compared to other formulations and the control without biostimulant (Figure 13a); and for root volume, where Bio 02 provided higher rates compared to other treatments (Figure 13e).

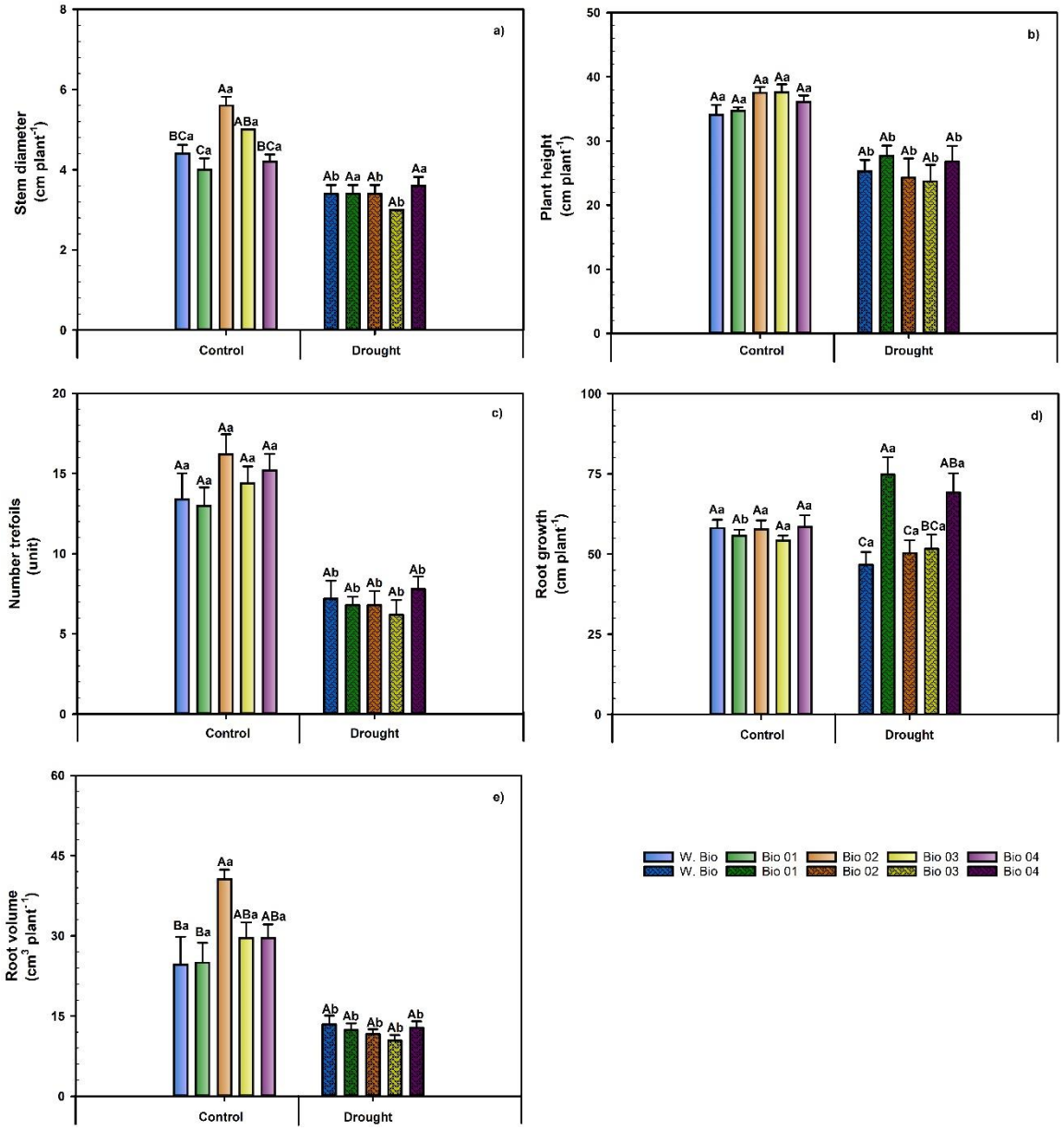



**Figure 13 –** Stem diameter (a), plant height (b), number of trifoliate leaves (c), root length (d), and root volume (e) of soybean plants, cultivar Extrema, under full irrigation (control) and water deficit conditions, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$).

Under water stress, no significant changes were recorded due to the biostimulant treatments for the variables stem diameter, plant height, number of trifoliate, and root volume. On the other hand, plants treated with the Bio 01 and Bio 04 formulations showed higher values of root length than those from the other treatments (Figure 13d).

Under well-irrigated conditions, significant changes in growth were observed only in plants treated with Bio 01, which showed lower values of root dry mass and leaf area compared to plants from Bio 02 treatments, although there was no significant difference compared to the control without biostimulant (Figure 13b, d). Water limitation significantly reduced the accumulation of shoot dry mass, root dry mass, total dry mass, and leaf area, resulting in lower relative stress tolerance indices compared to the control, regardless of the biostimulant treatments (Figure 14).

In the drought presence, plants from all biostimulant treatments showed similar values of dry mass and leaf area (Figure 14e). However, the application of biostimulants showed a trend towards reducing plant tolerance to water deficit, although a significant difference was only recorded in stressed plants from Bio 02 treatment (tolerance rate of 26.1%), which showed 41.8% lower tolerance indices compared to stressed plants without biostimulants (tolerance rate of 67.9%) (Figure 14e).

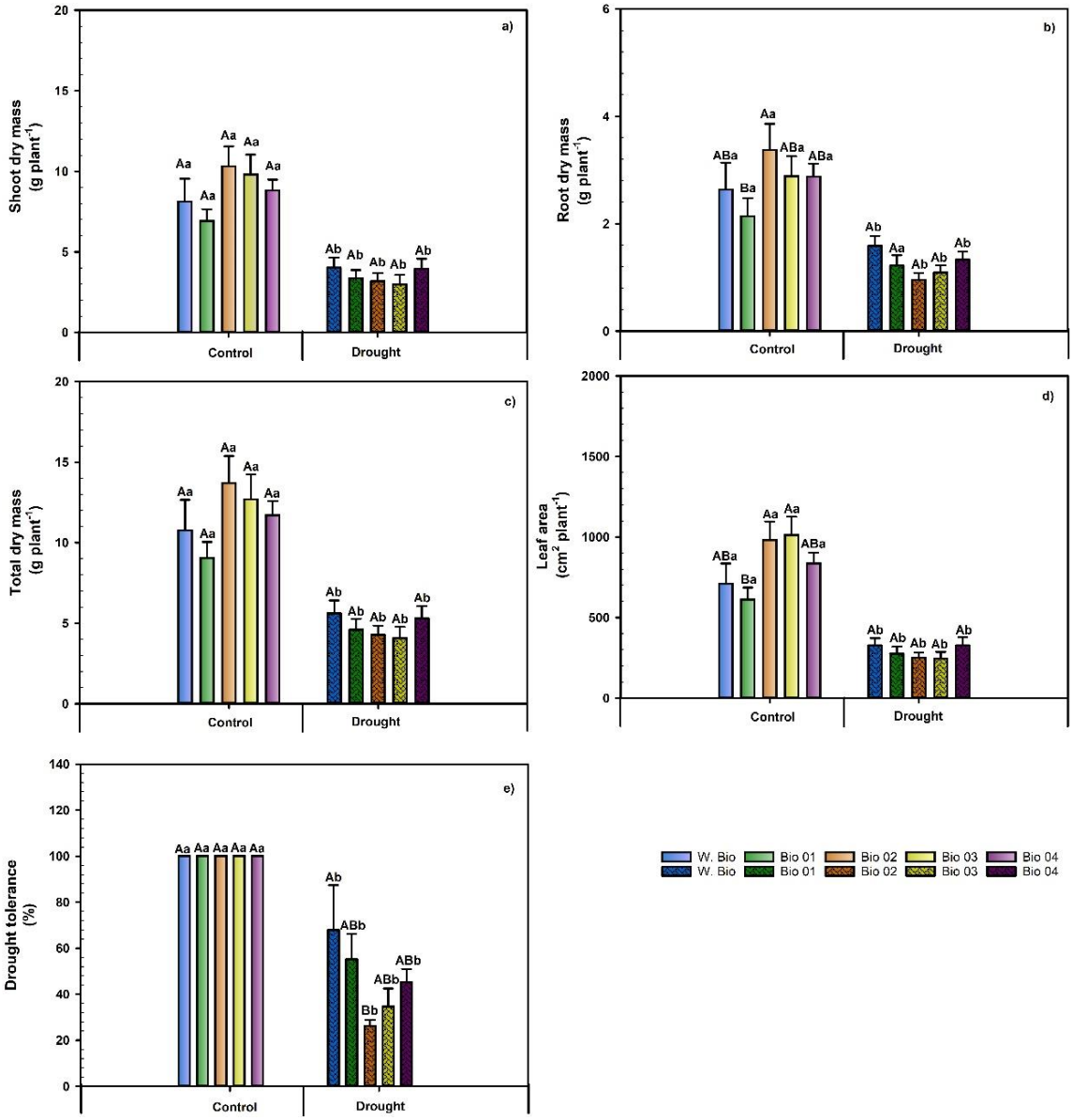



**Figure 14 –** Shoot dry mass (a), root dry mass (b), total dry mass (c), leaf area (d), and relative stress tolerance (e) of soybean plants, cultivar Extrema, with full irrigation (control) and under water deficit, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$).

### 5.3.2 Hydric status

Well-irrigated plants showed little or no significant changes in variables related to hydric status due to the application of biostimulants (Figure 15), except for the

parameters of osmotic potential and leaf succulence of Bio 04 plants, which showed lower and higher values, respectively, compared to those of plants from other treatments.

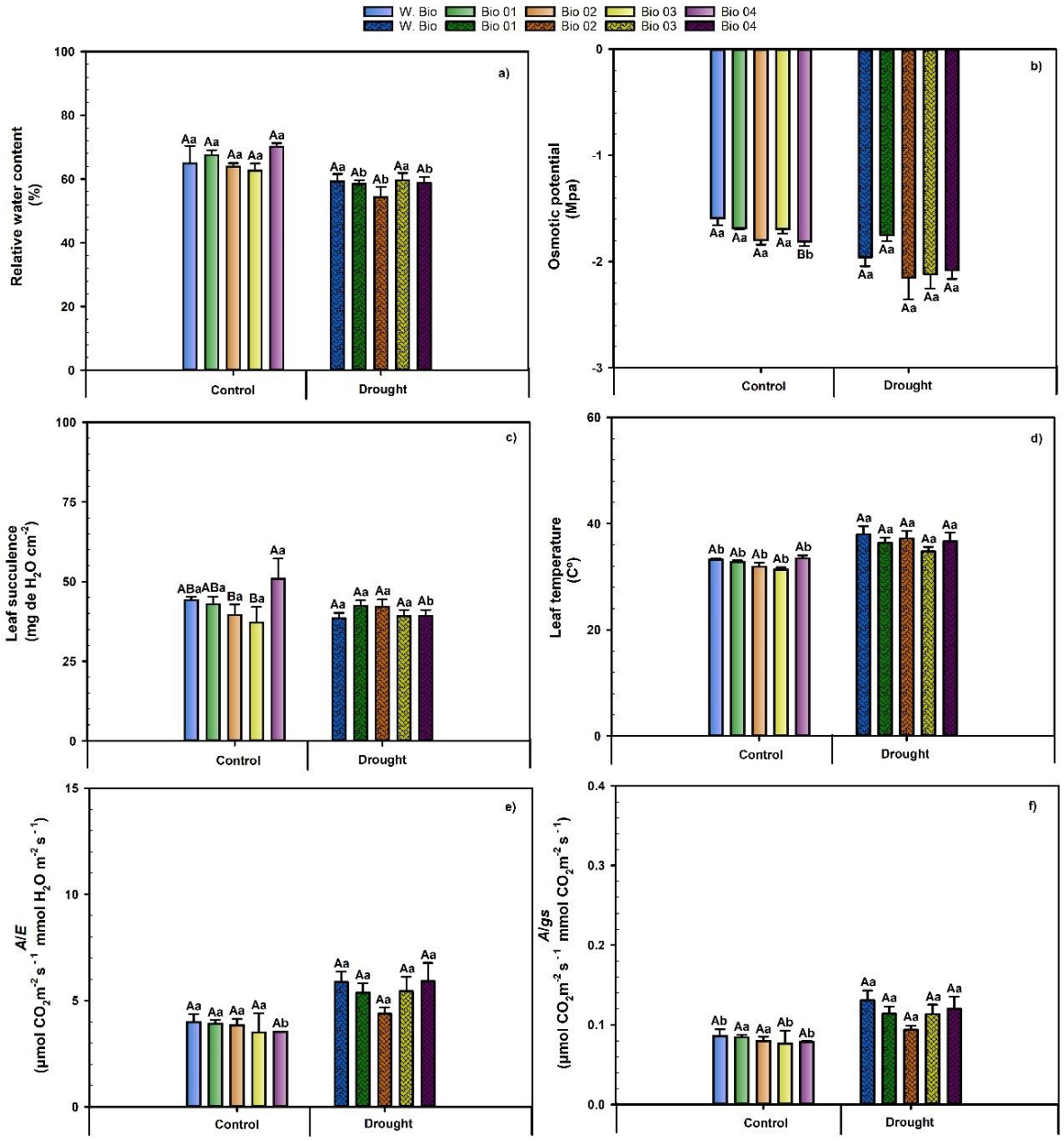



**Figure 15 –** Relative water content (a), osmotic potential (b), leaf succulence (c), leaf temperature (d), instantaneous (e) and intrinsic (f) water use efficiency of soybean plants, cultivar Extrema, with full irrigation (control) and under water deficit, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$).

Water deficit led to significant reductions in relative water content (RWC) in stressed plants treated with Bio 01, Bio 02, and Bio 04, while stressed plants growing without biostimulant and Bio 03 plants showed similar values to the control (Figure 15a). Conversely, leaf succulence of soybean plants remained unchanged due to water deficit, except for plants treated with Bio 04, which showed reductions compared to their respective control (Figure 15c).

The osmotic potential of plants growing under well-irrigated conditions was higher in those treated with Bio 04, which, in turn, was the only treatment to show reduced osmotic potential due to the imposed water deficit depth (Figure 15b). On the other hand, leaf temperature and intrinsic water use efficiency ($A/g_s$) increased due to water stress in plants from all treatments (Figure 15d, f), while instantaneous water use efficiency (*A/E*) increased under stress conditions only in Bio 04 plants (Figure 15e).

It is worth noting that under water deficit conditions, no changes were recorded due to biostimulant treatments in none of the variables related to the hydric status of soybean plants, regardless of the formulation studied. Similarly, leaf temperature increased due to water stress in all plants treated with biostimulants, as evidenced in thermal images, and no alteration by biostimulant was registered (Figure 16).

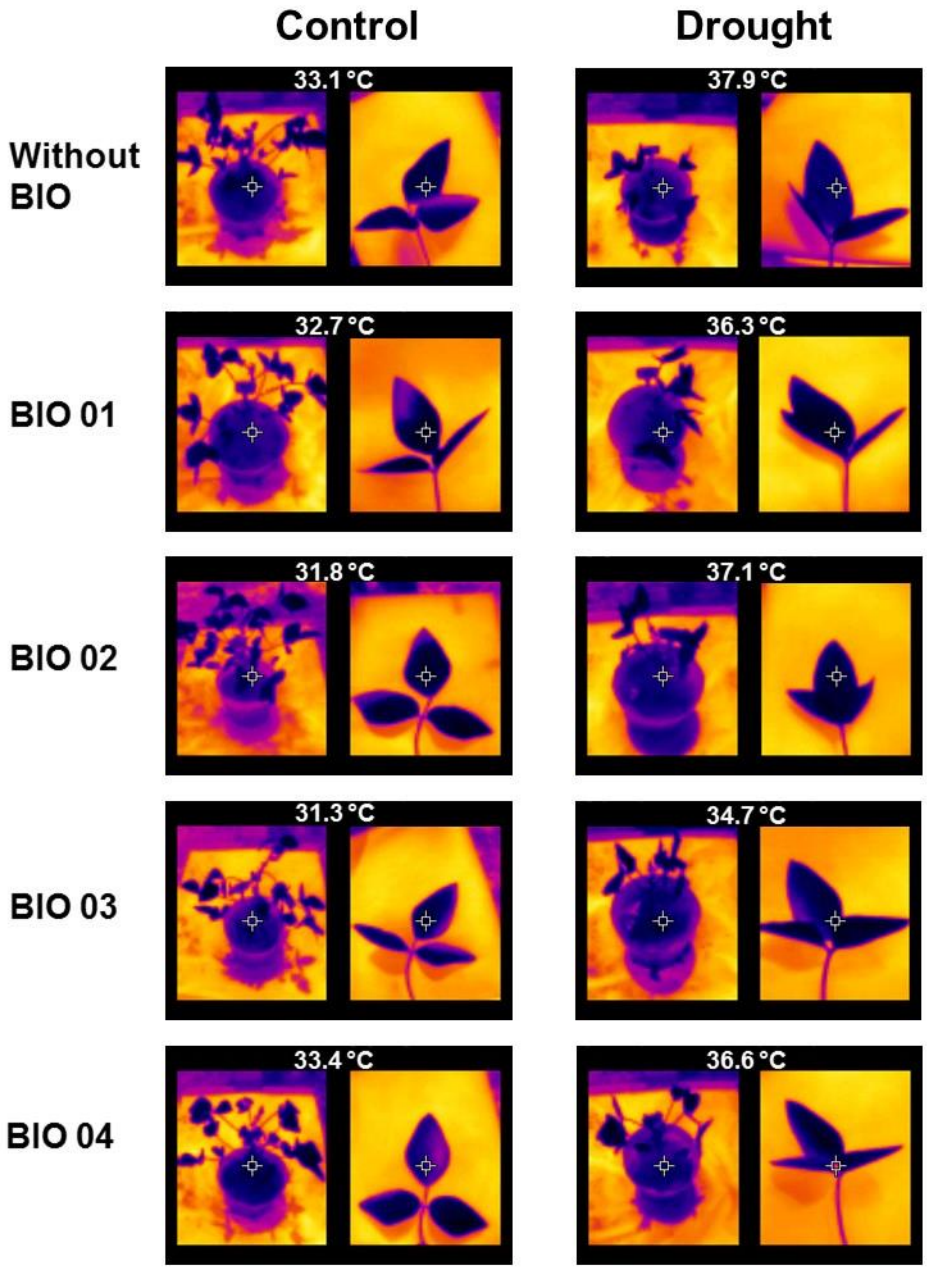



**Figure 16 –** Thermography of a representative soybean plant, cultivar Extrema, at 53 days of cultivation and 25 days of water deficit application, treated with (Bio 01, Bio 02, Bio 03, Bio 04) and without biostimulants.

### 5.3.3 Efficiency of the photosynthetic machinery

The gas exchange parameters showed distinct results with the imposition of water stress in most cases, and no differential responses were observed based on the applied biostimulant formulations, both in the control and drought treatments. Net photosynthesis remained unchanged, except for plants treated with Bio 02, which

showed a lower photosynthetic rate compared to their respective well-irrigated control (Figure 17a).

Transpiration rates were lower in stressed plants compared to the control (Figure 17b), which was associated with reductions in stomatal conductance rates (Figure 17c). On the other hand, the Rubisco carboxylation efficiency remained unchanged, regardless of water stress and the application of biostimulants (Figure 17d).

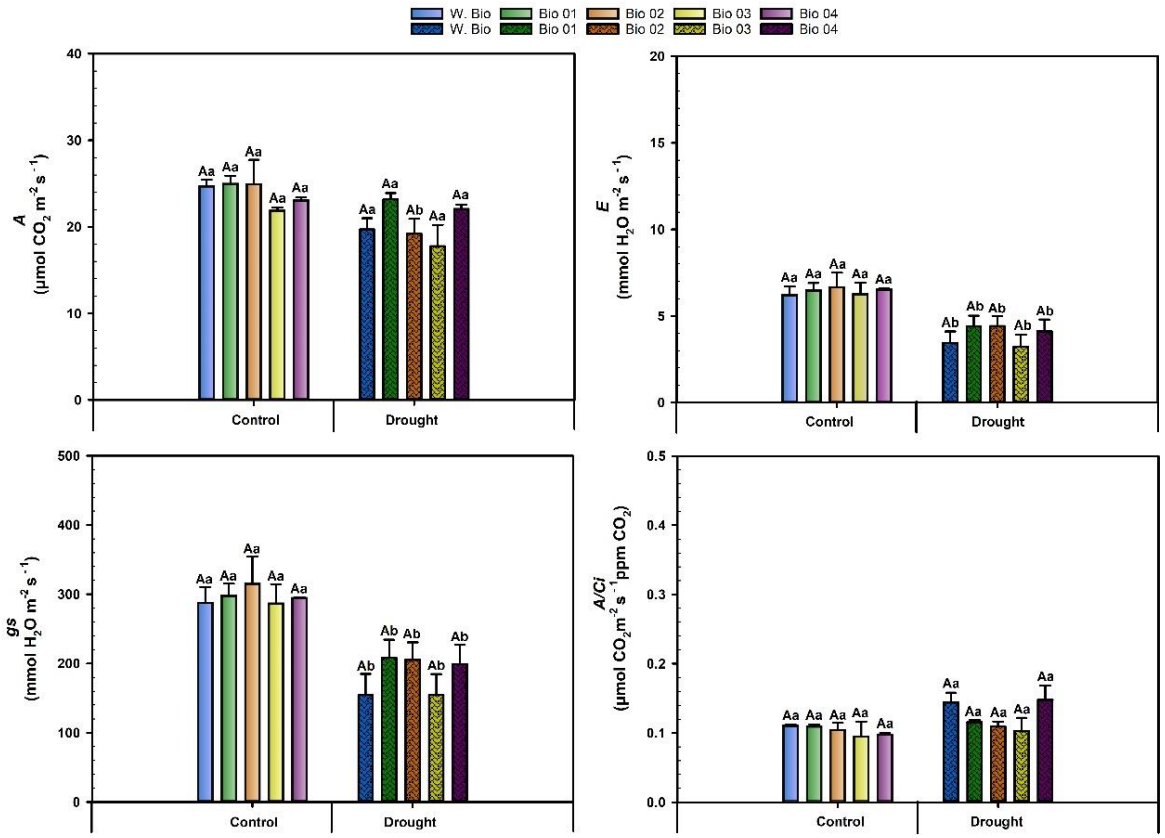



**Figure 17 –** Net photosynthesis (*A*, a), transpiration (*E*, b), stomatal conductance (*gs*, c), and Rubisco carboxylation efficiency (*A/Ci*, d) of soybean plants, cultivar Extrema, with full irrigation (control) and under water deficit, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$).

Overall, soybean plants showed photochemical efficiency, estimated by chlorophyll a fluorescence parameters, practically unchanged due to the studied treatments. The exception was non-photochemical quenching (NPQ), where drought-stressed plants treated with biostimulant 03 showed higher values than their respective control. Under drought, NPQ values of Bio 03 plants were statistically similar to Bio 01

and Bio 02 treatments but higher than those of Bio 04 plants and the negative control (Figure 18).

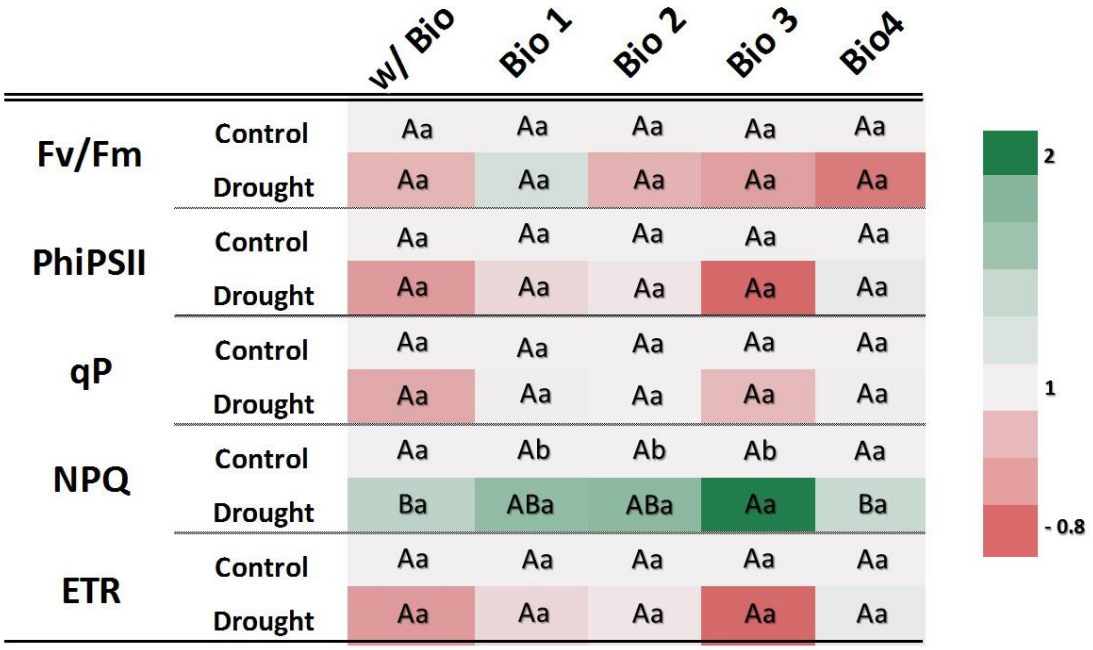



**Figure 18 –** Maximum quantum efficiency (Fv/Fm) and effective quantum efficiency of photosystem II (PhiPSII), photochemical (qP) and non-photochemical (NPQ) quenching, and electron transport rate (ETR) of soybean plants, cultivar Extrema, with full irrigation (control) and under water deficit, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$). Color changes to green indicate an increase and to red indicate a decrease in each variable analyzed.

The water deficit condition significantly reduced all photosynthetic pigments of soybean plants (Figure 19), except for chlorophyll b in plants from Bio 01 and Bio 02 treatments (Figure 19b). In all cases, there was no influence of biostimulants on the accumulation of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids, regardless of the water regime.

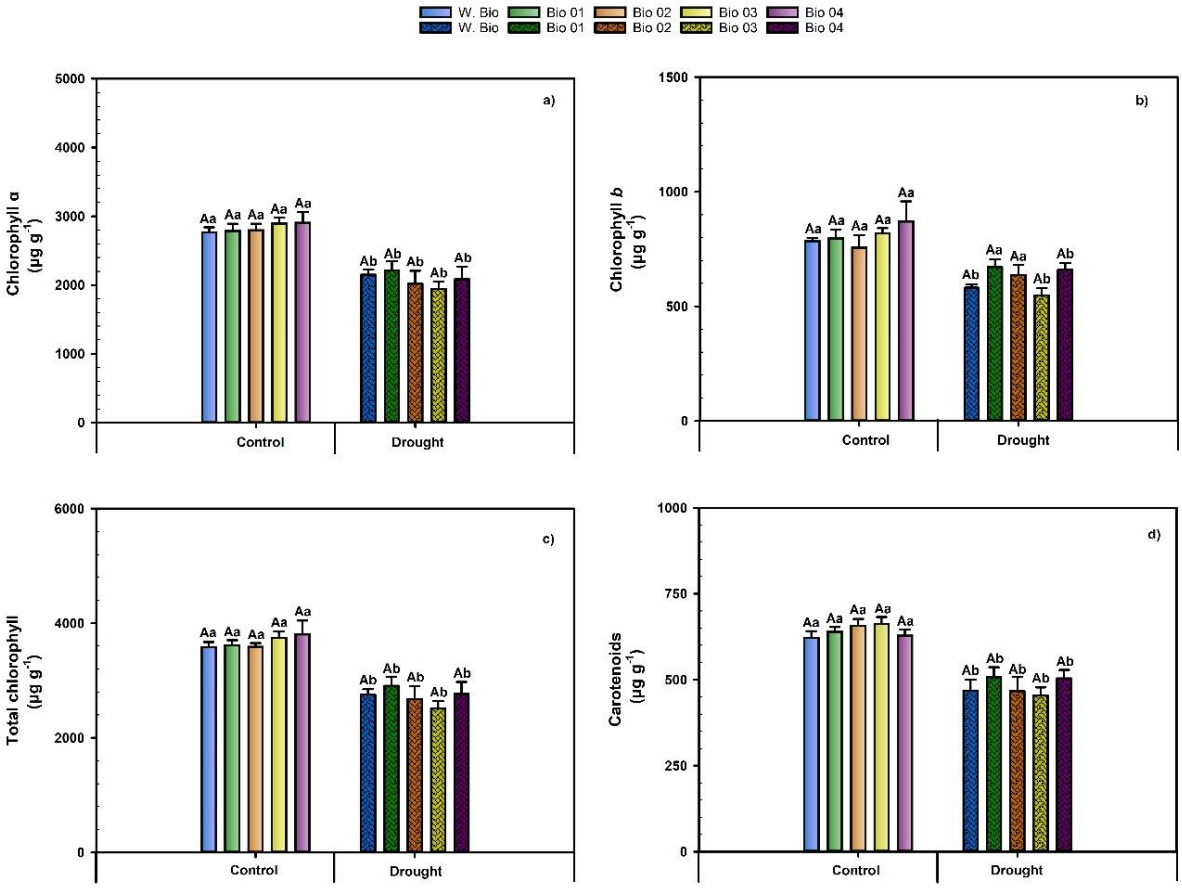



**Figure 19 –** Chlorophyll a content (a), chlorophyll b content (b), total chlorophyll content (c), and carotenoid content (d) of soybean plants, cultivar Extrema, with full irrigation (control) and under water deficit, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$).

### 5.3.4 Biochemical regulation, oxidative damage, and antioxidant system

Oxidative damage and antioxidant enzyme activity were differentially regulated by the treatments studied, with variations due to water deficit and the application of biostimulants (Figures 20, 21). In the absence of biostimulants, soybean plants showed increases in G-POD and SOD activity when exposed to water deficit, compared to the control, while they exhibited reductions in CAT activity (Figure 20).

The application of biostimulants differentially modulated the antioxidant enzymes (Figure 20). Stressed plants treated with Bio 01 showed increases in G-POD and SOD activity and reductions in APX activity compared to their respective control. On the other hand, plants sprayed with Bio 02 exhibited decreases in CAT and increases in G-POD when subjected to stress, compared to the well-irrigated control. Stressed plants treated with Bio 03 activated CAT and G-POD enzymes and reduced SOD activity under water deficit conditions. Lastly, under water limitation, plants treated with Bio 04 only activated G-POD activity and reduced APX and SOD activity compared to the respective well-irrigated control.

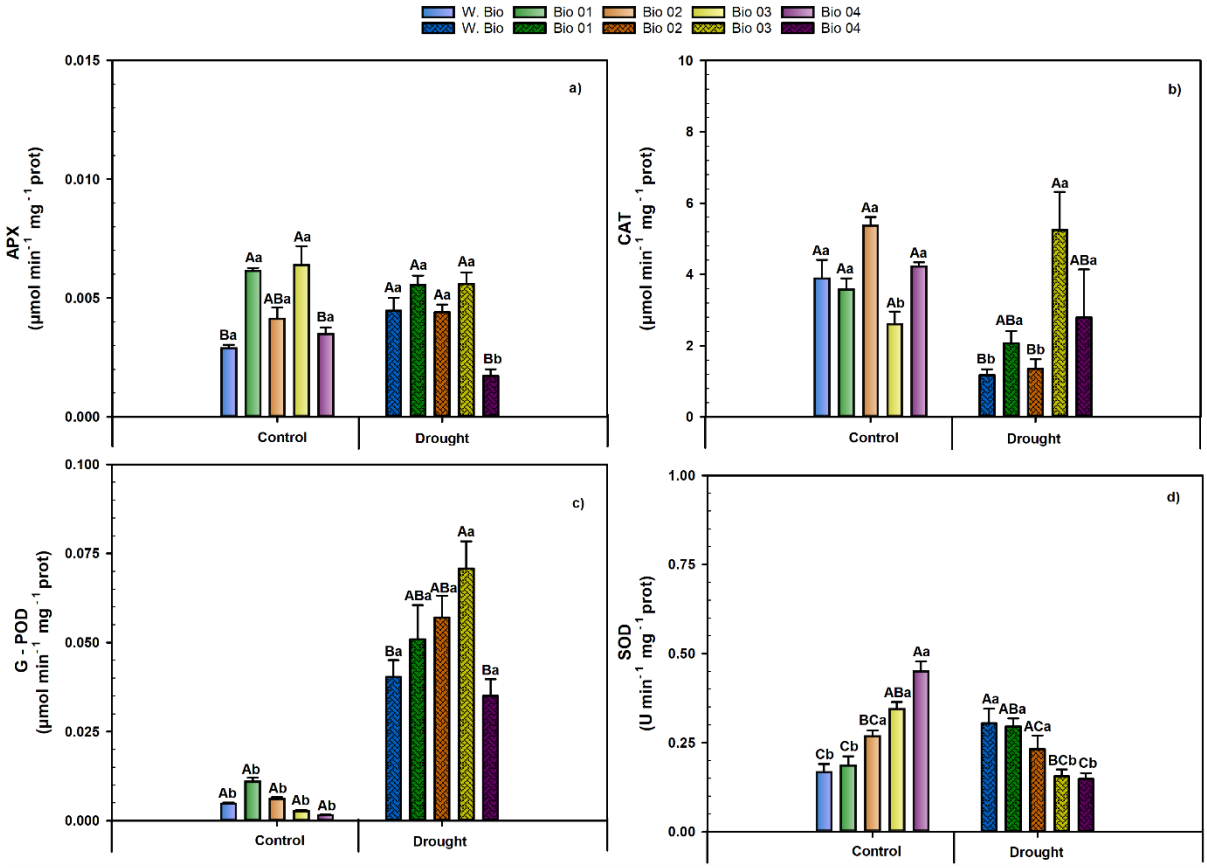



**Figure 20 –** Activity of ascorbate peroxidase (APX, a), catalase (CAT, b), guaiacol peroxidase (G-POD, c), and superoxide dismutase (SOD, d) enzymes in soybean plants, cultivar Extrema, with full irrigation (control) and under water deficit, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$).

The data revealed that the enzyme G-POD showed the highest increases in activity in the tissues of soybean plants under stress. Additionally, increases in the

activation of antioxidant enzymes due to biostimulants in plants subjected to water deficit were recorded only in the Bio 03 treatment. This group of plants showed higher CAT and G-POD activity compared to stressed plants not treated with biostimulants (Figure 20b, c).

In the leaves, malondialdehyde levels were similar in well-irrigated and stressed plants (Figure 21a), except for plants treated with Bio 03, which showed significant increases in MDA levels compared to the respective control. In the roots, water deficit increased MDA levels in plants from all treatments, and no effects of biostimulants were observed in stressed soybean plants (Figure 21b).

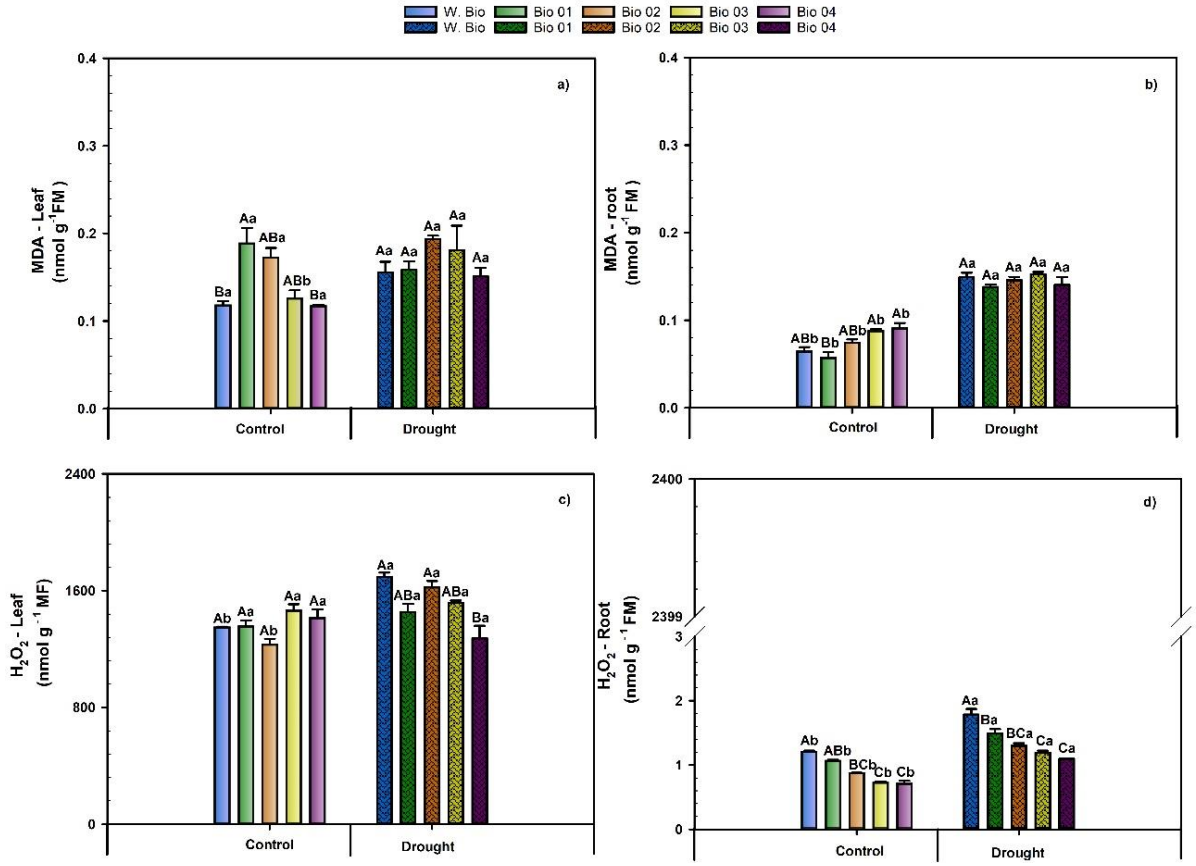



**Figure 21 –** Contents of malondialdehyde/lipid peroxidation (MDA) and hydrogen peroxide ($H_2O_2$) in the leaves (a, c) and roots (b, d) of soybean plants, cultivar Extrema, with full irrigation (control) and under water deficit, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$).

Foliar $H_2O_2$ levels were significantly increased by water stress only in plants growing without biostimulant and in those treated with Bio 02; while in the roots, significant increases were recorded in plants of all treatments compared to the well-

irrigated control treatments (Figure 21c, d). It is noteworthy that Bio 04 was effective in alleviating the accumulation of $H_2O_2$ in the leaves of stressed soybean plants; whereas, in the roots, all tested biostimulants promoted reductions in the accumulation of this reactive oxygen species compared to the treatment without biostimulant.

Overall, under well-irrigated conditions, treatments with biostimulants did not alter the levels of amino acids, carbohydrates, and proteins in soybean plants (Figure 22). Under water deficit, plants treated with Bio 01 and Bio 03 showed significant decreases in foliar levels of free amino acids (Figure 22a). In the roots, a similar pattern was observed in plants treated with Bio 02 and Bio 03, which showed significant reductions compared to their respective controls (Figure 22b). In any case, no significant increases in amino acid levels were observed in the leaves and roots of stressed plants due to the application of the evaluated biostimulants.

Alterations in foliar levels of soluble carbohydrates due to water deficit were recorded only in plants treated with Bio 04, which showed increases compared to the control (Figure 22c). In the roots, stress caused significant decreases in carbohydrate levels in plants growing without biostimulants and in those treated with Bio 02 and Bio 03, compared to their respective well-irrigated controls (Figure 22d). Similar to what was reported for amino acids, the biostimulants did not promote significant gains in carbohydrate accumulation in the tissues of soybean plants, with a tendency for reduction in some cases.

Water deficit significantly reduced protein levels in soybean plants without biostimulants (Figure 22e). However, no changes in protein levels were recorded as a result of biostimulant application, regardless of the water regime (Figure 22e).

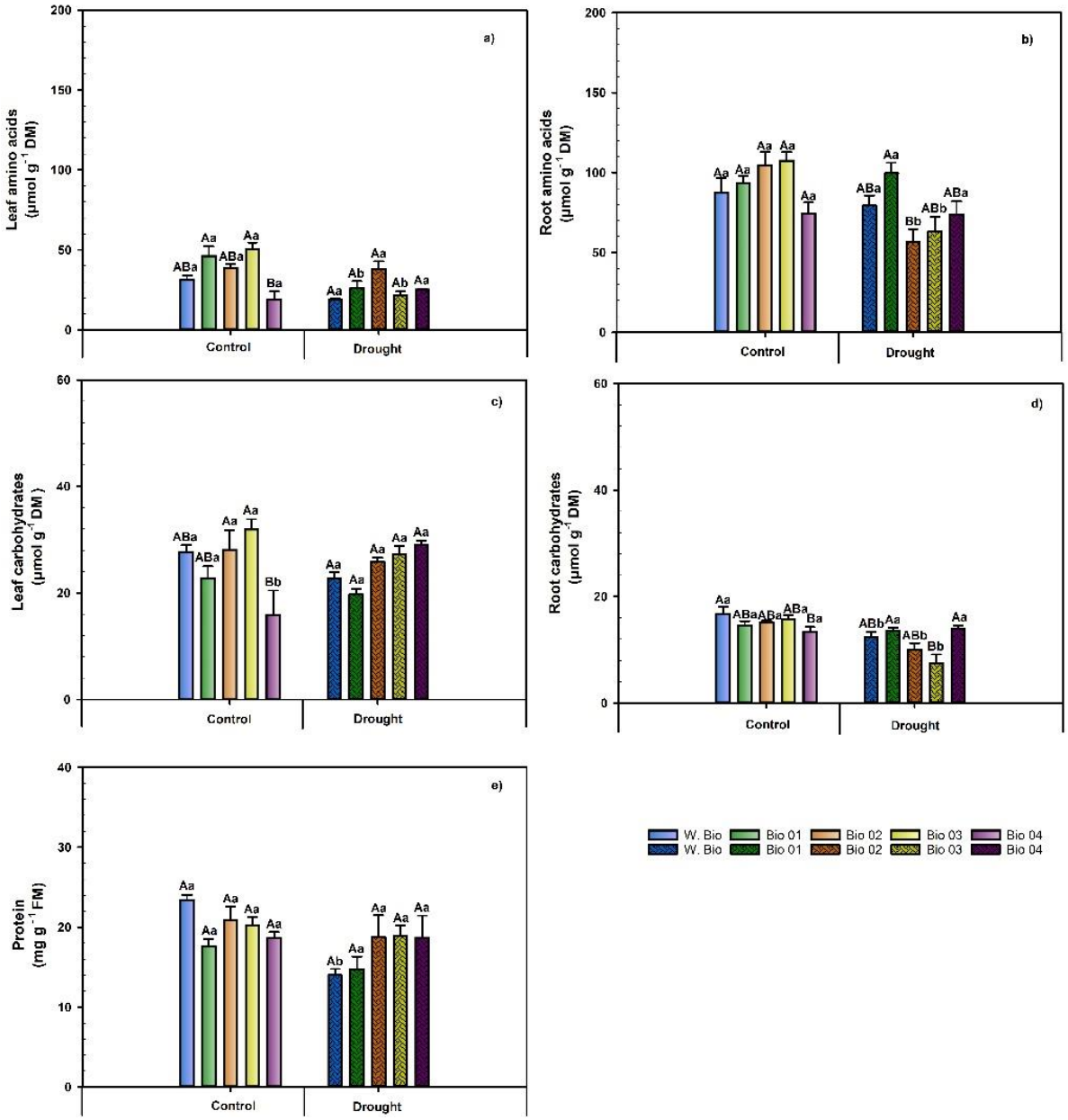



**Figure 22 –** Free amino acids in the leaf (a), amino acids in the root (b), carbohydrates in the leaf (c), carbohydrates in the root (d), and protein (e) of soybean plants, cultivar Extrema, with full irrigation (control) and under water deficit, in the absence and presence of biostimulants (Bio 01, Bio 02, Bio 03, Bio 04). Capital letters compare treatments based on the application of biostimulants at each water depth, while lowercase letters compare water treatments within each biostimulant treatment. Means followed by different letters differ statistically from each other, according to the Tukey test ($p \leq 0.05$).

5.3.5 Modulation of the metabolic profile

Through the analysis of the chromatogram and mass fragments for each peak detected by GCMS, 59 metabolites were identified, including 17 amino acids, 17 organic acids, 21 sugars and derivatives, and other organic compounds of primary metabolism (1 phenolic compound, 1 pyrimidine, 1 vitamin, and 1 amide) (Appendix E).

Figure 23 presents the principal component analysis (PCA), which explains 40.7% of the total variation for the leaf metabolites of soybean plants from different treatments. According to the ANOVA (one-way, followed by Tukey's test, $p < 0.01$), 39 metabolites were significant for the separation. The data clearly show the distribution of distinct groups, which together segregated the control treatment from the stress treatment. Under control conditions, the data show a differential behavior of plants treated with Bio 04 compared to those without biostimulant treatment.

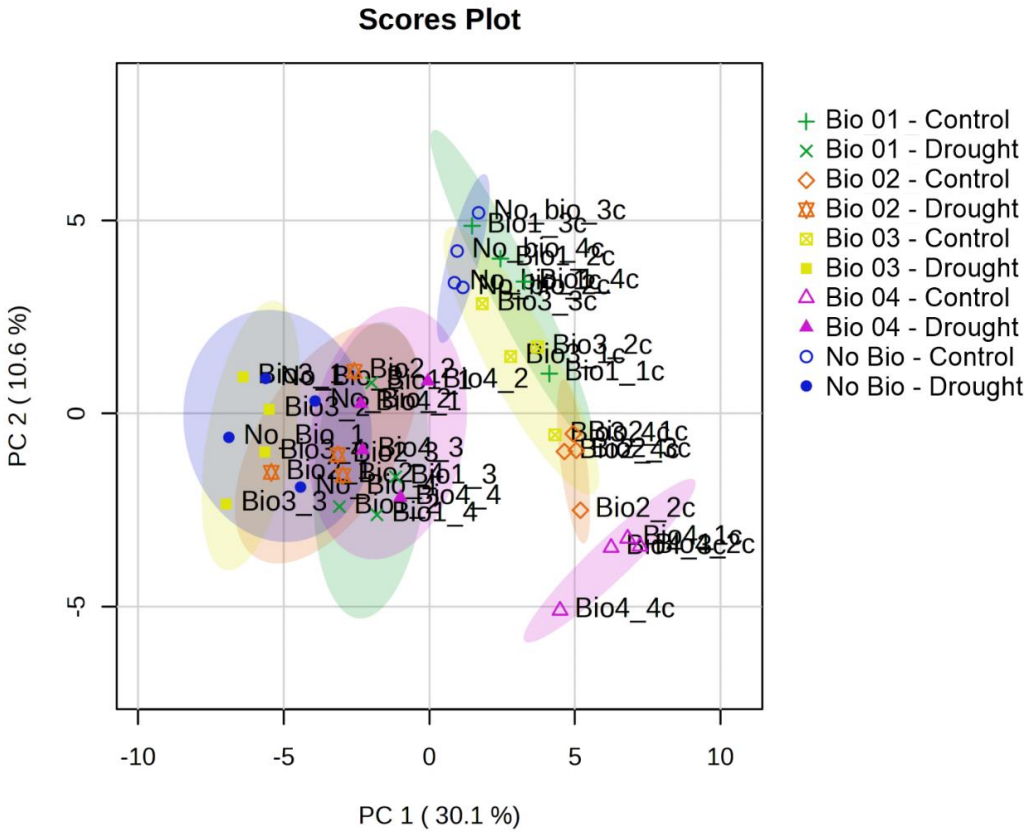



**Figure 23 -** Principal component analysis (PCA) of the metabolic profile in leaves of soybean plants (Extrema cultivar) in absence (control) and presence of drought, and subjected to different treatments with biostimulants.

In drought presence, distinct groups formed, and stressed plants grown with Bio 01 and Bio 04 tended to behave more similarly to the controls (Figure 23). Therefore, through the volcano plot, it can be observed that 10 metabolites were responsive to water deficit, regardless of biostimulant treatment (Figure 24). Under this condition, there was a significant increase in urea, phenylalanine, tryptophan, tyrosine, levodopa, and isoleucine; while 4 other metabolites were reduced, such as glyceric acid, uracil, erythronic acid, and isomaltose (Figure 24b).

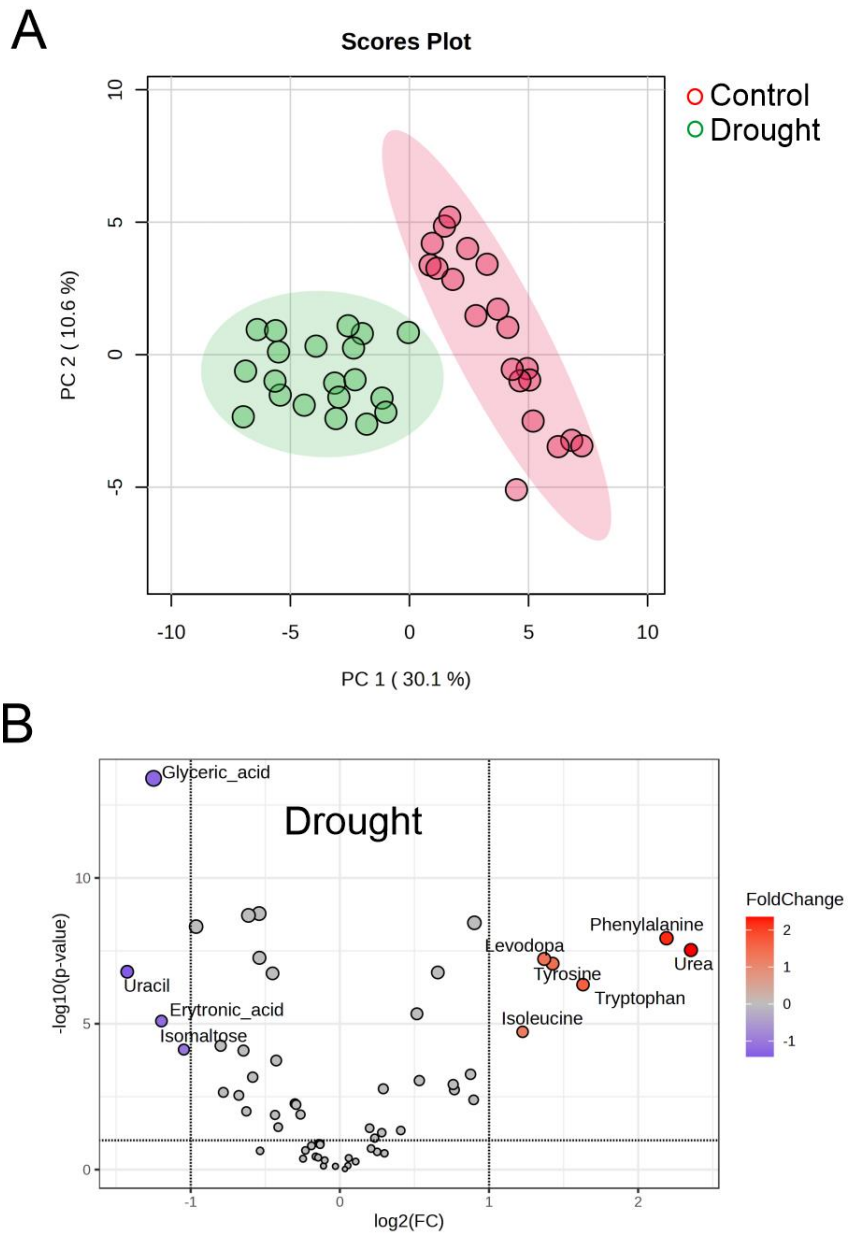



**Figure 24 -** Principal component analysis (PCA) of the metabolic profile in leaves of soybean plants (Extrema cultivar) in absence (control) and presence of drought, regardless of treatments with biostimulants (A). Volcano plot with the different metabolites responsive to water deficit, with red and blue showing positive and negative modulation, respectively (B).

Given the greater separation between the well-irrigated conditions, multivariate analysis was performed under water deficit conditions to identify the variation between the groups due to the application of biostimulants. Under stress conditions, PCA showed a 32.1% separation between the biostimulant treatments, indicating greater overlaps among the metabolic profiles analyzed (Figure 25a). However, it was observed that the Bio 01 treatment was more metabolically divergent, showing changes that separated it from the group treated without biostimulant (No Bio), as indicated by the cluster analysis (Figure 25b). This differential behavior was associated with a greater accumulation of specific metabolites, such as shikimic acid, arabinose, dehydroascorbic acid, erythronic acid, benzoic acid, threonine, GABA, and phenylalanine, when compared to the treatment without biostimulant.

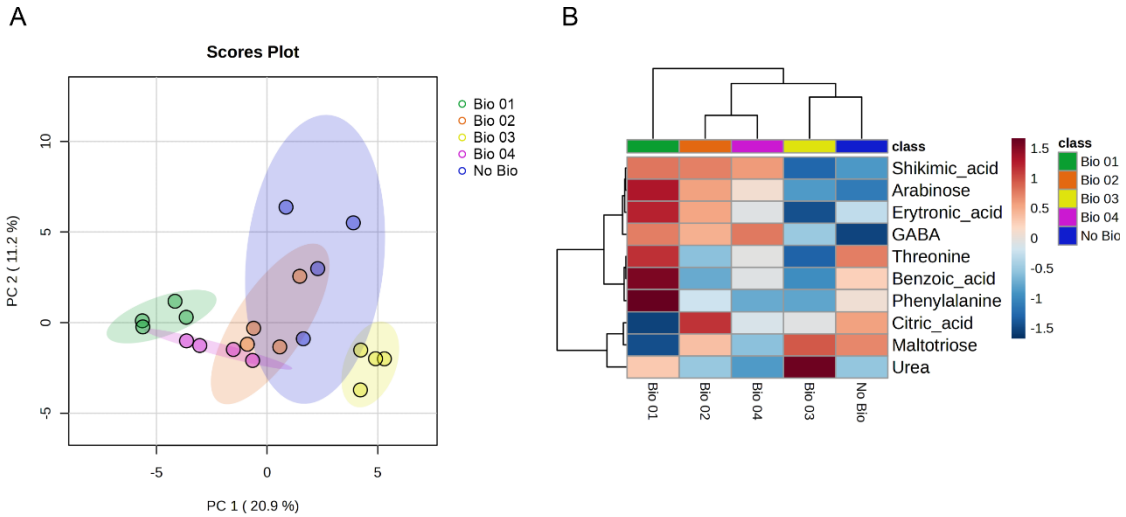



**Figure 25 -** Principal component analysis (PCA) of the metabolic profile with score plot (A), and hierarchical cluster analysis of metabolite variations with heat map and correlation network (B), showing relative abundance of different significant metabolites (Tukey test, p< 0.01) in leaves of soybean plants (Extrema cultivar) in presence of drought, and subjected to different treatments with biostimulants.

## 5.4 Discussion

### 5.4.1 Water deficit limits growth and biomass accumulation, and the tested biostimulants do not confer drought tolerance.

The data revealed that soybean plants under water deficit show reduced biometric parameters for all investigated treatments. Stem diameter, plant height, number of trifoliates, and root volume all showed significant reductions in plants grown under water deficit (Figure 13). A relevant fact is that treatments with biostimulants promoted slight increases in some variables studied under control conditions, but only Bio 01 and Bio 04 promoted improvements specifically in root length under water deficit (Figure 13a, b, c, d, e).

In previous studies, Rosa *et al*. (2021) reported lower biometric responses for soybean plants grown under water deficit and treated with *A. nodosum*-based biostimulant. Yang *et al*. (2021) suggested that under stressful conditions, plants tend to redirect growth energy toward activating defense mechanisms. Possibly, the high demand to cope with stress was limiting soybean performance, and the studied biostimulants were not able to mediate improvements in plant biometrics (Figure 13d). According to Verma *et al*. (2021), increased root growth is an important response to water deficit, aimed at enhancing water uptake, something that was intensified by biostimulants.

The same phenomenon was observed for biomass data, with similar responses among the studied treatments. Water restriction reduced leaf area index and dry mass indices, both for the root and shoot tissues (Figure 14a, b, c, d). Reduced growth commonly occurs due to decreased cell expansion resulting from low water absorption, leading to reduced leaf area and consequently dry mass production (Marin *et al*., 2020; Zia *et al*., 2021).

Our data showed that biostimulant treatments did not induce stress tolerance in soybean plants, at least during the studied period and with the formulations used. In fact, Bio 02 increased the sensitivity of soybean plants to water stress (Figure 14e). This result may indicate that this specific formulation does not provide additional

benefits for tolerance or that plants may have intrinsic stress tolerance mechanisms that were not significantly influenced by the biostimulant application. The biostimulant may contribute to reducing the synthesis of some compounds at the expense of producing others. This idea is supported by the study of Kocira *et al*. (2020), who reported that bean plants treated with algae-based biostimulant reduced the amount of stored starch, aiming to increase the antioxidant capacity of the seeds, as indicated by increased production of phenolic compounds, flavonoids, anthocyanins, and antioxidant activity.

### 5.4.2 The biostimulant formulations do not influence the water status and efficiency of the photosynthetic machinery of soybean plants under water deficit.

Soybean plants showed reductions in photosynthetic performance, which likely occurred due to stomatal and non-stomatal factors (Liao *et al*., 2022). Evidence of this was that, regardless of the biostimulants, there were severe reductions in stomatal conductance rates (Figure 17c), leading to lower transpiration rates (Figure 17b). As a consequence of the lower transpiration rate, the cooling system lost efficiency, and leaf temperature increased (Figures 15d, 16) (Marchin *et al*., 2021).

The findings also demonstrate that plants activated defense mechanisms, as there were reductions in the leaf osmotic potential of stressed plants (Figure 15b). This osmotic adjustment was extremely important to maintain water content in plant tissues under stress, as evidenced by unchanged values of transpiration rate (RWC) and leaf succulence compared to the control (Figure 15a, c). This adjustment was reflected in higher instantaneous and intrinsic water use efficiency under water limitation (Figure 15e, f).

Interestingly, the response related to osmotic adjustment likely occurred due to the accumulation of compatible solutes (Verma *et al*., 2021), as there was no accumulation of soluble carbohydrates and free amino acids in the leaves and roots of stressed plants (Figure 22a, b, c, d). Among the probable solutes, the compounds malic acid, glycine-betaine, and proline were determinants for the defense of maize, tomato, and peanut plants under water deficit (Hu; Chen, 2020; Ali *et al*., 2022; Jiménez-Arias *et al*., 2022; Elshamly *et al*., 2024).

As for non-stomatal factors, the negative effects of stress on the structural components of chloroplast electron transport chain (ETC) are noteworthy, particularly the decreases in chlorophyll and carotenoid levels (Figure 19). Such decreases may have been caused by the damaging action of reactive oxygen species, which were excessively accumulated in the tissues of stressed plants (Figure 21c) (Peng *et al*., 2023). This idea is supported by the increase in lipid peroxidation of plants growing under stress, indicative of membrane damage (Figure 21a). However, the data suggest that soybean plants showed some resilience to stress, as the reductions in $CO_2$ assimilation rates were not as pronounced (Figure 17a), there was maintenance in the Rubisco carboxylation efficiency (Figure 17d), and the variables of Photosystem II efficiency remained practically unchanged (Figure 18). This characteristic likely stems from intrinsic responses of the cultivar, which has been genetically improved for cultivation in semiarid regions (Miranda *et al*., 2023). Similar results were found by Jumrani *et al*. (2023) working with soybeans and assessing the combined effect of water and heat stress.

An interesting finding was that biostimulants did not influence modulating the physiological performance of soybean plants under stress, which, according to Melo *et al*. (2024), may be related to dose split at stages. In their study, physiological benefits were notable in soybean plants that received the full dose of the biostimulant, while inferior to plants without any biostimulant treatment or those that had the dose divided. Stressed plants treated with the studied biostimulants showed physiological responses virtually identical to those stressed without the application of algae-based formulations. Our results differ from other findings reported in the literature, where seaweed-based biostimulants increased chlorophyll levels in plants growing under normal or stressful conditions (Goñi *et al*., 2018; Carmody *et al*., 2020; Rosa *et al*., 2021; Souza *et al*., 2023).

### 5.4.3 Biostimulants promote relevant responses in the plant metabolome and activation of the antioxidant system under drought.

The data showed that soybean plants activated antioxidant defense pathways when exposed to water deficit. This activation differed depending on the applied

treatments, as the main activated enzymes were G-POD and SOD in non-treated plants with biostimulants (Figure 20c, d). The results also suggest that such enzymes were not sufficient to mitigate stress damage, as high levels of $H_2O_2$ and MDA were recorded (Figure 21), which were associated with lower growth rates under stress (Figures 11, 12) (Hasanuzzaman *et al*., 2021).

Soybean metabolism was altered when Bio 03 was applied, once drought-stressed plants primarily activated the CAT and G-POD enzymes (Figure 20b, c), which were possibly more effective in eliminating reactive oxygen species. This argument is supported by the less intense increase in $H_2O_2$ levels compared to those reported for stressed plants without biostimulants (Figure 21c, d).

The leaf metabolome data reported differential modulation due to biostimulant treatments, demonstrating that the formulations were able to alter the flow of molecules in plant tissues (Figures 23, 24, 25). Specifically, biostimulants Bio 01, Bio 02, and Bio 04 triggered the accumulation of shikimic acid and GABA (gamma-aminobutyric acid) in plant tissues under stress, an important component of secondary metabolism and stress response (Figure 25b) (Merewitz *et al*., 2011; Bown; Shelp, 2016; Li *et al*., 2021).

In plants treated with Bio 01, accumulation of phenylalanine and benzoic acid was also observed, which together with shikimate are precursors of a wide range of secondary metabolites, with an important stress defense response (Widhalm; Dudareva, 2015; Itam *et al*., 2020). Metabolites such as galactonic acid, arabinose, erythronic acid, threonic acid, mannitol, and isomaltose act in relevant plant defense pathways, as demonstrated in several crops (Jorge; António, 2018; Zhang *et al*., 2018; Galli *et al*., 2019; Chang *et al*., 2020; Zulfiqar *et al*., 2020; Liu *et al*., 2021, 2022; Xu *et al*., 2021; Lima *et al*., 2022; Singh; Roychoudhury, 2022; Alhudhaibi *et al*., 2024; Harsha *et al*., 2024), improving plant resistance and productivity (Desoky *et al*., 2021).

Based on the above, the data demonstrate that biostimulants can enhance defense responses of soybean plants to water deficit. Modulations in antioxidant pathways and metabolic profile are evidence that seaweed-based formulations are capable of triggering relevant defense mechanisms. The lack of benefits in physiological performance and plant growth was most likely due to the short timeframe for biostimulant action in the plants, as the final dose was applied 12 days (R2 application) after stress and plant harvest occurred 13 days later. Therefore, future studies should be conducted to better explore soybean plant responses to stress with

the use of biostimulants, especially focusing on long-term responses and investigating productive parameters in association with metabolic defense mechanisms.

## 5.5 Conclusion

The data reinforce that water deficit severely limits the growth and metabolism of soybean plants. Growth restrictions were associated with i) decreases in the efficiency of photosynthetic machinery, marked by reductions in photosynthetic pigments, stomatal conductance, and transpiration, and increases in non-photochemical energy dissipation; ii) increases in oxidative damage (MDA) due to massive accumulation of ROS; and iii) ineffective action of the antioxidant defense system. The data also revealed that plants activated pathways for osmotic adjustment to maintain water status.

Biostimulants promote relevant changes in the biochemical and molecular defense pathways of soybean under drought, with the reprogramming of antioxidant enzymes activation (such as CAT and G-POD) and defense metabolites (gamma-aminobutyric acid, benzoic acid, shikimic acid, phenylalanine, among others). However, none of the studied biostimulant formulations were efficient in alleviating the effects of stress on physiological defense pathways, with no influence on soybean tolerance to water deficit. Future studies should focus on biostimulant application strategies in stressed plants and long-term responses, promoting the investigation of productive parameters associated with defense mechanisms.

## REFERENCES


ALEXIEVIA, V.; SERGIO I.; MAPELLI, S.; KARANOV, E. The effect of drought and ultraviolet radiation on growth and stress markars in pea and wheat. **Plant, Cell and Environment**, n. 24, p. 1337-1344, 2001.

ALHUDHAIBI, A. M.; IBRAHIM, M. A.; ABD-ELAZIZ, S. M.; FARAG, H. R.; ELSAYED, S. M.; IBRAHIM, H. A.; SROUR, H. A. Enhancing salt stress tolerance in wheat (*Triticum aestivum*) seedlings: insights from trehalose and mannitol. **BMC Plant Biology**, v. 24, n. 1, p. 472, 2024.

ALI, B., WANG, X.; SALEEM, M. H.; SUMAIRA.; HAFEEZ, A.; AFRIDI, M.S.; ALI, S.; KHAN, S.; NISA, Z. U.; ULLAH, I.; AMARAL JÚNIOR, A. T.; ALATAWI, A.; ALI, S. PGPR-mediated salt tolerance in maize by modulating plant physiology, antioxidant defense, compatible solutes accumulation and bio-surfactant producing genes. **Plants**, v. 11, p. 345, 2022.

ALORI, E. T.; EMMANUEL, O. C.; GLICK, B. R.; BABALOLA, O. O. Plant–archaea relationships: a potential means to improve crop production in arid and semi-arid regions. **World Journal of Microbiology and Biotechnology**, v. 36, p. 133, 2020.

AMATUSSI, J. O.; MÓGOR, A. F.; MÓGOR, G.; LARA, G. B. Novel use of calcareous algae as a plant biostimulant. **Journal of Applied Phycology**, v. 32, n. 3, p. 2023-2030, 2020.

AMATUSSI, J. O.; MÓGOR, Á. F.; CORDEIRO, E. C. N.; MÓGOR, G.; MARQUES, H. M. C.; LARA, G. B. Synergic combination of calcareous algae and cyanobacteria stimulate metabolic alterations improving plant growth and yield. **Journal of Applied Phycology**, v. 35, n. 1, p. 483-493, 2022.

ANDERSON, M. D.; PRASAD, T. K.; STEWART, C. R. Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. **Plant Physiology**, v. 109, p. 1247-1257, 1995.

ASIF, Z.; CHEN, Z.; SADIQ, R.; ZHU, Y. Climate change impacts on water resources and sustainable water management strategies in north america. **Water Resources Management**, v. 37, n. 7, p. 2771-2786, 2023.

BANTIS, F.; KOUKOUNARAS, A. *Ascophyllum nodosum* and silicon-based biostimulants differentially affect the physiology and growth of watermelon transplants under abiotic stress factors: the case of drought. **Horticulturae**, v. 8, n. 12, p. 1177, 2022.

BASSO, S. M. S. **Caracterização morfológica e fixação biológica de nitrogênio de espécies de *Adesmia* DC. e *Lotus* L**. 1999. 268 f. Tese (Doutorado em Zootecnia) – Universidade Federal do Rio Grande do Sul, Porto Alegre, 1999.

BEYER, W. F.; FRIDOVICH, I. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. **Analytical Biochemistry**, v. 261, p. 559-566, 1987.

BOWN, A. W.; SHELP, B. J. Plant GABA: not just a metabolite. **Trends in Plant Science**, v. 21, n. 10, p. 811-813, 2016.

BRADFORD, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye binding. **Analytical Biochemistry**, v. 72, p. 248-254, 1976.

CARMODY, N.; GOÑI, O.; ŁANGOWSKI, Ł.; O'CONNELL, S. *Ascophyllum nodosum* extract biostimulant processing and its impact on enhancing heat stress tolerance during tomato fruit set. **Frontiers in Plant Science**, v. 11, p. 807, 2020.
ČATSKÝ, J. Determination of water deficit in disks cut out from leaf blades. **Biologia Plantarum**, v. 2, n. 1, p. 76-78, 1960.

CHANG, B.; MA, K.; LU, Z.; LU, J.; CUI, J.; WANG, L.; JIN, B. Physiological, transcriptomic, and metabolic responses of *Ginkgo biloba* L. to drought, salt, and heat stresses. **Biomolecules**, v. 10, n. 12, p. 1635, 2020.

COCETTA, G.; LANDONI, M.; PILU, R.; REPISO, C.; NOLASCO, J.; ALAJARIN, M.; FERRANTE, A. Priming treatments with biostimulants to cope the short-term heat stress response: A transcriptomic profile evaluation. **Plants**, v. 11, p. 1130, 2022.

DESOKY, E. S. M.; ELRYS, A. S.; MANSOUR, E.; EID, R. S. M.; SELEM, E.; RADY, M. M.; ALI, E. F.; MERSAL, G. A. M.; SEMIDA, W. M. Application of biostimulants promotes growth and productivity by fortifying the antioxidant machinery and suppressing oxidative stress in faba bean under various abiotic stresses. **Scientia Horticulturae**, v. 288, p. 110340, 2021.

DUBOIS, M.; GILLES, K. A.; HAMILTON, J. K.; REBERS, P. A.; SMITH, F. Colorimetric method for determination of sugars and related substances. **Analytical Chemistry**, v. 28, p. 350-356, 1956.

DUMANOVIĆ, J.; NEPOVIMOVA, E.; NATIĆ, M.; KUČA, K.; JAĆEVIĆ, V. The significance of reactive oxygen species and antioxidant defense system inplants: A concise overview. **Frontiers in Plant Science**, v. 11, p. 552969, 2021.

ELSHAMLY, A. M.; PARREY, Z. A.; GAAFAR, A. R. Z.; SIDDIQUI, M. H.; HUSSAIN, S. Potassium humate and cobalt enhance peanut tolerance to water stress through regulation of proline, antioxidants, and maintenance of nutrient homeostasis. **Scientific Reports**, v. 14, n. 1, p. 1625, 2024.

ENGEL, D. C. H.; FELTRIM, D.; RODRIGUES, M.; BAPTISTELLA, J. L. C.; MAZZAFERA, P. Algae extract increases seed production of soybean plants and alters nitrogen metabolism. **Agriculture**, v. 13, n. 7, p. 1296, 2023.

FAROOQ, M.; GOGOL, N.; BARTHAKUR, S.; BAROOWA, B.; BHARADWAJ, N.; ALGHAMDI, S. S.; SIDDIQUE, K. H. M. Drought stress in grain legumes during reproduction and grain filling. **Journal of Agronomy and Crop Science**, v. 203, n. 2, p. 81-102, 2016.

GALLI, V.; MESSIAS, R. S.; GUZMAN, F.; PERIN, E. C.; MARGIS, R.; ROMBALDI, C. V. Transcriptome analysis of strawberry (Fragaria × ananassa) fruits under osmotic stresses and identification of genes related to ascorbic acid pathway. **Physiologia Plantarum**, v. 166, n. 4, p. 979-995, 2019.

GENTY, B.; BRIANTAIS, J. M.; BAKER, N. R. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. **Biochimica et Biophysica Acta**, v. 990, p. 87-92, 1989.

GOÑI, O.; QUILLE, P.; O'CONNELL, S. *Ascophyllum nodosum* extract biostimulants and their role in enhancing tolerance to drought stress in tomato plants. **Plant Physiology and Biochemistry**, v. 126, p. 63-73, 2018.

GUL, M. U.; PAUL, A.; S, M.; CHEHRI, A. Hydrotropism: understanding the impact of water on plant movement and adaptation. **Water**, v. 15, n. 3, p. 567, 2023.

HARSHA, S. G.; GIRISH, B.; SHEELA, H. S.; DINSHA, M.; KANNAN, S.; LAXMAN, R. H.; PRATHIBHA, M. D. Sweet success: unraveling the role of root and shoot sugar dynamics in brinjal's short-term salinity stress resilience. **Journal of Plant Growth Regulation**, v. 43, n. 3, p. 871-888, 2024.

HASANUZZAMAN, M.; PARVIN, K.; BARDHAN, K.; NAHAR, K.; ANEE, T. I.; MASUD, A. A. C.; FOTOPOULOS, V. Biostimulants for the regulation of reactive oxygen species metabolism in plants under abiotic stress. **Cells**, v. 10, n. 10, p. 2537, 2021.

HAVIR, E. A.; MCHALE, N. A. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. **Plant Physiology**, v. 84, p. 450-455, 1987.

HE, J.; DU, Y.; WANG, T.; TURNER, N. C.; YANG, R. P.; JIN, Y.; XI, Y.; ZHANG, C.; CUI, T.; FANG, X. W. Conserved water use improves the yield performance of soybean (*Glycine max* (L.) Merr.) under drought. **Agricultural Water Management**, v. 179, p. 236-245, 2017.

HEATH, R. L.; PACKER, L. Photoperoxidation in isolated chloroplasts.Kinetics and stoichiometry of fatty acid peroxidation. **Archives of Biochemistry and Biophysics**, v. 125, p. 189-198, 1968.

HU, Y.; CHEN, B. Arbuscular mycorrhiza induced putrescine degradation into γ-aminobutyric acid, malic acid accumulation, and improvement of nitrogen assimilation in roots of water-stressed maize plants. **Mycorrhiza**, v. 30, n. 2-3, p. 329-339, 2020.

ITAM, M.; MEGA, R.; TADANO, S.; ABDELRAHMAN, M.; MATSUNAGA, S.; YAMASAKI, Y.; TSUJIMOTO, H. Metabolic and physiological responses to progressive drought stress in bread wheat. **Scientific Reports**, v. 10, n. 1, p. 17189, 2020.

JIMÉNEZ-ARIAS, D.; MORALES-SIERRA, S.; BORGES, A. A.; HERRERA, A. J.; LUIS, J. C. New biostimulants screening method for crop seedlings under water deficit stress. **Agronomy**, v. 12, n. 3, p. 728, 2022.

JOMOVA, K.; ALOMAR, S. Y.; ALWASEL, S. H.; NEPOVIMOVA, E.; KUCA, K.; VALKO, M. Several lines of antioxidant defense against oxidative stress: antioxidant enzymes, nanomaterials with multiple enzyme-mimicking activities, and low-molecular-weight antioxidants. **Archives of Toxicology**, v. 98, p. 1323-1367, 2024.

JUMRANI, K.; BHATIA, V. S.; KATARIA, S.; RASTOGI, A. The interactive effect of high temperature and water deficit stress on nitrogen fixation, photosynthesis, chlorophyll fluorescence, seed yield and quality in soybean (*Glycine max*). **Plant Physiology Reports**, v. 29, n. 1, p. 125-140, 2023.

KAR, M.; MISHRA, D. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. **Plant Physiology**, v. 57, p. 315-319, 1976.

KRAMER, D. M.; AVENSON, T. J.; EDWARDS, G. E. Dynamic flexibility in the light reactions of photosynthesis governed by both electron and proton transfer reactions. **Trends in Plant Science**, v. 9, p. 349-357, 2004.

KOCIRA, S.; SZPARAGA, A.; HARA, P.; TREDER, K.; FINDURA, P.; BARTOŠ, P.; FILIP, M. Biochemical and economical effect of application biostimulants containing

seaweed extracts and amino acids as an element of agroecological management of bean cultivation. **Relatórios Científicos**, v. 10, n. 1, p. 17759, 2020.

LI, L.; DOU, N.; ZHANG, H.; WU, C. The versatile GABA in plants. **Plant Signaling & Behavior**, v. 16, n. 3, p. 1862565, 2021.

LIAO, Q.; DING, R.; DU, T.; KANG, S.; TONG, L.; LI, S. Stomatal conductance drives variations of yield and water use of maize under water and nitrogen stress. **Agricultural Water Management**, v. 268, p. 107651, 2022.

LIMA, K. R. P; CAVALCANTE, F. L. P; PAULA-MARINHO, S. O.; PEREIRA, I. M. C.; LOPES, L. S.; NUNES, J. V. S.; COUTINHO, I. A. C.; GOMES-FILHO, E.; CARVALHO, H. H. Metabolomic profiles exhibit the influence of endoplasmic reticulum stress on sorghum seedling growth over time. **Plant Physiology and Biochemistry**, v. 170, p. 192-205, 2022.

LIU, L.; WU, X.; SUN, W.; YU, X.; DEMURA, T.; LI, D.; ZHUGE, Q. Galactinol synthase confers salt-stress tolerance by regulating the synthesis of galactinol and raffinose family oligosaccharides in poplar. **Industrial Crops and Products**, v. 165, p. 113432, 2021.

LIU, X.; WEI, R.; TIAN, M.; LIU, J.; RUAN, Y.; SUN, C.; LIU, C. Combined transcriptome and metabolome profiling provide insights into cold responses in Rapeseed (*Brassica napus* L.) genotypes with contrasting cold-stress sensitivity. **International Journal of Molecular Sciences**, v. 23, n. 21, p. 13546, 2022.

MANDAL, S.; ANAND, U.; LÓPEZ-BUCIO, J.; RADHA; KUMAR, M.; LAL, M. K.; TIWARI, R. K.; DEY, A. Biostimulants and environmental stress mitigation in crops: a novel and emerging approach for agricultural sustainability under climate change. **Environmental Research**, v. 233, p. 116357, 2023.

MANSOOR, S.; WANI, O. A.; LONE, J. K.; MANHAS, S.; KOUR, N.; ALAM, P.; AHMAD, A.; AHMAD, P. Reactive oxygen species in plants: From source to sink. **Antioxidants**, v. 11, n. 2, p. 225, 2022.

MANTOVANI, A. A method to improve leaf succulence quantification. **Brazilian Archives of Biology and Technology**, v. 42, 2011.

MARCHIN, R. M.; BACKES, D.; OSSOLA, A.; LEISHMAN, M. R.; TJOELKER, M. G.; ELLSWORTH, D. S. Extreme heat increases stomatal conductance and drought-induced mortality risk in vulnerable plant species. **Global Change Biology**, v. 28, n. 3, p. 1133-1146, 2021.

MARIN, M.; FEENEY, D. S.; BROWN, L. K.; NAVEED, M.; RUIZ, S.; KOEBERNICK, N.; BENGOUGH, A. G.; HALLETT, P. D.; ROOSE, T.; PUÉRTOLAS, J. Significance of root hairs for plant performance under contrasting field conditions and water deficit. **Annals of Botany**, v. 128, n. 1, p. 1-16, 2020.

MELO, G. B.; SILVA, A. G.; COSTA, A. C.; SILVA, A. A.; ROSA, M.; BESSA, L. A.; RODRIGUES, C. R.; CASTOLDI, G.; VITORINO, L. C. Foliar application of biostimulant mitigates water stress effects on soybean. **Agronomy**, v. 14, n. 3, p. 414, 2024.

MEREWITZ, E. B.; DU, H.; YU, W.; LIU, Y.; GIANFAGNA, T.; HUANG, B. Elevated cytokinin content in ipt transgenic creeping bentgrass promotes drought tolerance through regulating metabolite accumulation. **Journal of Experimental Botany**, v. 63, n. 3, p. 1315-1328, 2011.

MERTZ-HENNING, L.; FERREIRA, L.; HENNING, F.; MANDARINO, J.; SANTOS, E.; OLIVEIRA, M.; NEPOMUCENO, A.; FARIAS, J.; NEUMAIER, N. Effect of water deficit-induced at vegetative and reproductive stages on protein and oil content in soybean grains. **Agronomy**, v. 8, n. 1, p. 3, 2017.

MIRANDA, R. S.; FONSECA, B. S. F.; PINHO, D. S.; BATISTA, J. Y. N.; BRITO, R. R.; SILVA, E. M.; FERREIRA, W. S.; COSTA, J. H.; LOPES, M. S.; SOUSA, R. H. B.; NEVES, L. F.; PENHA, J. A. F.; SANTOS, A. S.; LIMA, J. J. P.; PAULA-MARINHO, S. O.; ALCANTARA NETO, F.; AGUIAR, E. S.; SANTOS, C. P.; GOMES FILHO, E. Selection of soybean and cowpea cultivars with superior performance under drought using growth and biochemical aspects. **Plants**, v. 12, p. 3134, 2023.

MIRANDA, R. S.; SOUZA, F. I. L.; ALVES, A. F.; SOUZA, R. R.; MESQUITA, R. O.; RIBEIRO, M. I. D.; GOMES-FILHO, E. Salt-acclimation physiological mechanisms at the vegetative stage of cowpea genotypes in soils from a semiarid region. **Journal of Soil Science and Plant Nutrition**, v. 21, n. 4, p. 3530-3543, 2021.

MÓGOR, A. F.; AMATUSSI, J. O.; MÓGOR, G.; GEMIN, L. G. Biostimulant action of *Lithothamnium* sp. promoting growth, yield, and biochemical and chemical changes on onion. **Journal of Applied Phycology**, v. 33, n. 3, p. 1905-1913, 2021.

NAKANO, Y.; ASADA, K. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. **Plant and Cell Physiology**, v. 22, p. 867-880, 1981.

NAWAZ, F.; SHEHZAD, M. A.; MAJEED, S.; AHMAD, K. S.; AQIB, M.; USMANI, M. M.; SHABBIR, R. N. Role of mineral nutrition in improving drought and salinity tolerance in field crops. *In:* HASANUZZAMAN, M. (ed.). **Agronomic Crops**, 3. ed. Singapure: Springer. 2020. p. 129-147.

OMIDBAKHSHFARD, M. A.; SUJEETH, N.; GUPTA, S.; OMRANIAN, N.; GUINAN, K. J.; BROTMAN, Y.; NIKOLOSKI, Z.; FERNIE, A. R.; MUELLER-ROEBER, B.; GECHEV, T. S. A biostimulant obtained from the seaweed *Ascophyllum nodosum* protects *Arabidopsis thaliana* from severe oxidative stress. **International Journal of Molecular Sciences**, v. 21, n. 2, p. 474, 2020.

JORGE, T. F.; ANTÓNIO, C. Plant metabolomics in a changing world: Metabolite responses to abiotic stress combinations. *In:* ANDJELKOVIC, V. (ed.). **Plant, abiotic stress and responses to climate change**. Serbia: InTech, 2018. 186 p.

OXBOROUGH, K.; BAKER, N. R. Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components–calculation of qP and Fv/Fm without measuring Fo. **Photosynthesis Research**, v. 54, n. 2, p. 135-142, 1997.

OZTURK, M.; UNAL, B. T.; GARCÍA-CAPARRÓS, P.; KHURSHEED, A.; GUL, A.; HASANUZZAMAN, M. Osmoregulation and its actions during the drought stress in plants. **Physiologia Plantarum**, v. 172, n. 2, p. 1321-1335, 2020.

PENG, M.; CHEN, Z.; ZHANG, L.; WANG, Y.; ZHU, S.; WANG, G. Preharvest application of sodium nitroprusside alleviates yellowing of chinese flowering cabbage via modulating chlorophyll metabolism and suppressing ROS accumulation. **Journal of Agricultural and Food Chemistry**, v. 71, n. 24, p. 9280-9290, 2023.

RAI, G. K.; KHANDAY, D. M.; KUMAR, P.; MAGOTRA, I.; CHOUDHARY, S. M.; KOSSER, R.; KALUNKE, R.; GIORDANO, M.; CORRADO, G.; ROUPHAEL, Y. Enhancing crop resilience to drought stress through CRISPR-Cas9 genome editing. **Plants**, v. 12, n. 12, p. 2306, 2023.

REPKE, R. A.; SILVA, D. M. R.; SANTOS, J. C. C.; SILVA, M. A. Alleviation of drought stress in soybean by applying a biostimulant based on amino acids and macro- and micronutrients. **Agronomy**, v. 12, n. 10, p. 2244, 2022.

RINNE, K. T.; SAURER, M.; STREIT, K.; SIEGWOLF, R. T. W. Evaluation of a liquid chromatography method for compound-specific δ13C analysis of plant carbohydrates in alkaline media. **Rapid Communications in Mass Spectrometry**, v. 26, p. 2173-2185, 2012.

ROESSNER, U.; LUEDEMANN, A.; BRUST, D.; FIEHN, O.; LINKE, T.; WILLMITZER, L.; FERNIE, A. R. Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. **The Plant Cell**, v. 13, p. 11-29, 2001.

ROSA, V. R.; SANTOS, A. L. F.; SILVA, A. A.; SAB, M. P. V.; GERMINO, G. H.; CARDOSO, F. B.; SILVA, M. A. Increased soybean tolerance to water deficiency through biostimulant based on fulvic acids and *Ascophyllum nodosum* (L.) seaweed extract. **Plant Physiology and Biochemistry**, v. 158, p. 228-243, 2021.

SACHDEV, S.; ANSARI, S. A.; ANSARI, M. I.; FUJITA, M.; HASANUZZAMAN, M. Abiotic stress and reactive oxygen species: generation, signaling, and defense mechanisms. **Antioxidants**, v. 10, n. 2, p. 277, 2021.

SELEIMAN, M. F.; AL-SUHAIBANI, N.; ALI, N.; AKMAL, M.; ALOTAIBI, M.; REFAY, Y.; DINDAROGLU, T.; ABDUL-WAJID, H. H.; BATTAGLIA, M. L. Drought stress impacts on plants and different approaches to alleviate its adverse effects. **Plants**, v. 10, n. 2, p. 259, 2021.

SHAHZAD, R.; BARKAT, M.; FAISAL, S.; AYUB, A.; NOUREEN, S.; ABBAS, G.; NIAZI, N. K.; LEE, I. The seaweed *Ascophyllum nodosum*-based biostimulant enhances salt stress tolerance in rice (*Oryza sativa* L.) by remodeling physiological, biochemical, and metabolic responses. **Journal of Plant Interactions**, v. 18, n. 1, p. 2266514, 2023.

SHUKLA, P. S.; PRITHIVIRAJ, B. *Ascophyllum nodosum* biostimulant improves the growth of *Zea mays* grown under phosphorus impoverished conditions. **Frontiers in Plant Science**, v. 11, p. 601843, 2021.

SILVA, A. C.; BRITO, F. M.; SANTOS, A. S.; BRITO, R. R.; LACERDA, J. J. J.; ALCANTARA NETO, F.; MIRANDA, R. S. Cowpea (*Vigna unguiculata* [L.] Walp.) plants display contrasting sulfur-mediated drought acclimation in greenhouse and field. **Archives of Agronomy and Soil Science**, v. 69, n. 15, p. 3550-3569, 2023.

SINGH, A.; ROYCHOUDHURY, A. Omics tools to understand abiotic stress response and adaptation in rye, oat and barley. *In:* ROYCHOUDHURY, A.; AFTAB, T.; ACHARYA, K. **Omics approach to manage abiotic stress in cereals.** Singapore: Springer, 2022. p. 513-529.

SOUSA, D. M. G.; LOBATO, E. (ed.). **Cerrado: correção do solo e adubação.** 2. ed. Planaltina: Embrapa Informação Tecnológica, 2004. 416 p.

SOUZA, S. J.; COSTA, R. S.; RODRIGUES, C. Y. A. C.; SILVA, B. A.; SOUSA, T. R. M.; CARDOSO, F. B.; MESQUISTA, R. O. The application of a biostimulant based on *Ascophyllum nodosum*, fulvic acid and nutrients mitigates water deficit in soybean. **Australian Journal of Crop Science**, v. 17, n. 7, p. 539-548, 2023.

STANIAK, M.; SZPUNAR-KROK, E.; KOCIRA, A. Responses of soybean to selected abiotic stresses photoperiod, temperature and water. **Agriculture**, v. 13, p. 146, 2023.

VEIHMEYER, F. J.; HENDRICKSON, A. H. The moisture equivalent as a measure of the field capacity of soils. **Soil Science**, V.32, p.181-194, 1931.

VERMA, N.; SAO, P.; SRIVASTAVA, A.; SINGH, S. Physiological and molecular responses to drought, submergence and excessive watering in plants. **Harsh Environment and Plant Resilience**, p. 305-321, 2021.

VILA, V. V. E.; MARQUES, P. A. A.; REZENDE, R.; WENNECK, G. S.; TERASSI, D. S.; ANDREAN, A. F. B. A.; NOCCHI, R. C. F.; MATUMOTO-PINTRO, P. T. Deficit irrigation with *Ascophyllum nodosum* extract application as a strategy to increase tomato Yield and quality. **Agronomy**, v. 13, n. 7, p. 1853, 2023.

WELLBURN, A. R. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. **Journal of Plant Physiology**, v. 144, p. 307-313, 1994.

WIDHALM, J. R.; DUDAREVA, N. A Familiar Ring to It: biosynthesis of plant benzoic acids. **Molecular Plant**, v. 8, n. 1, p. 83-97, 2015.

XU, Z.; CHEN, X.; LU, X.; ZHAO, B.; YANG, Y.; LIU, J. Integrative analysis of transcriptome and metabolome reveal mechanism of tolerance to salt stress in oat (*Avena sativa* L.). **Plant Physiology and Biochemistry**, v. 160, p. 315-328, 2021.

YADAV, S.; MODI, P.; DAVE, A.; VIJAPURA, A.; PATEL, D.; PATEL, M. Effect of abiotic stress on crops. **Sustainable Crop Production**, v. 3, n. 17, p. 5-16. 2020.

YANG, X.; LU, M.; WANG, Y.; LIU, Z.; CHEN, S. Response mechanism of plants to drought stress. **Horticulturae**, v. 7, n. 3, p. 50, 2021.

YASIR, T. A.; AHMAD, M.; WASAYA, A.; ATEEQ, M.; KANWAL, S.; WAHID, A.; AZIZ, M. Hydrogen sulfide ($H_2S$) signaling in plants responding to abiotic stresses. **Gasotransmitters Signaling in Plants under Challenging Environment**, p. 241-262, 2023.

YEMM, E. W.; COCKING, E. C. The determination of amino acids with ninhydrin. **Analyst**, v. 80, n. 1, p. 209-213, 1955.

ZANDALINAS, S. I.; BALFAGÓN, D.; GÓMEZ-CADENAS, A.; MITTLER, R. Plant responses to climate change: metabolic changes under combined abiotic stresses. **Journal of Experimental Botany**, v. 73, n. 11, p. 3339-3354, 2022.

ZHANG, Q.; SONG, X. E.; BARTELS, D. Sugar metabolism in the desiccation tolerant grass *Oropetium thomaeum* in response to environmental stresses. **Plant science**, v. 270, p. 30-36, 2018.

ZIA, R.; NAWAZ, M. S.; SIDDIQUE, M. J.; HAKIM, S.; IMRAN, A. Plant survival under drought stress: implications, adaptive responses, and integrated rhizosphere management strategy for stress mitigation. **Microbiological Research**, v. 242, p. 126626, 2021.

ZULFIQAR, F.; AKRAM, N. A.; ASHRAF, M. Osmoprotection in plants under abiotic stresses: New insights into a classical phenomenon. **Plant**, v. 251, n. 1, p. 3, 2020.

# 6 FINAL CONSIDERATIONS

Our data clearly evidence that, under well-irrigation conditions, the *A. nodosum*-based biostimulant at 1.5 L $ha^{-1}$ divided in V4 and R1 stages improved the photosynthetic efficiency. The elevated photosynthetic performance is attributed to control of water status, reinforcing that the biostimulants-treated plants maintain better hydration for physiological functions. The increased physiological conditioning resulted in a 12% increase in yield, a significant outcome, especially considering the soybean productivity in semiarid cultivation.

The findings of Chapter II demonstrate that the optimized application of biostimulants can translate into tangible economic benefits for farmers and that the dosage and timing of biostimulant application are crucial for obtaining positive physiological and productive responses in soybean plants.

The results also evidence the positive response for setup of biostimulants-treated well-irrigated plants is not observed under water deficit conditions. The findings of Chapter III demonstrate that the water limitation caused drastic implications on physiological performance of soybean plants, including the reduction of photosynthetic pigment levels, stomatal conductance, and transpiration. This impaired process was associated with increased non-photochemical quenching, which contributed to severe restrictions in plant growth. These negative effects were exacerbated by damage to cell membranes and increased levels of hydrogen peroxide, an indicator of oxidative stress.

The soybean defense responses appeared to rely more on intrinsic adjustments (genetic potential) than on biostimulant-mediated defense mechanisms. Under drought, plants activate pathways for osmotic adjustment and antioxidant defense, such as maintaining water status and increasing the activity of antioxidant enzymes. These responses seem not to be enough to effectively neutralize oxidative damage, once the plants still suffered significant consequences from the water deficit. Also, although the biostimulants promoted relevant responses like the accumulation of metabolites involved in defense pathways and activating the antioxidant system, they were not able to counteract drought damage in physiological and growth performance, at least during the studied experimental period (Chapter III).

Finally, our data evidence that, at least in the short-time experiment, biostimulants do not improve the performance of typical semiarid soybean cultivars under water deficit. To maximize the potential of these compounds, future research should focus on strategies for applying biostimulants under stressful conditions, considering long-term responses and investigating productive parameters related to defense mechanisms. It would be beneficial to investigate different combinations of doses with both full and split applications under water deficit conditions to select setups that may improve plant resilience to water stress.

# APPENDICES

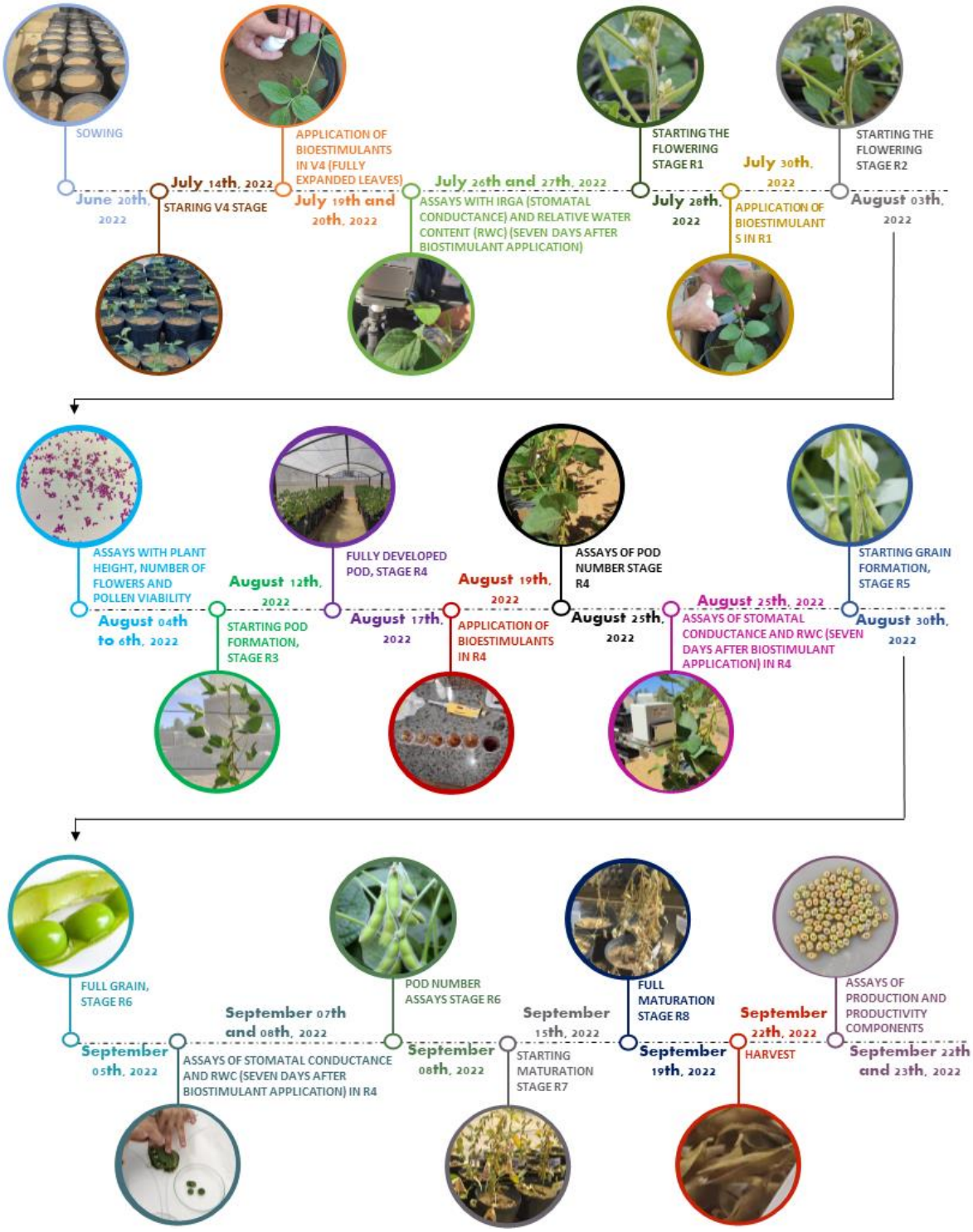
**APPENDIX A -** Chemical and physical analysis of soil before the experiments for growing soybean plants in greenhouse. A, B and C correspond to three individual soil samples.

Macronutrients

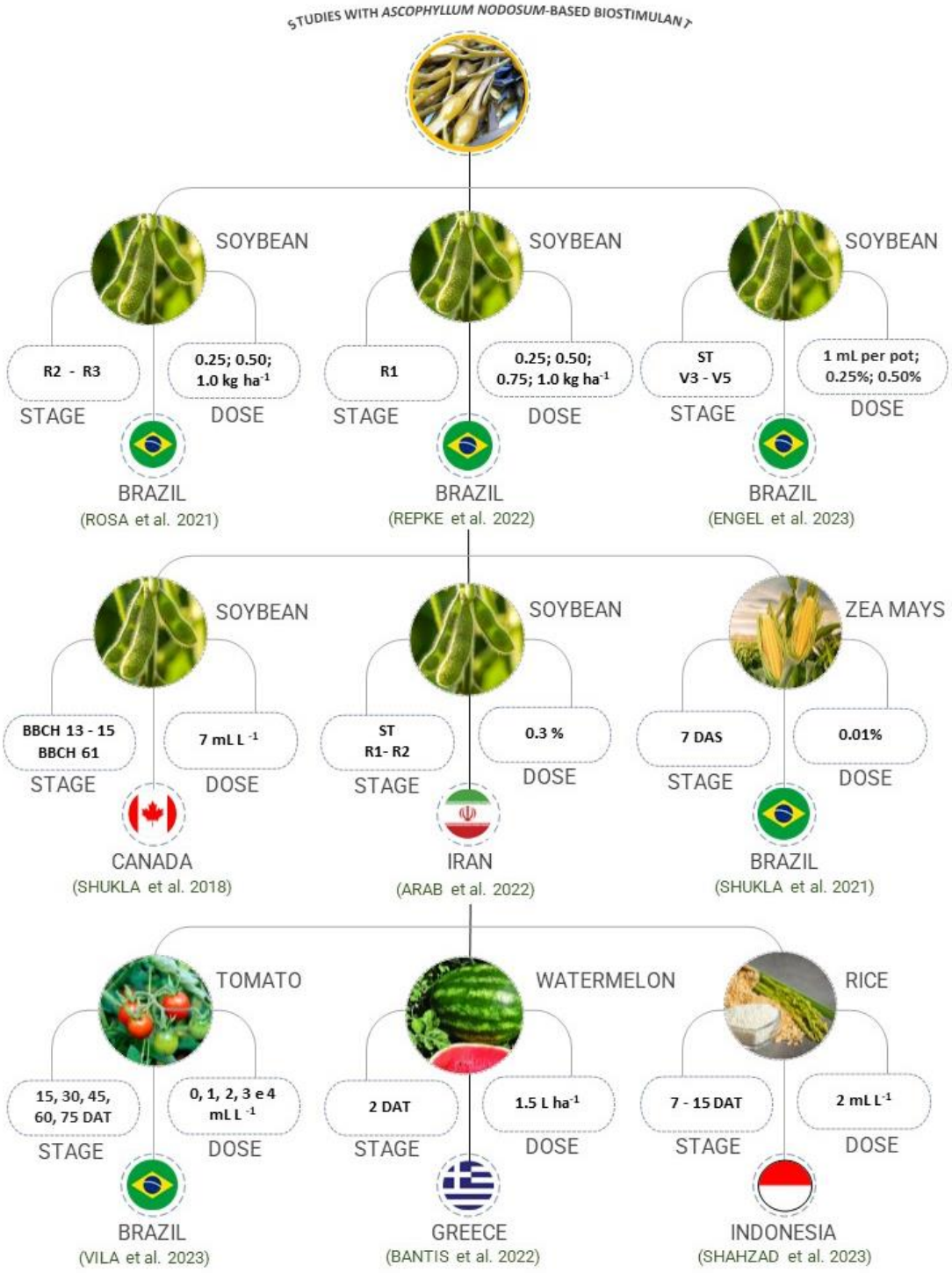
| Depth | Sample | pH | Ca | Mg | Al | H+Al | K | SB | T | P | V | OM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| m | | $H_2O$ | ----------$cmol_c\ dm^{-3}$---------- | | | | | | | $mg\ dm^{-3}$ | % | $g\ kg^{-1}$ |
| | A | 5.0 | 1.26 | 0.75 | 0.10 | 1.91 | 0.10 | 2.11 | 4.02 | 20.2 | 52.5 | 10.01 |
| 0.0 - 0.02 | B | 5.1 | 1.26 | 0.73 | 0.10 | 1.91 | 0.07 | 2.05 | 3.96 | 20.1 | 51.8 | 10.00 |
| | C | 5.0 | 1.25 | 0.72 | 0.10 | 1.81 | 0.08 | 2.06 | 3.87 | 23.2 | 53.2 | 10.37 |

Micronutrients

| | | B | Cu | Mn | Fe | Zn | m | Clay | Silt | Sand |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ----------$mg\ dm^{-3}$---------- | | | | | % | ----------$g\ kg^{-1}$---------- | | |
| | A | - | 0.94 | 3.27 | 142 | 2.38 | 4.5 | 166 | 28 | 806 |
| 0.0 - 0.02 | B | - | 0.91 | 12.51 | 144 | 2.37 | 4.6 | 186 | 25 | 789 |
| | C | - | 1.08 | 16.91 | 141 | 3.15 | 4.6 | 119 | 63 | 819 |




**APPENDIX B -** Timeline of greenhouse experiments with soybean plants treated with extract of *Ascophyllum nodosum* (ANE-biostimulants).




**APPENDIX C -** Summary of studies with doses and application timing for *Ascophyllum nodosum* biostimulants in cultivated crops. Source: Arab *et al*. (2022) South African Journal of Botany 148:510-518; Bantis and Koukounaras (2022) Horticulture 12:1177; Engel *et al*. (2023) Agriculture 13:1296; Repke *et al*. (2022) Journal of Applied Phycology 34:3205-3218; Rosa *et al*. (2021) Plant Physiology and Biochemistry 158:228-243; Shahzad *et al*. (2023) Journal of Plant Interactions 18: 2266514; Shukla and Prithiviraj B (2021) Frontiers in Plant Science 11:601843; Shukla *et al*. (2018) AoB Plants 10:51; Vila *et al*. (2023) Agronomy 13:1853.

**APPENDIX D -** Biostimulant formulations used in trials with soybean plants, under greenhouse conditions.

| | Name | Company | Recommended dose | Application stage | Route of application |
|---|---|---|---|---|---|
| BIO 01 | Terramar | Brandon Bioscience | 1.5 L $ha^{-1}$ | V4 - R1 | Foliar spray |
| BIO 02 | Stingray | Koppert | 1.5 L $ha^{-1}$ | Seed treatment, planting furrow, foliar application at various stages | Through soil or foliar spraying |
| BIO 03 | AlgenMax | Oceana Minerals | 2.0 a 5.0 L $ha^{-1}$ | In the seeding row, cover, split application until flowering or mid-cycle | Through soil or foliar spraying |
| BIO 04 | BioGain | Rigrantec | 0.5 a 1.0 L $ha^{-1}$ | In stage 3/5 leaves, pre-flowering, flowering, and grain filling. | Foliar spray |

**APPENDIX E -** List of detected metabolites in leaves of soybean with their classification in compound types, retention time, and mass fragment used for relative quantitative analysis of each compound, and Kyoto Encyclopedia of Genes and Genomes identifier number (KEGG ID)[a]

| | Name of metabolites | Compound type | Retention time (min) | Mass fragment (m/z) | Compound ID[a] |
|---|---|---|---|---|---|
| 1 | Pyruvic acid | Organic acid | 7.08 | 174 | C00022 |
| 2 | Oxalic acid | Organic acid | 7.34 | 219 | C00209 |
| 3 | Glycolic acid | Organic acid | 7.71 | 177 | C00160 |
| 4 | Malonic acid | Organic acid | 10.70 | 233 | C00383 |
| 5 | Valine | Amino acid | 10.95 | 144 | C00183 |
| 6 | Urea | Amide | 11.36 | 189 | C00086 |
| 7 | Benzoic acid | Organic acid | 11.50 | 179 | C00180 |
| 8 | Serine | Amino acid | 11.79 | 116 | C00065 |
| 9 | Leucine | Amino acid | 12.10 | 158 | C00123 |
| 10 | Glycerol | Sugar alcohol | 12.20 | 293 | C00165 |
| 11 | Nicotinic acid | Vitamins | 12.45 | 136 | C00253 |
| 12 | Isoleucine | Amino acid | 12.52 | 158 | C00407 |
| 13 | Glycine | Amino acid | 12.74 | 248 | C00037 |
| 14 | Succinic acid | Organic acid | 12.82 | 247 | C00042 |
| 15 | Glyceric acid | Organic acid | 13.27 | 189 | C00258 |
| 16 | Uracil | Pyrimidine | 13.36 | 241 | C00106 |
| 17 | Fumaric acid | Organic acid | 13.44 | 245 | C00122 |
| 18 | Alanine | Amino acid | 13.72 | 188 | C00041 |
| 19 | Threonine | Amino acid | 14.28 | 218 | C00188 |
| 20 | Glutaric acid | Organic acid | 14.58 | 261 | C00489 |
| 21 | Beta-Alanine | Amino acid | 14.88 | 248 | C00099 |
| 22 | Aspartic acid | Amino acid | 15.71 | 232 | C00049 |
| 23 | Malic acid | Organic acid | 15.95 | 233 | C00149 |
| 24 | Erythritol | Sugar alcohol | 16.34 | 217 | C00503 |
| 25 | Pyroglutamic acid | Amino acid | 16.48 | 156 | C01879 |
| 26 | Butyric acid (4-amino) | Amino acid | 16.55 | 174 | C00334 |
| 27 | Erythronic acid | Organic acid | 16.91 | 292 | - |
| 28 | Threonic acid | Organic acid | 17.18 | 292 | C01620 |
| 29 | Ornithine | Amino acid | 17.38 | 142 | C00077 |
| 30 | Benzoic acid (3-hydroxy) | Organic acid | 17.98 | 267 | C00587 |
| 31 | Phenylalanine | Amino acid | 18.05 | 218 | C00079 |
| 32 | Xylose | Sugar | 18.64 | 217 | C00181 |
| 33 | Arabinose | Sugar | 18.73 | 217 | C00259 |
| 34 | Xylitol | Sugar alcohol | 19.20 | 319 | C00379 |
| 35 | Ribose | Sugar | 19.52 | 189 | C00121 |

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|---|---|---|---|---|---|
| 36 | Tartaric acid | Organic acid | 20.17 | 292 | C02107 |
| 37 | Shikimic acid | Organic acid | 20.62 | 204 | C00493 |
| 38 | Citric acid | Organic acid | 20.79 | 273 | C00158 |
| 39 | Inositol-allo | Sugar alcohol | 21.11 | 305 | - |
| 40 | Fructose | Sugar | 21.60 | 217 | C02336 |
| 41 | Mannose | Sugar | 21.77 | 160 | C00159 |
| 42 | Galactose | Sugar | 21.84 | 160 | C00984 |
| 43 | Glucose | Sugar | 21.93 | 160 | C00031 |
| 44 | Lysine | Amino acid | 22.01 | 156 | C00047 |
| 45 | Tyrosine | Amino acid | 22.25 | 218 | C00082 |
| 46 | Mannitol | Sugar alcohol | 22.31 | 319 | C00392 |
| 47 | Dehydroascorbic acid | Organic acid | 22.47 | 316 | C00425 |
| 48 | Galactonic acid | Sugar | 23.15 | 292 | C00880 |
| 49 | Gluconic acid | Sugar | 23.20 | 319 | C00257 |
| 50 | Myo-inositol | Sugar alcohol | 24.23 | 265 | C00137 |
| 51 | Caffeic acid | Phenolic compound | 24.51 | 396 | C01481 |
| 52 | Tryptophan | Amino acid | 25.41 | 202 | C00078 |
| 53 | Levodopa | Amino acid | 27.41 | 267 | C00355 |
| 54 | Glucoheptose | Sugar | 30.20 | 217 | - |
| 55 | Cellobiose | Sugar | 30.76 | 361 | C06422 |
| 56 | Threalose | Sugar | 30.93 | 191 | C01083 |
| 57 | Maltose | Sugar | 31.44 | 204 | C00208 |
| 58 | Isomaltose | Sugar | 32.08 | 160 | C00252 |
| 59 | Maltotriose | Sugar | 34.10 | 204 | C01835 |